Title: The roles of B vitamins in phytoplankton nutrition: new perspectives and prospects

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Summary

B vitamins play essential roles in central metabolism. These organic water-soluble molecules act as, or as part of, coenzymes within the cell. Unlike land plants, many eukaryotic algae are auxotrophic for certain B vitamins. Recent progress in algal genetic resources and environmental chemistry have promoted a renewal of interest in the role of vitamins in governing phytoplankton dynamics, and illuminated amazing versatility in phytoplankton vitamin metabolism. Accumulating evidence demonstrates metabolic complexity in the production and bioavailability of different vitamin forms, coupled with identification of specialised acquisition strategies to salvage and remodel vitamin precursors. Here, I describe recent advances and discuss how they redefine our view of how vitamins are cycled in aquatic ecosystems and their importance in structuring phytoplankton communities.

Key Words: phytoplankton, algae, nutrient cycling, vitamins, vitamin B_{12}, cobalamin, pseudocobalamin, thiamine

Introduction:

The importance of vitamin-derived coenzymes (see **Box 1** for glossary of terms) in the aquatic realm, was first considered in the early 1900s, when it was recognised that the addition of natural seawater into artificial culture media improved considerably the growth of microalgae (Allen, 1914). Dr E. J. Allen, the then director of The Marine Biological Association (Plymouth, UK), inferred the presence of an organic substance in seawater, which he likened to the 'vitamine' thiamine from the husk of rice recently discovered by Casimir Funk (Funk, 1912). Indeed, he remarked that: *"The minute trace of substance added to the culture medium in the small percentage of natural seawater would seem to act as a catalytic agent, initiating the processes of metabolism but not being itself used up*".

The inference that exogenous organic micronutrients could support microalgal growth was at odds with existing views on the nutrition of these organisms, which were considered to be largely autotrophic, like higher plants (Droop, 1957a). That organic micronutrients could govern phytoplankton dynamics in the ocean ignited the interests of a new generation of phycologists, who pioneered the development of algal culturing techniques. As an aside, these scientists can be accredited with laying the foundations for the establishment of several culture collections, such as the Culture Collection of Algae and Protozoa founded by Ernst Pringsheim, and the National Centre for Marine Algae and Microbiota by Luigi Provasoli and Robert Guillard that serve an expanding community of microbiologists still today. Over the following years, the prevalence of auxotrophy (**Box 1**) amongst microalgae gradually became realised, with three vitamins being recognised in particular: vitamin B_1 (thiamine), B_7 (biotin) and B_{12} (cobalamin) (e.g. Droop, 1957a; Guillard & Ryther, 1962; Provasoli and Carlucci, 1974). The previous perception that microalgae were entirely autotrophic was attributed to an inherent bias in cultivation methods, remedied by the amendment of artificial media with organic extracts such as soil (Pringsheim, 1946) and even ox liver (Pringsheim, 1952)! These were shown subsequently to contain thiamine (Lwoff & Lederer, 1935), and cobalamin (Smith, 1948), respectively. The role of vitamins as important organic micronutrients for microalgae became firmly established.

Comparative genomics: filling in the blanks

A new era of interest sustained by genomic resources has allowed us to identify the genetic factors underpinning algal vitamin requirements, their synthesis and use. Contemporary studies have been informed enormously by advances made in understanding vitamin biosynthetic

pathways of bacteria, plants and fungi (Gerdes *et al.*, 2012). Genome mining for vitamin-related gene homologs, alongside culture-based surveys of vitamin dependencies has enabled molecular dissection of algal vitamin metabolism particularly for thiamine and vitamin B_{12}, which will be the focus of the remainder of the review.

Vitamin B_{12}, a cobalt-containing ring-contracted tetrapyrrole, is one of nature's most complex metabolites (**Fig. 1a**). In the first systematic examination of algal B_{12} requirements, compiled from assembled literature, over 50% of species surveyed (171/326) require exogenous B_{12} for growth (Croft et al. 2005). Bioinformatics searches for B_{12}-biosynthesis genes revealed that algae (including B_{12}-independent species) cannot synthesise B_{12} *de novo*, contrary to previous understanding (Carlucci & Bowes, 1970). Whether or not an alga requires B_{12} therefore, is dictated by its necessity as a cofactor not biosynthetic capacity. Three enzymes that have a B_{12} coenzyme are known in eukaryotes: methylmalonyl-CoA mutase (MCM), type II ribonucleotide reductase (RNRII) and methionine synthase (METH) (**Fig. 1b**). Higher plants and fungi do not encode MCM or RNRII, and have an alternative B_{12}-independent methionine synthase (METE), and so do not require cobalamin at all. Amongst algae, independence from requiring the vitamin is governed by the presence of *METE*, which appears to have been lost on multiple independent occasions in algal evolution (Helliwell et al. 2011) (**Fig. 1c**)

Through examining the distribution of biosynthetic genes across algal lineages, a picture of thiamine metabolism in algae is beginning to emerge too. Eukaryotic algae arose following a series of endosymbiotic events beginning with the primary endosymbiosis of a cyanobacterium by a heterotrophic eukaryote giving rise to the green, red and glaucophyte algae (Keeling, 2010). Photosynthesis later spread horizontally through secondary, and even tertiary/quaternary endosymbioses, founding the major extant algal lineages including: the haptophytes, stramenopiles and dinoflagellates. The opportunity for random mixing of genetic material resulting from serial endosymbioses has promoted the complex physiologies exhibited by photosynthetic eukaryotes, and thiamine metabolism is no exception. Essential for the activity of enzymes of carbohydrate and branch-chain amino acid metabolism, the biosynthetic pathway for this vitamin occurs via the condensation of pyrimidine and thiazole precursor moieties (**Fig. 2a**) to form thiamine monophosphate (TMP), which is phosphorylated to generate the active coenzyme form, thiamine pyrophosphate (TPP). The enzymes and substrates used to synthesise pyrimidine and thiazole differ between higher plants and bacteria. Whereas bacteria synthesise hydroxyethylthiazole (HET) via the deoxy-D-xylulose 5-phosphate (DXP) pathway, higher plants are thought to use precursors NAD+, glycine and a sulphur donor, like yeast (Chatterjee *et al.*, 2011) (Fig. 2a). The pyrimidine branch is similar

in higher plants and bacteria, except that enzymatic activities of ThiD and ThiE of bacteria are combined into a single bifunctional enzyme (TH1) in higher plants **(Fig. 2a)**. Amongst algae, representative chlorophyte species (*Chlamydomonas reinhardtii*, *Chlorella variabilis* NC64A and *Coccomyxa subellipsoidea* C169) encode a thiamine biosynthesis pathway similar to *A. thaliana* (Croft *et al.*, 2007) (**Fig. 2b**), whereas red algae have a biosynthesis gene set resembling the bacterial thiazole branch (Croft *et al.*, 2006). Sequenced haptophyte and diatom species also encode genes for thiazole biosynthesis resembling those of bacteria and red algae, suggesting they may have originated from their red algal plastid.

The emergence of vitamin auxotrophy

Whilst vitamins have long been recognised as important regulators of algal growth, gaining consensus on their ecological roles has been more challenging. Earlier work concluded ambient levels of B_{12} were likely sufficient to satisfy microalgal demands of natural populations (Droop, 1957b). However, studies since with refined analytical approaches in extensive areas of the coastal ocean have found levels often below detection limits ($\leq$0.18 pM for B_{12} and $\leq$0.81 pM for B_1) (Sañudo-Wilhelmy *et al.*, 2012). Vitamin amendment experiments indicate that B-vitamin availability can impact bloom formation, and phytoplankton productivity (Bertrand *et al.*, 2007; Koch *et al.*, 2012). That vitamin auxotrophs are widespread in nature therefore is somewhat of a paradox. Of species surveyed 50%, 22% and 5% require vitamin B_{12}, B_1 and B_7, respectively (Croft *et al.*, 2006). Moreover, the distribution of auxotrophy is scattered across the different algal lineages with variability existing even between strains of the same species (Provasoli and Carlucci, 1974), hinting that dependence must have evolved on multiple independent occasions. Recent evidence from experimental evolution indicates vitamin B_{12} auxotrophy can arise readily in the laboratory, in the presence of a reliable vitamin supply (Helliwell *et al.*, 2015). Evolution of a B_{12}-dependent clone of *C. reinhardtii* that rapidly displaced its ancestor, was observed in fewer than 500 generations. Insertion of a transposable element into the *METE* gene was found to underpin this shift in phenotype.

Studying patterns of requirement across algal lineages can also help us draw trends on how ecological lifestyle may influence a species tendency towards vitamin auxotrophy. For instance, the incidence of vitamin auxotrophy is disproportionately prevalent in dinoflagellates (Tang *et al.*, 2010), which invariably exhibit phagotrophic modes of life. This would allow such species to obtain organic nutrients from the prey on which they graze. Indeed the role of biotic interactions, including mutualistic interactions between algae and bacteria, in governing

vitamin acquisition strategies is an active area of research, in the laboratory (e. g. Croft *et al.*, 2005; Wagner-Döbler *et al.*, 2010; Durham *et al.*, 2015) and the field (Bertrand *et al.*, 2015), and has been reviewed recently (Kazamia *et al.*, 2016)

Beyond the genome: metabolic complexity in algal vitamin acquisition strategies

Through consideration of the biochemistry of different forms of vitamins and their precursors, we are gaining an appreciation of the metabolic innovations adopted by microbes to fulfil their nutrient demands. The genetic potential for B_{12} biosynthesis is confined to certain prokaryotes (Croft *et al.*, 2005). Dominant aquatic taxa implicated in its production include Cyanobacteria, Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes species (Sañudo-Wilhelmy *et al.*, 2014), and more recently members of the archaeal phyla Thaumarchaeota (Doxey *et al.*, 2015). However, diversity in the structural forms of B_{12} produced by different prokaryotes (and their bioactivity) may arise, based on the identity of the upper/lower ligands of the molecule **(Fig. 1a)**. To better understand specific processes dictating the biosynthesis of different forms and their bioactivity, it is crucial to delineate the metabolic capabilities of individual species. In this regard, a biochemical study of B_{12} production in strains of the marine cyanobacterium *Synechococcus* detected a B_{12} analog known as pseudocobalamin, in which the lower ligand base adenine replaces 5,6-dimethylbenzimidazole (DMB). Searches of over 100 cyanobacterial genomes for B_{12} biosynthesis genes (including lower ligand biosynthesis genes) suggests this is the form synthesized by cyanobacteria more broadly (Helliwell *et al.*, 2016). This is significant because pseudocobalamin alone is considerably less bioavailable to eukaryotic algae (Droop, 1957a; Helliwell *et al.*, 2016). However, intriguingly some species can convert pseudocobalamin to a bioavailable form when provided with exogenous DMB (**Fig. 3**). Whilst enzymes involved in lower ligand removal (CbiZ/CbiB) could not be found (Yi *et al.*, 2012) 'bacterial' genes encoding enzymes of lower ligand base assembly (*COBT*, *COBS* and *COBC*) (Anderson *et al.*, 2008) were identified in such algae (**Fig. 1c; Fig. 3**) providing a likely mechanism for aspects of this process of 'remodeling' (**Box 1**) (Helliwell *et al.*, 2016). These genes were identified in 11% of marine microbial eukaryote taxa surveyed, indicating that algal remodelers could be important players in the mobilisation of bioavailable B_{12}. This could be particularly important since pseudocobalamin concentrations can reach magnitudes equal to cobalamin in the marine environment (Heal *et al.*, 2017).

Whilst different structural forms, or vitamers (**Box 1**), of B_{12} clearly play distinctive roles in phytoplankton physiology, evidence of the importance of vitamin B_1 precursor compounds is accumulating. In particular, genome analyses have identified microalgae that possess many, but not all, thiamine biosynthesis genes. Notably, several prasinophyte species (of the *Micromonas* and *Ostreococcus* genus) lack pyrimidine and thiazole biosynthesis genes, but encode TH1*:* a plant-like enzyme necessary for the condensation of these two precursors (**Fig. 2b**) (Bertrand & Allen, 2012; Paerl *et al.*, 2015). Similarly, *Emiliania huxleyi* (haptophyte) and *Guilardia theta* (cryptophyte) lack just the pyrimidine branch (McRose *et al.*, 2014). This combination of gene presence/absence supports earlier reports that certain algae can satisfy their thiamine requirements with precursors (Provasoli and Carlucci, 1974). Indeed, *E. huxleyi* growth can be supported by feeding exogenous 4-amino-2-methyl-5-hydroxymethylpyrimidine (HMP) only (McRose *et al.*, 2014). However, contrary to predictions from pathway analyses, thiamine auxotrophic growth cannot be restored by supplementation with HET and HMP in *Micromonas* and *Ostreococcus* species (McRose *et al.*, 2014; Paerl *et al.*, 2015). Growth can however be rescued when HMP is provided alongside a cryptic thiazole-related precursor, produced by B_1-synthesising proteobacteria and found present in surface seawater samples (Paerl *et al.*, 2016), implying further complexities in algal vitamin B_1 salvage pathways.

Rethinking vitamin cycling in phytoplankton communities

Consideration of the different vitamin forms and precursors, their relative abundance, stability and preferential use, in the context of the unique environment in which phytoplankton reside is essential. Specialisation on certain vitamers/ precursors could play an important role in niche partitioning and/or species succession in phytoplankton blooms. Expression of B_{12}-remodelling genes during a dinoflagellate bloom (Gong *et al.*, 2017), lends support to the potential importance of remodelling strategies for competitive phytoplankton dynamics. That HMP levels can exceed thiamine in marine ecosystems (Carini *et al.*, 2014) is further evidence of the ecological relevance of precursors, especially considering thiamine degrades under UV radiation (Okumura, 1961). This goes to highlight the unique selective pressures shaping the nutrient acquisition strategies of microbes inhabiting the photic zone. However major gaps in our knowledge still exist. Over a dozen forms of $B_{12,}$ varying in lower ligand base identity, are produced in nature. While the remodelling machinery of bacteria exhibit versatility in attaching different lower ligands bases, albeit with different substrate specificities (Crofts *et al.*, 2013), little is known of the relevance of this diversity of forms in phytoplankton communities.

Environmental levels and algal specificity for lower ligands besides DMB and adenine are also unknown. Determining the relative abundances of vitamin forms, and how they govern community composition will be integral to understanding the roles of vitamins in natural aquatic ecosystems. Advances in analytical methodologies to measure particulate and dissolved B-vitamin pools including structural variants (Suffridge *et al.*, 2017), will help illuminate intricacies in the biogeochemical cycling of vitamins. Limited knowledge of uptake mechanisms for vitamins (and related molecules), and their specificity, also represents a major constraint. Currently just one protein involved in B_{12} uptake: cobalamin acquisition protein 1 (*CBA1*) confined to the stramenopile lineage (Bertrand *et al.*, 2012) (**Fig. 1c**) has been identified, and its function is not fully understood. Identification of novel vitamin-responsive genes will undoubtedly guide gene discovery efforts in the future.

More broadly, the apparent plasticity in microbial vitamin metabolism somewhat challenges the essence of how we define vitamins. The ability of precursor compounds to satisfy vitamin requirements obviates the need for exogenous supplementation with the vitamin *per se*, and has led researchers to ask: 'vitamin auxotrophy or precursor auxotrophy?' (McRose *et al.*, 2014). Indeed, given the amazing evolutionary diversity and varied ecological lifestyles exhibited by phytoplankton, we are left speculating on how reliably the principles of vitamin nutrition, generally built from animal fields, are readily transferable to the microbial realm. This is further highlighted by the recent discovery of a pathogenic bacterium that does not require thiamine as a coenzyme at all (Zhang *et al.*, 2016), providing the first evidence of metabolic independence from a molecule that was previously deemed essential for all cellular life. Such extraordinary examples perhaps emphasize more the constraints in our own knowledge that bias our understanding of what is typical. This echoes lessons learnt from the early perception that all photoautotrophic organisms can exist without organic nutrients, which later proved to be an artefact of our inability to culture those that cannot.

Conclusion

Whilst the importance of vitamins to phytoplankton nutrition has been known for decades, our understanding of vitamin cycling in aquatic ecosystems is far from complete. The picture emerging highlights the versatility of phytoplankton micronutrient synthesis and acquisition strategies, and critically, emphasises the need to explore microbial life with an open mind. Such metabolic complexity has undoubtedly been shaped by the unique lifestyles of phytoplankton: their diverse evolutionary history, fast generation times, and unicellular physiologies.

Moreover, since algae offer great potential applications, from use as nutritional food sources (Wells *et al.*, 2016) to biotechnology (Nguyen *et al.*, 2016), understanding these characteristics will be important for their exploitation. Decoding the distinct physiological roles of individual species will help us better understand the processes governing the productivity of phytoplankton populations, which drive the major biogeochemical processes and ecosystem services that we rely on.

Acknowledgements

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Box 1.

Vitamin: an essential micronutrient that must be taken up by an organism that cannot synthesize the compound itself

Coenzyme: an (non-protein) organic molecule that is essential for the activity of an enzyme

Auxotroph: an organism that requires an external source of an organic molecule that it cannot synthesise itself

Autotroph: an organism that does not require an external source of an organic molecule either because it can synthesise it itself, or because it does not require that compound

Vitamer: in human nutrition, this refers to a chemical variant of a vitamin that can be used by humans (and animals). However, in the context of microbial metabolism this is arguably too strict a definition, and as such in this manuscript it is defined simply as a chemical variant of a vitamin.

Remodeling: in the context of vitamin metabolism the process of chemically altering a vitamin form to improve its bioavailability or activity within the cell

Figure Legends:

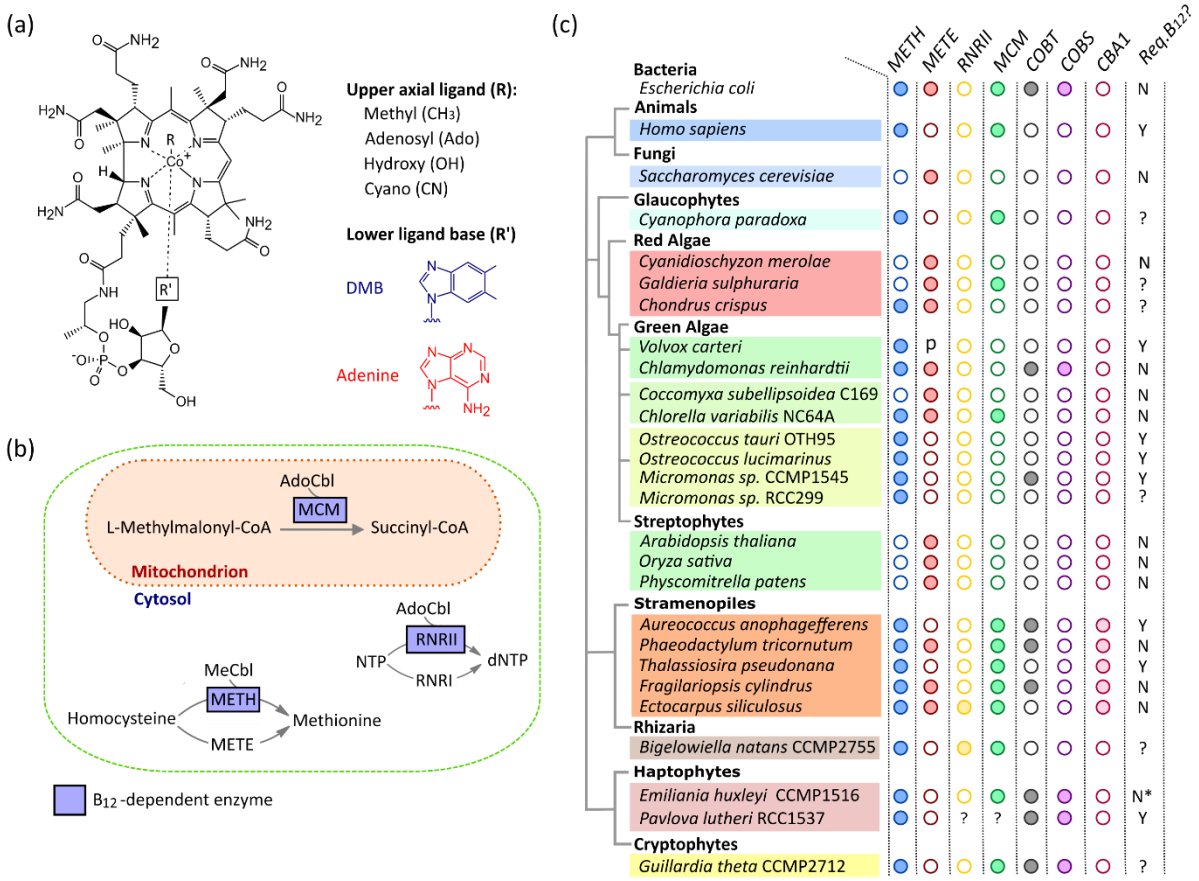


Figure 1a) Structural variants of B_{12} with two alternative lower ligand bases (R') displayed: DMB as in cobalamin, and adenine as in pseudovitamin B_{12}. In biological systems, two different upper axial ligands are found (adenosyl or a methyl group). The cyano group is an artificial ligand resulting from the extraction procedure (Warren *et al.*, 2002). **b)** The role of vitamin B_{12} as a cofactor in an algal cell. Eukaryotes are known to have three enzymes that require vitamin B_{12} as a cofactor: B_{12}-dependent methylmalonyl-CoA mutase (MCM) involved in odd-chain fatty acid metabolism, methionine synthase (METH) that catalyses the synthesis of methionine from homocysteine and methyl-tetrahydrofolate, and type II ribonucleotide reductase (RNRII) involved in deoxyribose biosynthesis. B_{12}-independent isoforms, METE and RNRI, exist for methionine synthase and ribonucleotide reductase, respectively. **c)** Distribution of B_{12}-related enzymes known in eukaryotes. The requirement for vitamin B_{12} by each species where known, as validated by experimental data, is shown. For further details, a record of the B_{12} requirements of over 300 species of algae as compiled from the literature is provided by Croft et al. (2005), and a survey of species with sequenced algal genome can be found in Helliwell et al. (2011). NB **Emiliania huxleyi* appears to grow in the absence of B_{12}, but does not encode *METE*. However, despite attempts to remove bacteria, it was not possible to obtain an axenic culture of this alga; bacterial contaminants could thus provide enough B_{12} to sustain the growth observed, see Helliwell et al. (2011)

for details. *COBS* and *COBT* encode enzymes of lower base activation and nucleotide-loop assembly, respectively (Anderson *et al.*, 2008; Helliwell *et al.*, 2016). CBA1 (Cobalamin Acquisition Protein 1) is thought to be involved in cobalamin uptake (Bertrand *et al.*, 2012). Key: closed circles (presence), open circles (absence), P (pseudogene), ? (unknown), Y (yes), N (no). In addition, the genomes of *C. paradoxa* (http://cyanophora.rutgers.edu/cyanophora/blast.php), *B. natans* (http://genome.jgi.doe.gov/Bigna1/Bigna1.home.html) and *G. theta* (http://genome.jgi.doe.gov/Guith1/Guith1.home.html) were searched via the Basic Local Alignment Search Tool (BLAST) with algal sequences using an e-value cut-off of $1E^{-5}$. The IDs for identified proteins are as follows METH: Contig6827 (*C. p*), 52176 (*B. n*), 159764 (*G. t*); RNRII: 41126 (*B. n*); MCM: Contig7838 (*C. p*), 45247 (*B. n*), 159175 (*G. t*); COBS: 100220 (*G. t*); COBT: 165718 (*G. t*).

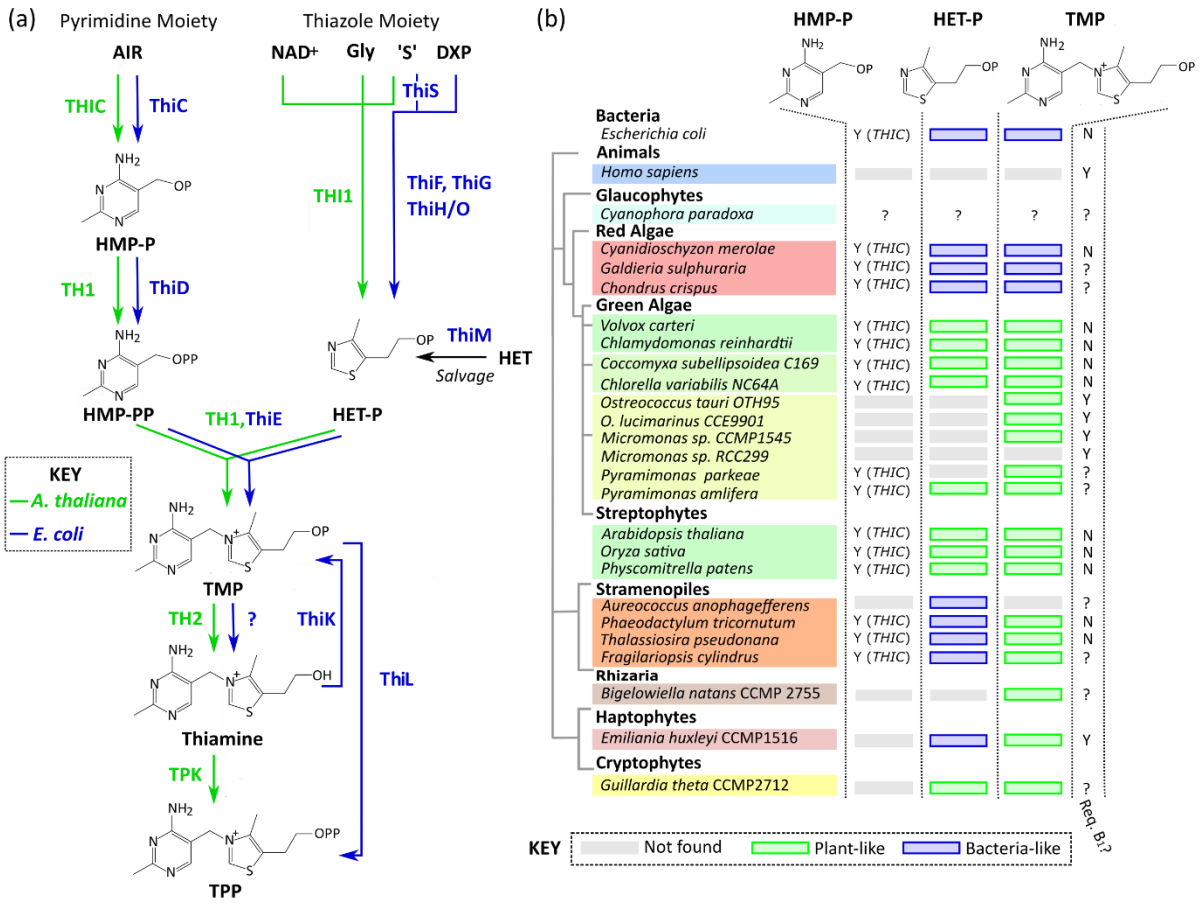
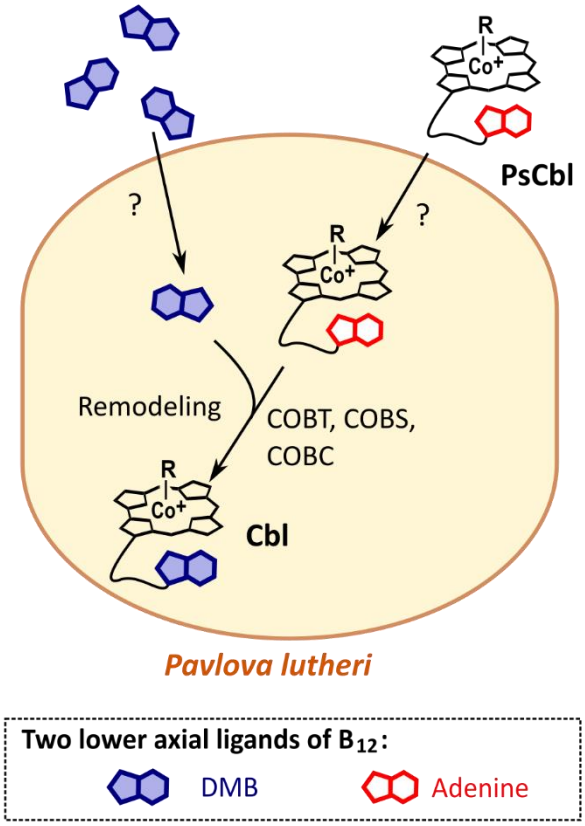


Figure 2. Metabolic diversity in Thiamine Pyrophosphate (TPP) biosynthesis. a) TPP biosynthetic pathway as described in *A. thaliana* (green) and *E. coli* (blue). In *A. thaliana* the thiazole moiety (HET-P) is thought to be synthesised from NAD^+, glycine and a sulphur donor ('S') like in fungi (Chatterjee *et al.*, 2011). *E. coli* synthesises HET-P via the deoxy-D-xylulose 5-phosphate (DXP) pathway. The pyrimidine biosynthesis pathway is similar in both plants and bacteria, except in *A. thaliana* the enzymatic activities of ThiD and ThiE are combined into a single bifunctional enzyme (encoded by the gene *TH1*) (abbreviations: AIR: 5-aminoimidazole ribonucleotide, HMP-P: 4-amino-2-methyl-5-hydroxymethylpyrimidine monophosphate, HMP-PP: 4-amino-2-methyl-5-hydroxymethylpyrimidine diphosphate, TMP: thiamine monophosphate). **b)** Distribution of thiamine biosynthesis genes in sequenced algal genomes. Similarity to branches of the pathway as described in higher plants or bacteria is indicated (Bertrand & Allen, 2012; McRose *et al.*, 2014; Paerl *et al.*, 2017). Since HMP-P biosynthesis is analogous in both plants and bacteria, whether or not an alga encodes *THIC* is indicated. In the case of HET-P biosynthesis, colour coding is assigned according to whether or not the alga encodes *THI1* homolog like in plants (and fungi; gene name *THI4*); or ThiG, as in bacteria. For TMP biosynthesis, algae encoding a single bi-functional protein with both phosphomethylpyrimidine kinase and thiamine monophosphate synthase domains, similar to *TH1* in *A. thaliana* are indicated with green boxes, whereas when these activities are divided between two proteins known as ThiD and ThiE like in *E. coli* they are blue. Notably, whilst for THIE and THID independent gene models can be found for *T. pseudonana* (262963 and 262964), *F. cylindrus* (153126 and 161112),and *O. tauri* (6224 and 20618) (Bertrand & Allen,

2012; McRose *et al.*, 2014), in every instance gene models were incomplete, adjacent to one another on the same chromosome, and could be extended using pairwise sequence alignment tool so that phosphomethylpyrimidine kinase and thiamine monophosphate synthase domains were on the same open reading frame (http://www.ebi.ac.uk/Tools/psa/genewise/). As such, these were taken as homologs of the single bi-functional *TH1* gene found in *A. thaliana*. The requirement for vitamin B_1 by each species, as validated by experimental data (McRose *et al.*, 2014; Paerl *et al.*, 2016, Croft et al. 2006), is also shown.



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453 **Figure 3. B_{12}-remodeling in an algal cell.** Pseudocobalamin (Pscbl), in which the lower ligand base is
454 adenine does not support growth of eukaryotic algae, but certain species (such as *P. lutheri* and *C.*
455 *reinhardtii*) can remodel it to a bioactive form when external DMB is provided in addition (Helliwell
456 *et al.*, 2016).

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