

Most harmful algal bloom species are vitamin B₁ and B₁₂ auxotrophs

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Eutrophication can play a central role in promoting harmful algal blooms (HABs), and therefore many HAB studies to date have focused on macronutrients (N, P, Si). Although a majority of algal species require exogenous B vitamins (i.e., auxotrophic for B vitamins), the possible importance of organic micronutrients such as B vitamins (B₁, B₇, B₁₂) in regulating HABs has rarely been considered. Prior investigations of vitamins and algae have examined a relatively small number of dinoflagellates ($n = 26$) and a paucity of HAB species ($n = 4$). In the present study, the vitamin B₁, B₇, and B₁₂ requirements of 41 strains of 27 HAB species (19 dinoflagellates) were investigated. All but one species (two strains) of harmful algae surveyed required vitamin B₁₂, 20 of 27 species required B₁, and 10 of 27 species required B₇, all proportions higher than the previously reported for non-HAB species. Half-saturation (K_s) constants of several HAB species for B₁ and B₁₂ were higher than those previously reported for other phytoplankton and similar to vitamin concentrations reported in estuaries. Cellular quotas for vitamins suggest that, in some cases, HAB demands for vitamins may exhaust standing stocks of vitamins in hours to days. The sum of these findings demonstrates the potentially significant ecological role of B-vitamins in regulating the dynamics of HABs.

Harmful algal blooms (HABs) are a significant threat to coastal ecosystems, public health, economies, and fisheries, and there are strong links between nutrient loading and HABs within ecosystems around the world (1–3). Most studies of HABs focused on nutrients have primarily investigated the importance of macronutrients (N, P, Si) (2, 3). In contrast, the importance of coenzymes and particularly vitamins (vitamins B₁, B₇, and B₁₂) in regulating and stimulating HABs has rarely been considered. This omission is despite the fact that exogenous B vitamins are essential compounds for phytoplankton species that lack the required biosynthetic pathways to produce B vitamins, i.e., vitamin B-auxotrophs (4–10).

Vitamin B₁₂ is essential for the synthesis of amino acids, deoxyriboses, and the reduction and transfer of single carbon fragments in many biochemical pathways (11, 12), whereas vitamin B₁ (thiamine) plays a pivotal role in intermediary carbon metabolism and is a cofactor for number of enzymes involved in primary carbohydrate and branched-chain amino acid metabolism (13, 14). More than half of 326 algal species surveyed are auxotrophs for B₁₂ (10–12, 15) and more than 20% of the 306 microalgal species surveyed are auxotrophs for B₁ [compiled in Croft et al. (14)]. In addition, 5% of 306 algae surveyed require biotin (vitamin B₇), a cofactor of several essential carboxylase enzymes, such as acetyl CoA (14).

Recently, there has been burgeoning interest in the ability of vitamins to regulate phytoplankton community growth and structure. Novel high performance liquid chromatography (HPLC) techniques for the direct measurement of vitamins B₁ and B₁₂ in seawater have been developed (16, 17). Studies measuring vitamin B₁₂ in the ocean have shown levels are lower than those estimated by prior bioassays (18) and in many cases, concentrations of levels are substantially less than the 7-pM threshold (18) putatively required by many phytoplankton in culture (15). A series of studies have demonstrated that vitamins can stimulate phytoplankton growth in Fe-limited, high-nutrient, low-chlorophyll (HNLC) regions of Antarctica (19, 20) and coastal zones where HABs commonly occur (21, 22).

Multiple observations suggest vitamins may be specifically important to the occurrence of HABs. HABs are caused primarily by dinoflagellates, and the percentage of vitamin B₁₂ auxotrophy among this algal class is greater than nearly all others (15). Field studies have observed a covariance of dissolved vitamin B₁₂ and the HAB species *Lingulodinium polyedrum* (23) and *Karenia brevis* (24–27). Gobler et al. (22) also observed the selective enhancement of large dinoflagellates by the enrichment of coastal waters with vitamin B₁ and B₁₂. Previous surveys of phytoplankton (15) and field studies, however, have examined a relatively small number of dinoflagellates and very few HAB species (namely, *Karenia brevis*, *Gymnodinium catenatum*, *Amphidinium operculatum*, and *Akashiwo sanguinea*). Investigations of phytoplankton requirements for vitamins B₁ and B₇ have been exceedingly rare (22, 28). Because there exists no discernible evolutionary pattern to vitamin auxotrophy among algae (15, 29), a more comprehensive investigation of the qualitative and quantitative vitamin requirements of HABs is needed to better understand the autoecology of these events.

Here we report an investigation of the vitamin B₁, B₇, and B₁₂ requirements of 41 strains of 27 species of marine microalgae. All but one species are HAB species the auxotrophy of which have yet to be established; a cryptophyte was investigated as a non-HAB species, as these algae are well-known dinoflagellate prey. In addition to establishing the auxotrophy of each strain for each vitamin, vitamin-dependent growth rates were measured for selected species to quantify half-saturation constants and cellular vitamin quotas. These results provide insight into the potential ecological importance of vitamins in the occurrence of harmful algal blooms.

Results

Qualitative Vitamin B₁, B₇, and B₁₂ Requirements. The results of the qualitative experiments are summarized in Table 1. All species examined except for *Symbiodinium* sp. SJNU were auxotrophs for vitamin B₁₂, including 18 species (29 strains) of dinophyceae, two species (three strains) of “brown tide” pelagophyceae, two species of raphidophyceae, two species of bacillariophyceae (three strains), and one species each of cryptophyceae and prymnesiophyceae. The two strains of the dinoflagellate *S. microadriaticum* displayed differing results regarding vitamin B₁₂ requirements. *S. microadriaticum* strain CCMP827 (origin unknown) required B₁₂, but the strain CCMP829 isolated from Australia did not (Table 1).

The large majority of strains (31 of 41) and species (20 of 27) examined were vitamin B₁ auxotrophs (Table 1). The exceptions were *H. triquetra* HT1, *A. minutum* CCMP113, *A. catenella* ACJNU, *S. microadriaticum* (strains CCMP827 and CCMP829), *Symbiodinium* sp. SJNU, the raphidophyte *F. japonica* Fibro1, the two strains of *P. multiseriis*, and one of the two strains of *Scrippsiella trochoidea* (MS3). Interestingly, the two strains of dinoflagellate *Scrippsiella trochoidea* isolated from the same New York

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Table 1. Vitamin requirements for different microalgae, mainly HAB species, including 27 species and 41 strains

Phylum, class, species, and strain	Origin	Requires cobalamin? (pM)	Requires thiamine? (nM)	Requires biotin? (pM)
Dinophyta; Dinophyceae				
<i>Alexandrium catenella</i> ACJNU	South China Sea, China	Y (>0.34)	N (<0.05)	N (<0.14)
<i>Alexandrium minutum</i> CCMP113	Ria de Vigo, Spain	Y (>0.08)	N (<1.0×10 ⁻⁸)	N (<2.9×10 ⁻⁸)
<i>Akashiwo sanguinea</i> AS2	Virginia, USA	Y (>0.11)	Y (0.05)	N (<6.7×10 ⁻¹⁸)
<i>Cochlodinium polykrikoides</i> CP1	New York, USA	Y (>0.001)	Y (>0.006)	N (<0.005)
<i>Gymnodinium aureolum</i> KA1	Virginia, USA	Y (>0.04)	Y (>0.15)	N (<0.42)
<i>Gymnodinium aureolum</i> KA2	Virginia, USA	Y (>1.0)	Y (>4.07)	N (<11)
<i>Gymnodinium aureolum</i> KA6	Virginia, USA	Y (>1.0)	Y (4.07)	Y (>11)
<i>Gymnodinium aureolum</i> KA7	Virginia, USA	Y (>1.0)	Y (>4.07)	N (<11)
<i>Gymnodinium instriatum</i> L2	Virginia, USA	Y (>1.0)	Y (>4.07)	N (<11)
<i>Gymnodinium instriatum</i> L3	Virginia, USA	Y (>0.001)	Y (>2.55×10 ⁻⁶)	Y (>1.0×10 ⁻⁶)
<i>Gymnodinium instriatum</i> L4	Virginia, USA	Y (0.34)	Y (>1.36)	N (<3.74)
<i>Gymnodinium instriatum</i> L6	Virginia, USA	Y (>1.0)	Y (4.07)	N (<11)
<i>Heterocapsa arctica</i> MS5	New York, USA	Y (>0.11)	Y (>1.36)	Y (>5.7×10 ⁻⁴)
<i>Heterocapsa</i> sp. AT1	Singapore	Y (>3.04)	Y (>12)	N (<0.14)
<i>Heterocapsa triquetra</i> HT1	Virginia, USA	Y (>0.11)	N (<7.65×10 ⁻⁶)	N (<2×10 ⁻⁵)
<i>Karenia brevis</i> CCMP2228	Florida, USA	Y (>1.0)	Y (4.07)	Y (>0.002)
<i>Karenia mikimotoi</i> ISO6	Singapore	Y (>1.0)	Y (>4.07)	Y (>2×10 ⁻⁵)
<i>Karlodinium veneficum</i> FR6*	New York, USA	Y (>3.04)	Y (>12.2)	Y (>33.7)
<i>Pheopolykrikos hartmannii</i> FR3	New York, USA	Y (>0.11)	Y (>0.45)	Y (>1.25)
<i>Pheopolykrikos hartmannii</i> FR4	New York, USA	Y (>0.0005)	Y (>0.002)	Y (>5.7×10 ⁻⁴)
<i>Prorocentrum donghaiense</i>	East China Sea, China	Y (>0.11)	Y (>1.36)	N (<5.7×10 ⁻⁴)
<i>Prorocentrum minimum</i> CCMP696	New York, USA	Y (>0.34)	Y (>1.4×10 ⁻¹¹)	Y (>1.4×10 ⁻¹¹)
<i>Prorocentrum minimum</i> PB3	Singapore	Y (>0.04)	Y (>0.15)	Y (>0.002)
<i>Scrippsiella trochoidea</i> CCPO3	Virginia, USA	Y (>0.01)	Y (>0.05)	N (<0.14)
<i>Scrippsiella trochoidea</i> CCPO4	Virginia, USA	Y (>5×10 ⁻⁴)	Y (>2.3×10 ⁻⁵)	N (<8.8×10 ⁻⁸)
<i>Scrippsiella trochoidea</i> MS1	New York, USA	Y (>0.001)	Y (2.3×10 ⁻⁵)	Y (>2.6×10 ⁻⁷)
<i>Scrippsiella trochoidea</i> MS3	New York, USA	Y (>2×10 ⁻⁵)	N (<3.3×10 ⁻¹⁹)	N (<9.0×10 ⁻¹⁹)
<i>Symbiodinium microadriaticum</i> CCMP827	Unknown	Y (>0.34)	N (<2.4×10 ⁻¹⁶)	N (<6.7×10 ⁻¹⁶)
<i>S. microadriaticum</i> CCMP829	Australia	N (<5.5×10 ⁻¹⁶)	N (<2.2×10 ⁻¹⁵)	N (<6.1×10 ⁻¹⁵)
<i>Symbiodinium</i> sp. SJNU	South China Sea, China	N (<1.7×10 ⁻⁵)	N (<6.9×10 ⁻⁵)	N (<1.9×10 ⁻⁴)
<i>Takayama acrotricha</i> CCMP2960*	Singapore	Y (>6.83)	Y (>3.05)	N (<0.015)
Stramenopiles; Pelagophyceae				
<i>Aureococcus anophagefferens</i> CCMP1984	New York, USA	Y (>3.04)	Y (>12)	N (<33.7)
<i>Aureococcus anophagefferens</i> CCMP1707	New York, USA	Y (>9.11)	Y (>0.05)	N (<5.7×10 ⁻⁴)
<i>Aureocoumbra lagunensis</i> CCMP1681	Gulf of Mexico, USA	Y (>1.01)	Y (>0.002)	N (<3.2×10 ⁻⁹)
Cryptophyta; Cryptophyceae				
<i>Rhodomonas salina</i> CCMP1319	New York, USA	Y (>3.04)	Y (>12)	N (<0.015)
Ochrophyta; Raphidophyceae				
<i>Chattonella marina</i> ChatM1	Singapore	Y (>4×10 ⁻³)	Y (>1.36)	N (<6.7×10 ⁻¹⁶)
<i>Fibrocapsa japonica</i> Fibro 1	Singapore	Y (>3.6×10 ⁻¹²)	N (<2.0×10 ⁻¹⁴)	N (<5.5×10 ⁻¹⁴)
Haptophyta; Prymnesiophyceae				
<i>Phaeocystis globosa</i>	South China Sea, China	Y (>0.01)	Y (>0.017)	N (<3.6×10 ⁻¹⁰)
Bacillariophyta; Bacillariophyceae				
<i>Pseudo-Nitzschia multiseries</i> CLNN16	Bay of Fundy, Canada	Y (>0.01)	N (<5.6×10 ⁻³)	N (<1.5×10 ⁻²)
<i>Pseudo-Nitzschia multiseries</i> CLNN21	Bay of Fundy, Canada	Y (>0.01)	N (<5.6×10 ⁻³)	N (<1.5×10 ⁻²)
<i>Pseudo-Nitzschia pungens</i>	China	Y (>0.34)	Y (>1.36)	Y (>2.1×10 ⁻⁵)

Numbers in parentheses indicate the estimated vitamin concentrations when the culture growth ceased or when the experiment was terminated. Information regarding species identifications is given in *SI Materials and Methods*. N, no (vitamin auxotrophy was not observed); Y, yes (auxotrophy for vitamin). *Two cultures for which antibiotics solution was not used because cultures could not survive antibiotics.

estuary differed in their vitamin B₁ requirement: Strain MS1 was a vitamin B₁ auxotroph, whereas strain MS3 was not (Table 1). Ten of 27 species, including 12 of 41 strains, required biotin (vitamin B₇) (Table 1). Biotin auxotrophs included the dino-flagellates *G. aureolum* KA6, *G. instriatum* L3, *K. brevis* CCMP2228, *K. mikimotoi* ISO6, *K. veneficum* FR6, *P. hartmannii* FR3 and FR4, *H. arctica* MS5, *S. trochoidea* MS1, *P. minimum* CCMP696 and PB3, and the diatom *P. pungens* PPJNU. All species from Pelagophyceae (*A. anophagefferens* CCMP1984 and CCMP1707, *A. lagunensis* CCMP1681), Cryptophyceae (*R. salina* CCMP1319), Raphidophyceae (*F. japonica* Fibro 1 and *C. marina* ChatM1), Prymnesiophyceae (*P. globosa*) and the diatoms *P. multiseries* were not vitamin B₇ auxotrophs (Table 1). Three auxotrophs of biotin, *G. aureolum* KA6, *K. veneficum* FR6, and *P. hartmannii* FR3, ceased growth at relatively high estimated biotin levels (1.4–34

pM; Table 1), whereas the other nine biotin auxotrophs survived substantially lower concentrations of biotin (<0.01 pM; Table 1). Twelve of 41 strains required all three vitamins (*G. aureolum* KA6, *G. instriatum* L3, *K. brevis* CCMP2228, *K. mikimotoi* ISO6, *K. veneficum* FR6, *P. hartmannii* FR3 and FR4, *H. arctica* MS5, *S. trochoidea* MS1, *P. minimum* CCMP696 and PB3, and *P. pungens* PPJNU; Table 1). Nineteen strains required vitamins B₁ and B₁₂ but not B₇ (*A. sanguinea* AS2, *G. aureolum* KA1, KA2, and KA7, *G. instriatum* L2, L4, and L6, *P. donghaiense*, *T. acrotricha* CCMP2960, *C. polykrikoides* CP1, *S. trochoidea* CCPO3 and CCPO4, *Heterocapsa* sp. AT1, *A. anophagefferens* CCMP1984 and CCMP1707, *A. lagunensis* CCMP1681, *R. salina* CCMP1319, *C. marina* ChatM1, *P. globosa*; Table 1). Eight strains required vitamin B₁₂ only (*H. triquetra* HT1, *S. trochoidea* MS3, *A. minutum* CCMP113, *A. catenella* ACJNU, *S. microadriaticum* CCMP827,

F. japonica Fibro1, and the two strains of *P. multiseriis*; Table 1). *S. microadriaticum* CCMP829 and *Symbiodinium* sp. SJNU required none of the three vitamins. No strain demonstrated auxotrophy for B₁ or B₇ only, or B₁ and B₇ but not B₁₂.

Quantitative Assessment of Vitamin B₁, B₇, and B₁₂ Requirements. The final cell yields of six phytoplankton species, *A. anophagefferens* CCMP1984, *K. mikimotoi* ISO6, *S. trochoidea* MS1, *C. marina* Chatt1, *F. japonica* Fibro1, and *R. salina* CCMP1319 at different initial concentrations (0–740 pM) of vitamin B₁₂ confirmed their vitamin auxotrophy (Fig. S1). Each level of B₁₂ yielded significantly higher cell yields than lower concentrations for each species (ANOVA, $P < 0.05$), although concentrations required to saturate yields varied: 74 pM for *A. anophagefferens* (Fig. S1A), *K. mikimotoi* (Fig. S1B), and *R. salina* (Fig. S1F), 15 pM for *C. marina* (Fig. S1D), 7.4 pM for *S. trochoidea* MS1 (Fig. S1C), *F. japonica* (Fig. S1E), *P. minimum* PB3 (Fig. S1G), and *G. instriatum* L3 (Fig. S1H).

Vitamin B₁₂ half-saturation constants (K_s) for maximal growth rates varied considerably among algal species (Table 2). The dinoflagellate *K. mikimotoi* exhibited the largest B₁₂ half-saturation constant of any of the species surveyed (13.1 pM; Table 2) as well as high content of B₁₂ per unit biovolume of biomass (3.31×10^{-1} pmol- μL^{-1} ; Table 2), followed by the pelagophyte *A. anophagefferens* (3.49 pM and 5.89×10^{-1} pmol- μL^{-1} , respectively; Table 2), the cryptophyte *R. salina* (0.36 pM and 2.04×10^{-3} pmol- μL^{-1} , respectively; Table 2), and the raphidophytes *F. japonica* (0.28 pM and 4.10×10^{-3} pmol- μL^{-1} , respectively; Table 2) and *C. marina* (0.19 pM and 4.61×10^{-4} pmol- μL^{-1} , respectively; Table 2). The K_s for *P. minimum* was lower (0.02 pM; Table 2), thus the lower content of B₁₂ per unit biovolume of biomass (3.46×10^{-4} pmol- μL^{-1} ; Table 2).

Cell yields of the dinoflagellate *G. aureolum* strain KA6 and *R. salina* were both strongly dependent on vitamin B₁ (ANOVA, $P < 0.05$ (Fig. S2 A and B), and did not grow at levels below ~4 and 12 nM, respectively (Table 1). The half-saturation concentrations for maximal growth rates and cellular contents of vitamin B₁ for the cultures of *A. anophagefferens* CCMP1984, *P. minimum* CCMP696, *S. trochoidea* MS1, *G. aureolum* KA6, and *R. salina* CCMP1319 varied but demonstrated that vitamin B₁ is required at much higher concentrations than vitamin B₁₂ (Table 2). The culture *R. salina* had a K_s of 184 pM and a cellular content of B₁ of 3.43 pmol- μL^{-1} , followed by *S. trochoidea* (131 pM and 2.85×10^{-1} pmol- μL^{-1} biomass, respectively), *G. aur-*

eolum (96.9 pM and 5.65 pmol- μL^{-1}), *P. minimum* (86.3 pM and 5.45×10^{-1} pmol- μL^{-1}), and *A. anophagefferens* (5.94 pM and 1.16 pmol- μL^{-1} ; Table 2). The cellular quotas of B₁ per unit biomass among the five species investigated were relatively similar (0.285–5.65 pmol- μL^{-1} ; Table 2).

The cellular yields of both strains of *P. minimum* (CCMP696: New York strain; PB3: Singapore strain) were strongly dependent on vitamin B₇ (ANOVA, $P < 0.05$; Fig. S2) although the two strains differed in their quantitative requirements: CCMP696 reached its maximum yield at 82 pM (Fig. S2C) but the growth of PB3 was further slightly enhanced when the concentration of B₇ was 820 pM (Fig. S2D). The dinoflagellate *G. instriatum* had the highest K_s and cellular content of vitamin B₇ per unit of biovolume among the three species quantitatively investigated for B₇ auxotrophy (0.28 pM and 2.42×10^{-2} pmol- μL^{-1} , respectively; Table 2), whereas the two strains of *P. minimum* had very similar K_s and cellular contents of B₇ (0.09 pM and 9.76×10^{-4} pmol- μL^{-1} for CCMP696 and 0.06 pM and 1.60×10^{-3} pmol- μL^{-1} for PB3, respectively; Table 2).

Discussion

Most HAB Species and Dinoflagellates Require Vitamins. Almost all HAB species/strains (96/95%) investigated in the current study were shown to be auxotrophs of vitamins B₁₂, whereas 74% and 37% of all species were observed to be auxotrophs of vitamins B₁ and B₇, respectively (Table 1), much higher percentages than those summarized by Croft et al. (14) for all phytoplankton species (52%). The auxotrophic status of nearly all of these species has not previously been reported. Among the 45 species of dinoflagellates investigated during this and prior studies, the numbers of auxotrophs for vitamins B₁₂, B₁, and B₇ are now 41 (91%), 22 (49%), and 17 (38%), respectively (Table 3), increasing from 24 (86%), 7 (25%), and 7 (25%), respectively reported in Croft et al. (14). The commonality of vitamin auxotrophy among dinoflagellates is consistent with the well-known osmotrophic abilities and mixotrophic tendencies displayed by these phytoplankton (1, 3, 45, 46), suggesting that vitamins are among a suite of organic compounds that dinoflagellates exploit for nutrition. Because dinoflagellates are notorious for their ability to form HABs (e.g., most of the species examined in the study), this study suggests that vitamins are key organic compounds that may influence the occurrence of HABs of dinoflagellates. This study also reports on vitamin auxotrophy in four species of ochrophyta (two pelagophytes and two raphidophytes)

Table 2. Half-saturation constants of growth rates (K_s) and cellular vitamin quotas expressed per unit biovolume and per cell for representative species of microalgae for vitamins B₁, B₇, and B₁₂

Vitamin	Strain	K_s (pM)	R	pmol- μL Biomass ⁻¹	pmol-Cell ⁻¹
B₁₂					
<i>A. anophagefferens</i>	CCMP1984	3.49 ± 0.75	0.95	5.89×10^{-1}	3.25×10^{-9}
<i>P. minimum</i>	PB3	0.02 ± 0.01	0.97	3.46×10^{-4}	3.98×10^{-10}
<i>K. mikimotoi</i>	ISO6	13.1 ± 1.11	0.98	3.31×10^{-1}	1.66×10^{-6}
<i>C. marina</i>	Chatt1	0.19 ± 0.11	0.55	4.61×10^{-4}	3.00×10^{-9}
<i>F. japonica</i>	Fibro1	0.28 ± 0.02	0.71	4.10×10^{-3}	1.64×10^{-8}
<i>R. salina</i>	CCMP1319	0.36 ± 0.02	0.93	2.04×10^{-3}	1.79×10^{-10}
B₁					
<i>A. anophagefferens</i>	CCMP1984	5.94 ± 1.36	0.71	1.16	6.52×10^{-9}
<i>P. minimum</i>	CCMP696	86.3 ± 3.76	0.99	0.545	6.27×10^{-7}
<i>S. trochoidea</i>	MS1	131 ± 30.2	0.85	0.285	2.41×10^{-6}
<i>G. aureolum</i>	KA6	96.9 ± 4.01	0.74	5.65	1.94×10^{-5}
<i>R. salina</i>	CCMP1319	184 ± 81.5	0.80	3.43	3.02×10^{-7}
B₇					
<i>P. minimum</i>	CCMP696	0.09 ± 0.02	0.82	9.76×10^{-4}	1.12×10^{-9}
<i>P. minimum</i>	PB3	0.06 ± 0.01	0.93	1.60×10^{-3}	1.84×10^{-9}
<i>G. instriatum</i>	L3	0.28 ± 0.08	0.79	2.42×10^{-2}	3.19×10^{-7}

Correlation coefficient (R) represents fit of growth rate versus vitamin concentration data to the Michaelis-Menten model. All models presented were statistically significant.

Table 3. Number and percentage of species requiring vitamins from different phylum of algae combining the data of this study and prior studies (5, 10, 14, 15, 30–44)

Phylum	No. of species surveyed	No. (%) of species requiring:		
		Cobalamin	Thiamine	Biotin
Chlorophyta	148	44 (30%)	19 (13%)	0
Rhodophyta	13	12 (92%)	0	0
Cryptophyta	7	6 (86%)	6 (86%)	1 (14%)
Dinophyta	45	41 (91%)	22 (49%)	17 (38%)
Euglenophyta	15	13 (93%)	11 (73%)	1 (7%)
Haptophyta	18	11 (61%)	15 (83%)	0
Heterokontophyta	82	49 (60%)	12 (15%)	6 (7%)
Ochrophyta	4	4 (100%)	3 (75%)	0
Total	332	180 (54%)	88 (27%)	25 (8%)

Updated numbers are in boldface type.

that all required B₁₂ and all but *Fibrocapsa japonica* strain Fibro 1 were auxotrophic for vitamin B₁. Finally, all three *Pseudonitzschia* species and strains were B₁₂ auxotrophs and *Pseudonitzschia pungens* strain PPJNU was auxotrophic for all three vitamins.

Among the 19 species of dinoflagellates investigated in this study, four (*G. aureolum*, *G. instriatum*, *S. trochoidea* and *S. microadriaticum*) displayed differential auxotrophy among strains. Also, our results for a strain of *Phaeocystis globosa* that was isolated from the South China Sea differed from the two strains described by Peperzak et al. (47): The South China Sea strain required vitamins B₁ and B₁₂, whereas the strains described by Peperzak et al. (47) required B₁ only. Similar intraspecific differences in B₁ and B₁₂ auxotrophy were reported by Hargraves and Guillard (48) for *Bellerochea spinifera* (B₁) and *Fragilaria pinnata* (B₁₂). This demonstrates that intraspecific differences in vitamin auxotrophy among strains are not rare and can exist even within strains isolated from the same estuary (e.g., *S. trochoidea* MS1 and MS3). The two strains of *P. hartmannii*, FR3 and FR4, and the two strains of *A. anophagefferens*, CCMP1984 and CCMP1707, however, displayed consistent patterns of auxotrophy among strains for each vitamin. Regarding *Symbiodinium*, some species within this genus form HABs (*SI Text*) while others live endosymbiotically within coral (49). The general lack of auxotrophy within this genus reported here (*S. microadriaticum* CCMP827 required B₁₂ only; all other strains did not require vitamins; Table 1) and by Provasoli and Carlucci (a *S. microadriaticum* strain required none of the three B-vitamins) (10) suggests that high levels of vitamins are not typically present in the symbiotic space *Symbiodinium* occupies within coral, an organism that is incapable of synthesizing vitamins (14).

HAB Species Require Large Quantities of Vitamins. Our quantitative results demonstrate that vitamins B₁ and B₁₂ can limit the accumulation of microalgal biomass over a wide range of concentrations, and that the limiting concentrations for some species in culture are higher than levels present in some coastal waters. For example, the growth of the toxic dinoflagellate *Karenia mikimotoi* and the “brown tide” species *Aureococcus anophagefferens* displayed half-saturation constants of 13.1 and 3.49 pM, respectively, for vitamin B₁₂ (Table 2), which are similar to concentrations typically found in coastal systems (0.5–20 pM) (18, 22). In the case of *A. anophagefferens*, the genomic presence of a vitamin B₁₂-dependent methionine synthase (*metH*) and absence of the vitamin B₁₂-independent methionine synthase (*metE*) confirms its absolute requirement of this vitamin (15).

The frequency of vitamin B₁ auxotrophy among HAB species studied was high. The K_s values of B₁ for *Scrippsiella trochoidea*, *Prorocentrum minimum*, and *Gymnodinium aureolum* (131, 86.3, and 96.9 pM, respectively; Table 2) were similar to and, in some cases, higher than concentrations recorded in coastal ecosystems [e.g., 23.7–44.5 pM in Provasoli and Carlucci (10); 9–190 pM in Gobler et al. (22)], and these K_s values were one to four orders of

magnitude higher than K_s values for B₁₂ (this study). Although the importance of vitamin B₁ to phytoplankton has been infrequently studied compared with B₁₂, both the qualitative and quantitative data presented here suggests that, for those species that are auxotrophs of both vitamins B₁ and B₁₂, the former would be more likely to be limiting at equimolar concentrations. Typically, however, vitamin B₁ is present in coastal waters at concentrations greater than is B₁₂ (22), a scenario that may have evolutionarily facilitated the larger demand for this vitamin.

A smaller proportion of HAB species were vitamin B₇ auxotrophs (10 in 27 species and 12 in 41 strains) and the concentrations required were generally lower than vitamin B₁ and B₁₂ with K_s values ranging from 0.06 to 0.28 pM (Table 2). Importantly, dinoflagellates have, by far, the highest frequency of vitamin B₇ auxotrophy among all classes of microalgae (38% of 45 species surveyed) and represent two-thirds of all known vitamin B₇ auxotrophs (Table 3). Because dinoflagellates are distinct in their vitamin B₇ auxotrophy, this vitamin is more likely than any other to exert selective pressure exclusively on this class of phytoplankton.

In productive coastal waters, the occurrence of high biomass HABs with large vitamin demands could drive such ecosystems into vitamin limitation even in areas where vitamins concentrations have been measured at relatively high concentrations (18, 22). For example, assuming modest growth rates (doubling per day), moderate blooms of *Karenia mikimotoi* (5×10^3 cells·mL⁻¹ (50)), *Prorocentrum minimum* (1×10^4 cells·mL⁻¹) (51), *Scrippsiella trochoidea* (4×10^3 cells·mL⁻¹) (52), and *Gymnodinium aureolum* (5×10^3 cells·mL⁻¹) (53) with vitamin quotas as described in Table 2 would display vitamin assimilation rates of 8.3 pM B₁₂·d⁻¹ (*K. mikimotoi*), 6.3 pM B₁·d⁻¹ (*P. minimum*), 9.6 pM B₁·d⁻¹ (*S. trochoidea*), and 97 pM B₁·d⁻¹ (*G. aureolum*), respectively. Such rates could deplete standing stock of vitamins found in coastal waters (0.5–20 pM vitamin B₁₂; 9–190 pM vitamin B₁) (18, 22) within hours to days (Table 2). Given previously published N quotas for the HAB species of *A. anophagefferens* (54), *K. mikimotoi* (55), *P. minimum* (56), and *S. trochoidea* (57), concurrent daily N demands would exhaust typical inorganic nitrogen pools, but not total dissolved N pools (22). This suggests that mixotrophic HABs that access both organic and inorganic forms of N could deplete similar proportions of dissolved nitrogen and vitamin pools during bloom events. Collectively, these observations are consistent with the occurrence of N and vitamin B₁₂ colimitation in productive coastal waters (21) and during HABs (22). Given the frequent occurrence of HABs, processes such as algal/bacterial symbiosis (15), delivery from external sources (e.g., benthic fluxes, terrestrial run-off) (22), regeneration from microbial processes, and/or vitamin assimilation via phagotrophy must be crucial processes for maintaining vitamin replete conditions for HABs. Although dinoflagellates are well known phagotrophs (58, 59), the stability and subsequent bioavailability of large, complex macromolecules such as vitamins following the digestion of algal prey is unknown.

Ecological Significance of Vitamins in HAB Dynamics. The results obtained in this study suggest that the auxotrophic vitamin requirements of most HAB species are substantial, and that previous studies underestimated the proportion of dinoflagellates and HAB species requiring vitamins (14, 15). Many field observations support the hypothesis that vitamins can have important ecological relevance for HABs. For example, blooms of the auxotrophic dinoflagellate *Lingulodinium polyedrum* in waters off the coast of California have been correlated with a disappearance of dissolved vitamin B₁₂ in the water column (23). In Japan, the growth rates of the dinoflagellates *Gymnodinium* sp (60), and *Prorocentrum micans* (61) during blooms have been found to be significantly (e.g., up to 15-fold) stimulated by additions of vitamins B₁ or B₁₂ in combination with N, P, and/or Fe. In China, nutrient addition experiments using mesocosms revealed that additions of vitamin B₁, or B₁₂, or a combination of B₁ and B₁₂ significantly increased the growth of dinoflagellates (62) and *P. micans* (28). Previous studies have also suggested a sequential link between HABs and vitamins

where initial blooms of *Skeletonema costatum* supply cobalt, vitamin B₁₂, or both to subsequent blooms of *Chrysochromulina poly-lepis* (63). It was suggested 40 y ago that blooms of the toxic, vitamin B₁₂-auxotrophic dinoflagellate, *Karenia brevis* in the Gulf of Mexico were caused by the delivery of vitamins (24–27). Recent oceanographic studies have documented the off-shore initiation of these blooms (64) putatively due to the delivery of excess N production by the diazotrophic cyanophyte *Trichodesmium* (65). As more recent studies indicate that the *K. brevis* blooms obtain N from other sources (66), *Trichodesmium* may be supplying blooms with other compounds such as vitamins.

The analytical breakthrough of direct measurements of vitamins B₁ and B₁₂ (16, 17) coupled with the monitoring of phytoplankton dynamics in coastal waters has demonstrated a strong covariation of vitamin B₁₂ and the biomass of large (>5 μm) phytoplankton (21, 22). Consistent with the large demand of HABs, concentrations of vitamin B₁ and B₁₂ have been shown to be drawn down by 90% to limiting levels by blooms of *C. polykrikoides*, *K. veneficum*, and *P. minimum* (22), all vitamin B₁ and B₁₂ auxotrophs (Table 1). The sum of these field observations and field-based amendment experiments (22, 28, 60–62) unambiguously demonstrate the ecological importance of B₁ and B₁₂ on phytoplankton community dynamics. The results of the present study together with previous field work suggest vitamins may, like macronutrients such as nitrogen, play a key role in the occurrence of HABs.

Materials and Methods

Cultures. The microalgae investigated in the present study were primarily HAB species and included 19 species (30 strains) of dinoflagellates, two species (three strains) of pelagophytes (*Aureococcus anophagefferens* and *Aureocumbra lagunensis*), two species of raphidophytes (*Chattonella marina* and *Fibrocapsa japonica*), two species (three strains) of bacillariophyceae (*Pseudo-Nitzschia pungens* and *P. multiseriata*), one species each of cryptophyte (*Rhodomonas salina*) and prymnesiophyceae (*Phaeocystis globosa*). In cases in which strains were not obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP; West Boothbay Harbor, ME), species identifications were made by PCR amplification of large-subunit ribosomal DNA, sequencing, and alignment with GenBank sequences (SI Text). All species names, strain numbers, and origins are listed in Table 1. Cultures were maintained in GSe medium (G medium supplemented with 1×10^{-8} M selenium) (67), made with autoclaved and sterile filtered (0.22 μm) artificial seawater with a salinity of 32–33 PSU that was prepared with Sea Salts (Sigma Chemicals). Cultures were maintained at 21 °C in an incubator with a 12:12-h light:dark cycle, illuminated by a bank of fluorescent lights that provided a light intensity of $\sim 100 \mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ to cultures.

Qualitative Tests. To qualitatively establish whether the microalgae required vitamins, four types of GSe media made from artificial seawater (ASW GSe) were prepared: full strength of ASW GSe containing 2.97×10^6 pM B₁ (thiamine hydrochloride; ACROS Organics), 8.19×10^3 pM B₇ (biotin; ACROS Organics), and 7.38×10^2 pM B₁₂ (cobalamin; MP Biomedicals); ASW GSe minus B₁; ASW GSe minus B₇; and ASW GSe minus B₁₂. Cultures were initially

grown in cell culture well plates (Corning) under conditions described above with an antibiotic-antimycotic solution (a mixture of 10,000 IU penicillin, 10,000 μg·mL⁻¹ streptomycin, and 25 μg·mL⁻¹ amphotericin B; Mediatech) added to a final concentration of 1–2% to discourage contamination by bacteria and fungi that can synthesize vitamins (15). Triplicate wells were inoculated with cultures and medium and maintained as described above. Cell densities of cultures were monitored microscopically and cultures were transferred into fresh media with antibiotics-antimycotics solution once stationary growth stage was reached, typically within less than 2 weeks. Cultures were continually transferred until culture growth ceased in one of the media treatments (Table 1) or after cultures were transferred 40 times without each vitamin. Auxotrophy for a vitamin was declared when the following occurred: (i) A culture ceased to grow in the absence of a vitamin whereas growth in parallel control treatments with the vitamin persisted; and (ii) growth within the putatively vitamin limited cultures resumed upon the addition of the limiting vitamin.

Establishing Vitamin-Dependent Growth Rates, K_s, and Vitamin Cellular Quotas.

To clarify the ecological relevance of vitamin auxotrophy, the quantitative effects of vitamins on the growth rates and cell yields of multiple microalgal strains were investigated. Historically, determination of vitamin-dependent growth rates and half-saturation constants (K_s) within phytoplankton cultures has been challenging. Batch culture-derived K_s and growth rates can lead to overestimates of K_s if there is significant depletion of vitamin concentrations or if large volumes of culture inoculum are used (68). Although continuous cultures avoid these issues, this approach has been often compromised by the accumulation of vitamin B₁₂-binding proteins that inhibit B₁₂ availability and thus prohibit accurate determination of vitamin-dependent growth rates and K_s values (68). Moreover, many dinoflagellates are highly sensitive to turbulence (1, 69) and thus often cannot be continuously cultured. Given these collective shortcomings, batch cultures were used for this study. To guard against problem inherent in such an approach, inoculum volumes of all quantitative assays were <2% of the total volumes and hence did not alter total vitamin concentrations or affect K_s determinations. In addition, growth rate data were always collected during the earliest stages of exponential growth to minimize vitamin depletion. Finally, we also present cell yields for the study because (i) HABs are known to form dense blooms despite their low growth rates (1, 2, 3), (ii) the harmful effects of most HABs are typically proportional to their cell densities (70), and (iii) the succession of bloom events is often framed in terms of cell densities (1, 2, 3). Further details regarding the experimental procedures, calculations for cellular growth rates, half-saturation constants (K_s), and cell quotas of vitamins can be found in the SI Text.

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