The ISME Journal (2014), 1–10

www.nature.com/ismej

ORIGINAL ARTICLE

Direct exchange of vitamin B_{12} is demonstrated by modelling the growth dynamics of algal–bacterial cocultures

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The growth dynamics of populations of interacting species in the aquatic environment is of great importance, both for understanding natural ecosystems and in efforts to cultivate these organisms for industrial purposes. Here we consider a simple two-species system wherein the bacterium *Mesorhizobium loti* supplies vitamin B_{12} (cobalamin) to the freshwater green alga *Lobomonas rostrata*, which requires this organic micronutrient for growth. In return, the bacterium receives photosynthate from the alga. Mathematical models are developed that describe minimally the interdependence between the two organisms, and that fit the experimental observations of the consortium. These models enable us to distinguish between different mechanisms of nutrient exchange between the organisms, and provide strong evidence that, rather than undergoing simple lysis and release of nutrients into the medium, *M. loti* regulates the levels of cobalamin it produces, resulting in a true mutualism with *L. rostrata*. Over half of all microalgae are dependent on an exogenous source of cobalamin for growth, and this vitamin is synthesised only by bacteria; it is very likely that similar symbiotic interactions underpin algal productivity more generally.
The ISME Journal advance online publication, 13 February 2014; doi:10.1038/ismej.2014.9
Subject Category: Microbe-microbe and microbe-host interactions
Keywords: algae; mutualism; mathematical modelling; nutrient exchange

Introduction

The diverse photosynthetic eukaryotes collectively known as algae are found in all marine and freshwater environments, and may account for up to 50% of the world's carbon fixation (Field, 1998). Primary production in aquatic systems is generally at equilibrium, and the community composition of the algae, as well as the abundance of individual species, is dependent on the available nutrients. The role of phosphorus and nitrogen has been studied extensively, largely because eutrophication by these elements (for example, by run-off from agriculture, or sediment discharge from rivers) alters the rate of production and community composition in most systems, inducing an algal bloom (Heisler *et al.*, 2008). Iron has also been shown to have a key role, particularly in high-nutrient, low-chlorophyll regions (HNLC) as in the subarctic Northeast Pacific and Southern Oceans (for example, Moore *et al.*, 2001). The importance of iron in limiting productivity was demonstrated experimentally by enriching surface waters of the Southern Ocean with acidic iron sulphate dissolved in sea water, eliciting a strong response in phytoplankton productivity monitored by satellite imaging of chlorophyll fluorescence (Coale *et al.*, 2004).

In addition to these inorganic nutrients, it is becoming increasingly recognised that organic micronutrients in the form of vitamins may also have a role in controlling algal growth. Over half of all microalgal species surveyed (from fresh, marine and brackish habitats) are auxotrophic for cobalamin (vitamin B_{12}) that is, they are unable to grow in its absence. Similarly, 20% require thiamine (vitamin B_1) and 5% require biotin (vitamin B_7) (Croft *et al.*, 2006). Several studies have demonstrated that fertilisation by B vitamins of natural systems has consequences equivalent to Fe (for example, Sañudo-Wilhelmy *et al.*, 2006; Gobler *et al.*, 2007). Vitamin auxotrophy is randomly distributed across the algal lineages, with no phylogenetic relationship between those organisms that require the nutrients. For example, even within a single genus there are both requirers and nonrequirers: the heterokont *Navicula pelliculosa* requires thiamine but other members of the genus do not; *Chlamydomonas reinhardtii* is vitamin B_{12} independent, whereas *C. nivalis* is an auxotroph (Provasoli and Carlucci,

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Received 17 September 2013; revised 22 December 2013; accepted 6 January 2014

1974; Croft *et al.*, 2005). The widespread prevalence of auxotrophy would suggest that there is a readily available source of these nutrients in natural ecosystems; otherwise, B_{12} requirers would suffer from a fitness disadvantage. In fact, the opposite appears to be the case: in a survey of 29 different bloom-forming species the majority were found to be auxotrophs for both thiamine and cobalamin (Tang *et al.*, 2010), suggesting that these species are ubiquitous.

The majority of algae surveyed can synthesise thiamine, and those that require it appear to be missing one or more of the genes for thiamine-biosynthesis enzymes (Croft *et al.*, 2006; Worden *et al.*, 2009; Bertrand *et al.*, 2012). In contrast, vitamin B_{12}, one of the most complex primary metabolites in nature (Figure 1a), is synthesised only by bacteria, requiring more than 20 enzyme-catalysed reactions to do so (Warren *et al.*, 2002). Algal B_{12} auxotrophs appear to have lost a key enzyme, cobalamin-independent methionine synthase (METE), and instead rely on a form of methionine synthase, METH, which needs B_{12} as a cofactor (Helliwell *et al.*, 2011). Laboratory studies indicate that a minimum of 20–50 ng l^{-1} exogenous cobalamin is necessary to sustain algal growth axenically (Bertrand *et al.*, 2012; Kazamia *et al.*, 2012). An important question that arises therefore is how, in the natural environment, do algal auxotrophs obtain cobalamin in sufficient quantities for growth from the bacteria that synthesise it? In this context, it should be mentioned that only about one-third of prokaryotes encode genes for the complete cobalamin biosynthesis pathway (Warren *et al.*, 2002).

Measurement of cobalamin levels free in solution in the natural environment indicate that it is in the range of 10–70 ng l^{-1} (Sañudo-Wilhelmy *et al.*, 2006, 2012; Bertrand *et al.*, 2007; Panzeca *et al.*, 2009), although in certain lakes or in some coastal waters, where there are high bacterial loads and/or contamination by sewage, up to 600 ng l^{-1} have been measured (Okbamichael and Sañudo-Wilhelmy, 2004). Nonetheless, in most habitats there is insufficient vitamin B_{12} free in solution to support the growth of algal auxotrophs in the photic zone. It has been proposed that vitamin B_{12} is delivered passively to algae as part of the microbial loop (Karl, 2002). In this model, vitamin B_{12}-synthesising bacteria grow at depth on organic material that sinks from surface waters after organisms in the photic zone die. The bacteria that grow at depth lyse and release macronutrients and micronutrients (including vitamin B_{12}), forming nutrient-rich deep water that is periodically transported back to the surface by upwelling, thus fertilising algal production and closing the loop. An alternative possibility is that algae obtain the vitamin directly from synthesising bacteria associated with them in the same environment, and that the heterotrophic bacteria get photosynthate in return. Symbiosis between algae and bacteria has been widely described, and ranges from obligate interactions, such as that described between the cyanobacterium UCYN-A and a unicellular prymnesiophyte (Thompson *et al.*, 2012), to

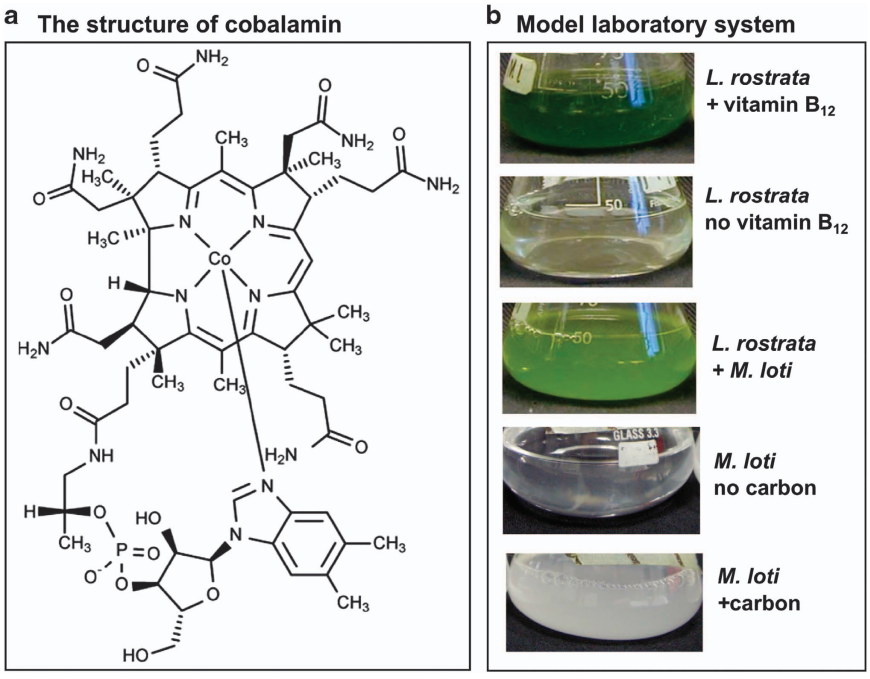

Figure 1 Vitamin B_{12}-requiring algae are only able to grow in the presence of cobalamin, which can only be synthesised by some bacteria. (**a**) The structure of cobalamin (vitamin B_{12}). (**b**) The model laboratory system developed by Kazamia *et al.* (2012) to study this interaction. *L. rostrata*, a vitamin B_{12} auxotroph, grows in the presence of *M. loti* in autotrophic medium that does not contain cobalamin. Controls indicate that this is because of the exchange of a carbon source for vitamin B_{12}.

facultative mutualisms centred on nutrient exchange. Amin *et al.* (2009) showed that bacteria within the boundary layer of dinoflagellate algae promoted algal assimilation of Fe by facilitating photochemical redox cycling of this critical nutrient. Similarly, growth of several species of B_{12}-dependent marine algae has been shown to be supported by the presence of the bacterium *Halomonas spp.* (Croft *et al.*, 2005), and the symbiotic *Dinoroseobacter shibae* delivered both vitamin B_{12} and vitamin B_1 to its dinoflagellate host (Wagner-Döbler *et al.*, 2010).

The biological implications of the two methods of vitamin B_{12} delivery—passive exchange versus direct symbiosis—are widely different. Within the microbial loop, there is no interaction between algae and bacteria, and no need for them to coordinate metabolism. In contrast, if the symbiosis model prevails, it implies that organisms must have a way of recognising each other in order to undergo targeted nutrient exchange. It may also mean that bacteria need to synthesise more vitamin B_{12} to support the growth of algae in addition to satisfying their own requirement. The provision of fixed carbon would thus compensate for this, whereas in the microbial loop there is no guarantee that B_{12} producers would have preferential access to algal photosynthate.

Studying interactions between algae and bacteria in natural environments is challenging because of the open and highly complex nature of such systems. With reference to vitamin auxotrophy, which shows no phylogenetic relationship, species-specific interactions are the most important and yet the hardest to infer. For example, it was only after a large-scale metagenomic analysis that it was possible to identify in natural samples the nitrogen-fixing cyanobacterium UCYN-A, whose highly reduced genome indicated that it must have an obligate symbiotic lifestyle. Identifying facultative mutualists would be impossible in this way, as the genomic markers would be more subtle. In this regard, model laboratory systems offer the opportunity to study species-specific interactions that are likely to parallel those in natural systems, but under defined experimental conditions. We have previously developed such a model system between a vitamin B_{12}-dependent freshwater green alga *Lobomonas rostrata* and a vitamin B_{12}-producing bacterium *Mesorhizobium loti* (Kazamia *et al.*, 2012), which has the following characteristics: (1) The two organisms can grow in coculture in medium that lacks both B_{12} and fixed carbon, whereas neither can grow separately in this medium (Figure 1b). (2) In cocultures, algal and bacterial cells reached a steady-state ratio of $\sim$1:30, regardless of the starting population densities of the two organisms. This is true both for batch cultures and semi-continuous cultures where the medium was replenished regularly. (3) The carrying capacity reached in coculture was lower for both algae and bacteria than in axenic controls supplemented with vitamin B_{12} and a source of carbon, respectively. (4) It was possible to perturb the established equilibrium in cocultures by supplementation with either vitamin B_{12} or a source of carbon. When vitamin B_{12} was added to the cocultures, this favoured algal growth without concomitant increase in bacterial densities. Conversely, when a source of carbon was added, bacterial growth was exponential and did not lead to an increase in algal density.

Here, we formulate a mathematical description of the cocultures of *L. rostrata* with *M. loti* based on the physiological observations outlined above. The quantitative model arrived at allowed us to test specific hypotheses, particularly whether exchange is due to regulated transfer of nutrients, or is instead mediated by the death and lysis of organisms, as implied by the microbial loop. Although there have been some previous quantitative studies of algal uptake of vitamin B_{12}, these examined certain aspects only, such as the impact of vitamin B_{12} concentration on algal growth rate (Droop, 1968), and did not consider the environmental source of vitamin B_{12}. The mathematical models presented here take a radical step away from this approach, by considering directly and with minimal complexity the impact of *M. loti* on the growth of *L. rostrata*, and *vice versa*. By considering the biological relevance of the necessary parameters in the model, it is possible to distinguish between mutualism and lysis as a means of delivery of vitamin B_{12}. Shedding light on algal–bacterial dynamics is important both for our understanding of natural aquatic systems and for industrial-scale cultivation of algae. Currently, algae are grown commercially for high-value products and there is considerable interest in cultivating algae for biofuels (Scott *et al.*, 2010). However, the scale at which the latter will be necessary will obviate the possibility to maintain sterile conditions, and thus contamination by bacteria is inevitable. Our model of algal–bacterial interactions could be used to predict quantitatively growth dynamics in such systems.

Materials and methods

Numerical integration

The way in which the mathematical model was developed is detailed in Supplementary Materials Section 1. The model equations were integrated using a custom C++ implementation of the fourth-order Runge-Kutta method.

Measurement of vitamin B_{12} levels

To determine the amount of vitamin B_{12} produced by *M. loti*, axenic cultures of the bacterium and cocultures with *L. rostrata* were grown in batch culture in TP^+ medium, as described in Kazamia *et al* (2012). Cells and medium were separated by centrifugation for 5 min at 13 000 *g*. The supernatant was filtered through a 0.22-μm filter to remove any

remaining cells, whereas the pellet was resuspended in the equivalent volume of TP^+ medium and boiled at 100 °C for 15 min to lyse cells and release contents. For measurement of total vitamin B_{12}, the cells were not separated from the medium, and the cultures were boiled to lyse all cells directly. For all boiled samples, there was a filtration step to remove the cell debris. Vitamin B_{12} levels were determined using the *Salmonella typhimurium* bioassay (Raux *et al.*, 1996), which provides a semiquantitative estimation of the amount of B_{12} in a sample by reference to a standard curve.

Results

A mathematical model provides an objective way in which to understand better the interactions between the algal and bacterial cells. To establish such a model, it is necessary to construct equations that are able to describe the behaviour of the *L. rostrata*/*M. loti* cocultures (Kazamia *et al.*, 2012), but the most instructive form will have a minimal set of terms, which will elucidate the main processes. To proceed, it was first necessary to formalise mathematically the experimental criteria listed previously. Thus:

1. *L. rostrata*, a, and *M. loti*, b, cannot grow axenically (by themselves, as a monoculture) in a medium that lacks vitamin B_{12}, V, and a carbon source, C, respectively: $a \to 0$ if b, $V = 0$ and $b \to 0$ if a, $C = 0$.
2. The algae and bacteria reach a steady ratio, r: $a/b \to r$.
3. The carrying capacity (subscript 'max') reached in coculture (superscript 'c') is lower for both algae and bacteria than in axenic controls (supplemented with vitamin B_{12} and carbon, respectively—superscript 'm' (monoculture)): $a_{\max}^{(c)} < a_{\max}^{(m)}$, $b_{\max}^{(c)} < b_{\max}^{(m)}$.
4. The coculture is perturbed by nutrient add-back. When the system is perturbed by supplementing the medium with vitamin B_{12}, the algal population reaches a higher carrying capacity. If a carbon source is added, then the equilibrium ratio is broken in favour of bacteria, which reach a greater carrying capacity: $a/b > r$ if $V > 0$, $C = 0$. $a/b < r$ if $C > 0$, $V = 0$.

Starting from a logistic equation for each of *L. rostrata* and *M. loti*, as described in Supplementary Materials Section 1, it was immediately apparent that, to satisfy the conditions, the model cannot have independent equations, as neither the algae nor the bacteria can grow alone without a source of vitamin B_{12} or fixed carbon, respectively. We then added further terms until, by construction, a minimal form was achieved that can describe the experimental results. In the following, a and b denote the number (population) of algae and bacteria, α and β their respective growth rates and K_a and K_b their carrying capacities. Two models were able to satisfy the experimental observations:

Model 1

This model features a carrying capacity term for the culture that is dependent on the population density of each organism, and which saturates at high numbers. There is also an independent term (K_v or K_c) for when there is add-back of nutrients (vitamin B_{12} or fixed carbon, respectively). In this model, provision of the nutrient is maximal at equilibrium; an increase in numbers of bacteria will have no effect on algal growth, and vice versa. This 'unregulated' model can be interpreted in different ways, and fits the suggestion that algae can grow using vitamin B_{12} released by bacteria following death and cell lysis (Droop, 2007). A form of Model 1 that reflects this explicitly, and that satisfies conditions 1–4, is

$$\dot{a} = \alpha a \left[1 - a \left(\frac{K_a \varepsilon b}{b_c + \varepsilon b} + K_v \right)^{-1} \right], \qquad (1)$$

$$\dot{b} = \beta b \left[1 - b \left(\frac{K_b a}{a_c + a} + K_c \right)^{-1} \right] - \varepsilon b. \qquad (2)$$

The lysis term, ε, describes the fraction of the bacterial cells that die per unit time, and it is these cells that enable the growth of *L. rostrata* in cocultures with *M. loti*. The composite term $K_a \varepsilon b/(b_c + \varepsilon b)$ defines the carrying capacity of the algae that is due to bacterial presence (following lysis). This approach of modelling the carrying capacity of an organism as a function of its symbiont was first proposed by Yukalov *et al.* (2012). K_a is the maximum number of bacteria that the algae are able to support and εb is the total number of bacteria in the culture that die per unit time. When vitamin B_{12} is provided in the medium, growth is dependent on K_v, which is the maximum number of algae that any externally added vitamin B_{12} provided can support.

Model 2

This model is similar to Model 1, but it includes an additional term to describe the dynamics during nutrient add-back. Effectively, it allows for a change in behaviour between algae and bacteria when nutrients are externally supplied. This is achieved through the use of the Heaviside function. For both algal and bacterial growth, the Heaviside step function, defined as

$$\tilde{H}(x) = \begin{cases} 0, & x \leqslant 0 \\ 1, & x > 0, \end{cases} \qquad (3)$$

is incorporated to reflect the hypothesis that vitamin B_{12} is only made available to algae when there is no additional carbon in the medium. In other words, when fixed carbon is provided in

the medium, the Heaviside function takes on the value of 0 (zero) and the size and influence of nutrient exchange in the carrying capacity shrinks.

The motivation for inclusion of this term is that the experimental data suggest a reduction of the algal population when bacterial growth is stimulated by the addition of fixed carbon. Model 2 takes the following form:

$$\dot{a} = \alpha a\left[1 - a\left(\frac{K_a b\left(1-\tilde{H}(K_c)\delta_b\right)}{b_c + b} + K_v\right)^{-1}\right], \quad (4)$$

$$\dot{b} = \beta b\left[1 - b\left(\frac{K_b a\left(1-\tilde{H}(K_v)\delta_a\right)}{a_c + a} + K_c\right)^{-1}\right]. \quad (5)$$

Here, the carrying capacity of the algae is modelled with two terms. A composite term $K_a b(1-\tilde{H}(K_c)\delta_b)/(b_c+b)$ defines the carrying capacity of the algae that is due to the presence of bacteria. When there is no vitamin B_{12} added to the medium, Model 2 assumes that algal growth is dependent entirely on this composite term; however, when exogenous B_{12} is added, growth is also dependent on K_v, which is the maximum number of algal cells that can be supported by the concentration of the added vitamin.

Bacterial growth is modelled similarly, with K_c representing the carrying capacity when carbon is added externally into the medium, and the composite term $K_b a(1-\tilde{H}(K_v)\delta_a)/(a_c+a)$ representing the carrying capacity due to algal presence. It should be noted that Model 2 reduces to Model 1, upon rescaling some of the parameters, and setting $\delta_a = \delta_b = 0$.

Choosing parameter values. The parameters in Table 1 were fitted to the data, using values from the literature where possible and varying the rest until a satisfactory qualitative fit was achieved. The results are shown in Figure 2a, where the values of numbers of algal and bacterial cells that would arise from the two models with four different starting values of a and b are shown as lines. These trend lines fit the experimental data (shown as individual symbols) very closely, indicating that the models are able to describe the regulation of growth within the coculture as it tends to the equilibrium ratio of $\approx$1:30 algal to bacterial cells. Both models fit equally well. Figure 2b shows the trend lines of the models for the algal and bacterial cells after the addition of nutrients. Again the models are able to recreate the qualitative effects seen in the experimental data (shown as symbols), namely that addition of the carbon source results in an increase in bacterial numbers but no increase in algal cells, whereas addition of vitamin B_{12} causes an increase in algal cells with no corresponding increase in bacterial numbers. However, the experimental data suggest that the algal and bacterial numbers might actually decrease when carbon and vitamin B_{12}, respectively, are added to the medium. Model 2 has the additional parameters δ_a and δ_b that enable it to capture such a decrease (Figure 2b Model 2, top panel), whereas Model 1 does not (Figure 2b Model 1, top panel).

Distinguishing between the two models. Because of the biological variability associated with the experimental measurements, we carried out further laboratory experiments to enable us to distinguish with more certainty which model best describes the

Table 1 List of parameters used in the models

Parameter Symbol	*Description*	*Value*[a]	*Reference*
α	Growth rate of *L. rostrata*	1.5 log2 days^{-1}	Harris (2001)
K_a	Maximum number of algae that the bacteria can support	4×10^6 ml^{-1}	Fitted
b_c	Number of bacteria required to reach half the maximum carrying capacity, K_a	10^4 ml^{-1} (M1) 10^8 ml^{-1} (M2)	Fitted Fitted
δ_a	Fractional decrease in carbon production by algae when vitamin B_{12} is provided externally in the medium	0.4	Fitted
K_v	Maximum number of algae when vitamin B_{12} is provided externally in the medium	4×10^6 ml^{-1}	Kazamia *et al.* (2012)
β	Growth rate of *M. loti*	4 log2 days^{-1}	Vincze and Bowra (2006)
K_b	Maximum number of bacteria that the algae can support	10^7 ml^{-1}	Fitted
a_c	Number of algae required to reach half of the maxiumum carrying capacity, K_b	5×10^3 ml^{-1}	Fitted
δ_b	Fractional decrease in the production of vitamin B_{12} by bacteria if carbon is provided externally in the medium	Experimentally determined (Equation (8))	
K_c	Maximum number of bacteria if carbon is provided externally in the medium	2×10^8 ml^{-1}	Kazamia *et al.* (2012)
ε	Proportion of lysed cells	Varied	

b_c takes different values in Model 1 (M1) and Model 2 (M2).
Values were taken from the literature where possible, and then the rest varied until a satisfactory qualitative fit was achieved. The numerical values shown are those used in the solutions plotted in Figure 2.
[a]Parameter values used in the solutions plotted in Figure 2.

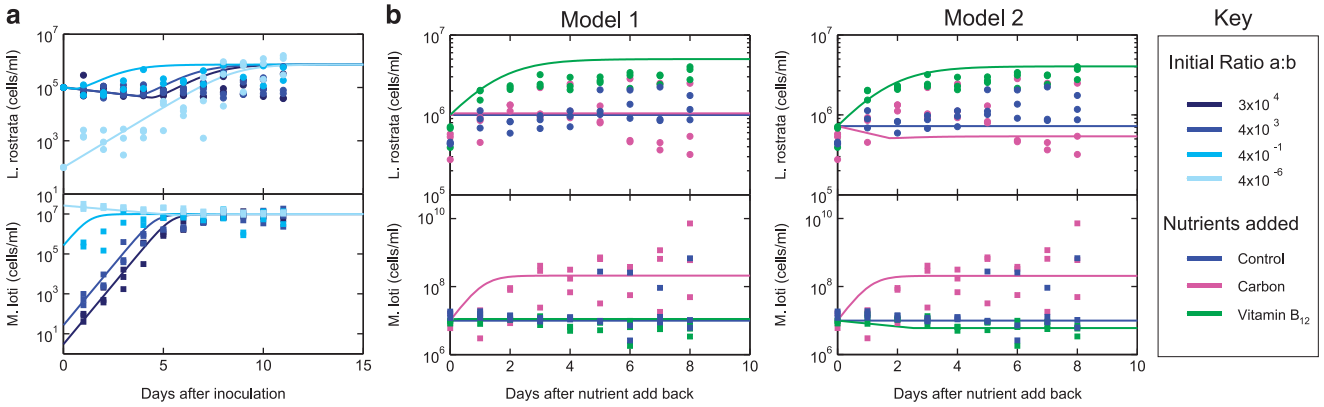

Figure 2 The models describe the growth of *L. rostrata* and *M. loti* seen in experiments. In all graphs, the symbols represent the experimental data from Kazamia *et al.* (2012), collected with three biological replicates per time point per treatment, whereas the lines are output of the models. (**a**) The lines show the trends given by Model 2 at different initial conditions (different starting values of a and b), without add-back of nutrients. The algae (top panel) and bacteria (bottom panel) grow until they reach the carrying capacity. Model 1 gives a similar fit. (**b**) The equilibrium is broken by adding back either a carbon source in the form of glycerol (magenta lines and symbols) or vitamin B_{12} (green lines and symbols). The control (i.e. no addition) is shown in blue. The unregulated model (Model 1) recreates the increase in the number of algae when B_{12} is added to the medium (top panel), and the increase in the number of bacteria when carbon is added to the medium (bottom panel). The regulated model (Model 2) similarly recreates the increase in the number of algae and bacteria after the addition of B_{12} or carbon, respectively. It is also able to capture the corresponding decrease in algal numbers seen upon the addition of carbon (top panel, magenta line), and bacteria after addition of B_{12} (bottom panel, green line). Note that the scale of the y axis is logarithmic.

observed algal–bacterial interactions. By design, and with an appropriate choice of parameter values, both models can fit the data in a satisfactory manner, but it is possible to ask which of the two is the most biologically plausible. To answer this, two approaches were taken: first, Model 2 predicts the presence of the parameter δ_b, representing a change in production of vitamin B_{12} by *M. loti* in the presence of the algae. If δ_b is non-zero, this means the bacteria regulate the production of vitamin B_{12}, increasing it in the presence of the algae. This can be tested by measuring the levels of vitamin B_{12} in monocultures of *M. loti* and in cocultures of *L. rostrata*. Second, it is possible to estimate the number of bacteria that would need to lyse in order to support the algal growth, and consider if this figure is realistic. As little is known about the form of carbon provided by the algae, it is not possible to test experimentally the value of the parameter δ_a, which represents a change in the supply of carbon by the algae to the bacteria.

To determine the amount of B_{12} produced by *M. loti* in the different cultures, we measured the total B_{12} content in the culture, and also the B_{12} in the cells and the medium (Figure 3). There was good agreement between the measurements (i.e. medium + cells = total) indicating that the bioassay was reliable. It is clear that when grown with *L. rostrata* the amount of B_{12} produced per bacterial cell is ~10-fold higher than in monoculture.

Using the method described in full in Supplementary Materials Section 2, the rate of vitamin uptake per algal cell, u, can be estimated to be

$$u = 2\times10^{-7}\,\mathrm{ng\ alga^{-1}\ day^{-1}} \qquad (6)$$

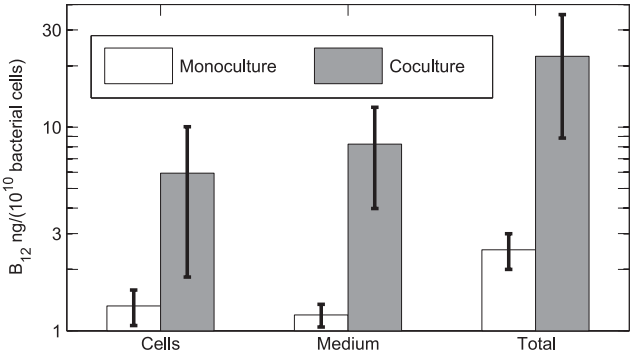

Figure 3 Levels of B_{12} in monoculture of *M. loti* versus coculture with *L. rostrata*. B_{12} was measured separately in the cell pellet, the medium and the total (i.e. before centrifugation to pellet the cells). In the monoculture, the medium has been supplemented with carbon. In the coculture, the cell fraction includes the *L. rostrata* cells. The values, normalised to the numbers of bacterial cells, are the average of three biological replicates. Note that the scale is logarithmic.

which is in good agreement with that obtained by Droop (1968).

Disagreement with the 'unregulated' Model 1. It is possible to estimate the total rate of uptake of vitamin B_{12} by all the algal cells in a coculture, u_{tot}. Using the value of u mentioned above (equation (6)), together with the average carrying capacity of 3×10^8 cells per L (shown in Figure 2a, top panel), this is calculated to be:

$$u_{tot} = 60\ \mathrm{ng\ l^{-1}\ day^{-1}} \qquad (7)$$

From the experimental data in Figure 3, and noting that there are approximately 10^{10} bacterial

cells per litre in coculture (Figure 2a, bottom panel), it is apparent that there is $<10\,\mathrm{ng\,l^{-1}}$ stored in all the cells in coculture. Thus, the amount of vitamin B_{12} required by the algae per day is greater than the amount of vitamin B_{12} stored in all the bacterial cells. As a result, the lysis interpretation of the unregulated model, Model 1, is unrealistic, as even in the implausible scenario in which all the bacteria in the culture lysed in a single day not enough vitamin B_{12} would be released to support the number of algae present in a coculture for that day. This suggests that the vitamin B_{12} is produced by the bacteria and taken up by the algae in a continuous manner, rather than being produced by the *M. loti* and retained by them, only to be transferred when they lyse.

Agreement with 'regulated' Model 2. Model 2 predicts the presence of a parameter δ_b, which reflects the hypothesis that the bacteria decrease the production of vitamin B_{12} when there is carbon present in the medium. To probe the value of this parameter, and test if it is non-zero, we took the values shown in Figure 3 (details in Supplementary Materials Section 3), obtaining the value:

$$\delta_b \approx 0.9. \tag{8}$$

This value describes a situation in which, in a monoculture, the production rate of vitamin B_{12} by *M. loti* is a factor of ten lower than in coculture. The precise value is less important than the fact that it appears to be non-zero, which lends weight to the scenario in which the production of vitamin B_{12} by *M. loti* is reduced upon introduction of carbon into the medium.

Discussion

The dynamics of *M. loti* and *L. rostrata* growth observed in cocultures is not simple, as seen in the detailed experimental observations over a range of experimental conditions (Kazamia *et al.*, 2012). An equilibrium in the number of cells of the two organisms is reached in media in which neither can grow alone. The equilibrium is broken when the exchanged nutrients are added externally, but enhanced growth of one organism does not lead to a corresponding increase in the other, even though, before nutrient add-back, there is a strong indicator of co-dependence. The 'regulated' Model 2 presented here can capture these dynamic interactions in a relatively simple set of equations by expressing the carrying capacity of each organism as a function of the other's abundance, while incorporating a 'switch' to a state where that is not the case when either fixed carbon or vitamin B_{12} is added to the medium. The model fits the data very well (Figure 2); there are only slight discrepancies in the perturbed region, where the data become noisy beyond the description of any deterministic model. However, the key features of the perturbed system, such as the observation that the algal numbers do not increase when a carbon source is added (and vice versa for the bacteria with B_{12}), are captured by the model.

Previous attempts have been made to describe quantitatively the aspects of algal B_{12} auxotrophy. It was observed by Droop (1966) that the growth rate of algae did not depend on the concentration of vitamin B_{12} in the classic manner of Monod dynamics. To circumvent this failure of standard growth models, additional terms were included alongside the usual Monod description to give a better empirical fit to the data. The form of these terms led Droop to hypothesise that a 'vitamin-binding protein' was secreted by the algae, which sequestered, and thus prevented access to, the vitamin (Droop, 1968). However, in evolutionary terms, the benefit of rendering an essential nutrient inaccessible seems questionable, and while B_{12}-binding proteins have been characterised from different algal species these are much more likely to be involved in the uptake of the vitamin. Work with *Euglena gracilis* (Watanabe *et al.*, 1988) and *Thalassiosira pseudonana* (Sahni *et al.*, 2001) found high-affinity vitamin B_{12}-binding factors in the growth medium of these two organisms. Similarly, a candidate B_{12}-binding protein named CBA1 (for cobalamin acquisition) has recently been identified based on a transcriptomic and proteomic analysis of *T. pseudonana* and a second diatom *Phaeodactylum tricornutum* (Bertrand *et al.*, 2012). The protein was up to 160 times more abundant in vitamin B_{12}-deficient medium, suggesting a role in acquiring this compound.

The benefit of the approach we have used here, describing the interaction of the species through their population dynamics alone, means that, for example, knowledge of the precise growth rate dependence of the algae on vitamin B_{12} concentration is not required; only the dependence on the number of bacteria is needed. This enabled us to test the models against the wide set of data from different experimental protocols keeping additional assumptions to a minimum; in particular, we were able to test whether the provision of nutrients could be explained by lysis. First, the levels of B_{12} produced by *M. loti* in both cocultures and when grown in monoculture were measured (Figure 3). Even though these are much higher in the former, more than the total number of bacteria present would have to lyse in order to satisfy the observed growth requirements for vitamin B_{12} of *L. rostrata* (equations (6) and (7)), leading us to reject conclusively the hypothesis that release of the vitamin by bacterial cell lysis could account for its provision in the coculture. Moreover, the data show that *M. loti* actively increased the rate of vitamin B_{12} synthesis to satisfy algal requirements. Together, these observations support the conclusion that true mutualism through direct and

regulated exchange of vitamin B_{12} is occurring between *L. rostrata* and *M. loti*.

While our model describes the exchange of nutrients in a laboratory context, we believe that similar associations of algae with bacteria are likely to be found in the natural environment, particularly as vitamin B_{12} auxotrophy is widespread and not confined to particular habitats or algal phyla. Indeed, there have been previous reports of algal–bacterial assemblages for the provision of this micronutrient (Gillespie and Morita, 1972; Haines and Guillard, 1974). More recent oceanographic studies have reported the significance of vitamin B_{12} in shaping algal growth and dynamics (Sañudo-Wilhelmy *et al.*, 2006; Gobler *et al.*, 2007; Koch *et al.*, 2011), but the likely role of bacteria as symbionts to the algae has been overlooked. A recent study examined concentrations of vitamins in the ocean, arguing that the distribution of B-vitamins can be explained by seasonal patterns and ocean currents (Sañudo-Wilhelmy *et al.*, 2012). Profiles for average vitamin B_{12} abundance showed that the vitamin was present at depth, with little accumulating in the surface. The role of bacteria as B_{12} producers was acknowledged, but presumed to happen at depth, whereas in surface waters bacteria are described as 'scavengers' that compete with algae for the available vitamin B_{12}. Similarly, in a study of nutrient supplementation on primary production in the Ross Sea (one of the most productive areas in the Southern Ocean), the addition of vitamin B_{12} was investigated alongside iron (Bertrand *et al.*, 2007). Nutrient add-back experiments conducted on collected sea water samples showed that significantly higher chlorophyll *a* concentrations were measured upon the addition of iron and B_{12}, relative to iron additions alone, in two out of three experiments. In the third experiment that did not show this stimulation, initial bacterial abundances were significantly higher. Despite these observations, no study to date has analysed whether there is a species-specific correlation of vitamin B_{12} requirers and vitamin B_{12} producers in the photic zone.

The biological implications of vitamin B_{12} delivery via symbiosis or lysis are also widely different at the molecular and physiological levels. The symbiosis model implies that organisms must have a way of recognising each other in order to undergo targeted nutrient exchange. It may also mean that bacteria actively synthesise more vitamin B_{12} to support the growth of algae in addition to satisfying their own needs. Conversely, if vitamin B_{12} were provided via lysis, there would be no interaction between algae and bacteria, and thus the latter would not alter their level of vitamin B_{12} production in response to the presence of algae; clearly, in our system this is not the case.

Moreover, direct vitamin B_{12} exchange via symbiosis would also explain why vitamin B_{12} auxotrophy has evolved so many times throughout the algal kingdom, and in all habitats (Croft *et al.*, 2005). The molecular basis of this auxotrophy was uncovered by analysis of sequenced algal genomes, revealing that it is the result of loss of a single gene, *METE*, which encodes vitamin B_{12}-independent methionine synthase (Helliwell *et al.*, 2011). Expression of *METE* is repressed by the addition of vitamin B_{12} to the external medium of diverse algae (Croft *et al.*, 2005; Helliwell *et al.*, 2011), which then rely on the activity of cobalamin-dependent methionine synthase, encoded by *METH*. In the absence of selective pressure, *METE* can accumulate deleterious mutations over time, and indeed *METE* pseudogenes have been found in algal auxotrophs (Helliwell *et al.*, 2011). Thus, to account for the widespread occurrence of vitamin B_{12} auxotrophy by this process, there must be sustained supplies of cobalamin in the natural environment. These conditions would be obtained if there were direct algal–bacterial interactions to allow exchange of nutrients. Passive exchange via lysis would be unlikely to provide cobalamin free in solution for extended periods of time at sufficient levels to repress METE expression. In contrast, direct algal–bacterial interactions would allow a sustained exchange of nutrients. Interestingly, there is evidence that auxotrophy for other vitamins may also have arisen as a result of repression of gene expression, including for thiamine (B_1) and biotin (B_7) (Helliwell *et al.*, 2013). Vitamins might be considered the ideal signalling currency between microbes: because they provide essential cofactors, they are ubiquitously required and recognised.

Our findings add to the tide of evidence of close bacterial associations with algae that are often highly evolved (e.g. Grossart *et al.*, 2005; Wagner-Döbler *et al.*, 2010; Gärdes *et al.*, 2011; Hollants *et al.*, 2011). Recently, Goecke *et al.* (2013) argued that algae may be viewed as an important environment for bacterial growth. They conducted a phylogenetic study based on 16S rRNA for 101 described bacterial species isolated from eukaryotic macro- and micro-algae from marine and freshwater environments, and found that bacterial species and strains that carried out similar metabolic functions were likely to colonise similar algal taxa or algal groups. This echoes earlier studies, such as that by Cole *et al.* (1988), which looked at bacterial production in fresh and saltwater ecosystems and found that those with high rates of algal production such as coral reefs have bacterial biomass greater than that predicted by the amount of bioavailable organic carbon alone. The data were interpreted as suggesting either competition between algae and bacteria and hence their growth in response to common factors or that phytoplankton were an important substrate for bacterial growth.

In summary, the work presented here illustrates how regulated symbiosis can be modelled and will make it possible to explore, and better understand, more complex interactions present in biology and biotechnology.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

EK was in receipt of a studentship from the UK Energy Research Centre funding under the Natural Environment Research Council award NE/C513169/1. We also acknowledge financial support from the Winton Fund and EPSRC. We are grateful to Dr O Croze and Dr KE Helliwell for their critical reading of the manuscript.

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