

Direct determination of vitamin B₁ in seawater by solid-phase extraction and high-performance liquid chromatography quantification

Mussie Okbamichael* and Sergio A. Sañudo-Wilhelmy

Marine Sciences Research Center, Stony Brook University, Stony Brook, NY 11794-5000, USA

Abstract

Vitamin B₁ (thiamine) is one of the organic micronutrients essential for growth of algae. However, there is no direct method for the quantification of this vitamin in natural waters. Bioassay is the only protocol currently available to measure B₁ in the marine environment. We have developed a new method for the direct determination of B₁, along with vitamin B₁₂ (cobalamin) in seawater. Solid-phase extraction with a column of C₁₈ resin followed by elution with methanol was used to extract dissolved B₁ from seawater. The eluted vitamin was then analyzed by reversed phase high-performance liquid chromatography (HPLC) using ammonium acetate (0.05 M) and methanol as mobile phases in pressure gradient mode. Ultraviolet-visible (UV-Vis) detection at 270 nm was used to quantify B₁ levels. The calibration curve was linear from 0 to 0.60 μM and the detection limit of the protocol was on the order of 0.083 pM. This new method was successfully applied to the determination of vitamin B₁ in filtered coastal seawater, porewater, and river water samples. Concentrations of vitamin B₁ in those aquatic environments ranged from 0.15 nM in river to 0.75 nM in porewaters, with spike recoveries ranging from 93% to 98%.

Along with light, temperature, and nutrient availability (e.g., N, P, Si, Fe, Zn, Co), phytoplankton growth in the marine environment depends on the presence of vitamins (e.g., Provasoli 1963). Those essential vitamins include vitamin B₁₂ (cobalamin), vitamin B₁ (thiamine), and biotin. Vitamins B₁ and B₁₂ appear to be required alone or in combination by most algal species (Provasoli 1963). Among the four phytoplankton functional groups that dominate the global ocean (diatoms, coccolithophores, chlorophytes, and cyanobacteria; Gregg et al. 2003), cyanobacteria require only vitamin B₁₂, while diatoms and chlorophytes may also require B₁ for optimum growth (Provasoli and Carlucci 1974). Coccolithophores such as *Coccolithus huxleyi* produce vitamin B₁₂ but require B₁ (Carlucci and Bowes 1970; Turner 1979). The different vitamin requirements of the distinct phytoplankton groups suggest that species composition and distribution in the world ocean may be influenced by the bioavailability of these organic compounds.

While extensive research on the organic regulation of phytoplankton fertility was carried out between the 1960s and the early 1980s (e.g., Vishniac and Riley 1961; Provasoli 1963;

Natarajan and Dugdale 1966; Gold et al. 1966; Natarajan 1968, 1970; Carlucci and Silbernagel 1969; Carlucci and Bowes 1970; Ohwada and Taga 1969, 1972; Carlucci and Cuhel 1975; Swift 1981), no recent study has experimentally addressed the importance of vitamins in the world ocean. The lack of a simple method for the direct determination of vitamins in seawater has hindered our understanding of the ecological role of these compounds in the marine environment. Although several methods for vitamin separation and quantification in pharmaceuticals, food, and biological fluids have been published (e.g., Ivanovic et al. 1999; Moreno and Salvado 2000; Markopoulo et al. 2002; Lynch and Young 2002), vitamin determinations in seawater have been carried out only by an indirect, time-consuming microbial assay (Carlucci 1966). In this paper, we describe a new direct method for determining vitamin B₁ levels in seawater. This protocol is similar to the vitamin B₁₂ method that we have recently developed (Okbamichael and Sañudo-Wilhelmy 2004). In fact, the present protocol allows for the simultaneous measurements of vitamins B₁ and B₁₂ in the same water sample.

Materials and procedures

Equipment and reagents—A high-performance liquid chromatography (HPLC) (Shimadzu Corporation) equipped with two pumps (LC-10AD), a degassing unit (DGU-14A), an ultraviolet-visible (UV-Vis) spectrometer (SPD-10AV), an auto-sampler (SIL-10A), along with a class VP program were used to record chro-

*Phone: (631) 632-8615. Fax: (631) 632-8820. E-mail address: mokbamic@ic.sunysb.edu

Acknowledgments

This research was fully supported by the National Science Foundation (OCE-0351999).

matographs and calculate peak heights of vitamin B₁. The HPLC column used was a reversed-phase Premier C₁₈ (150 × 4.6 mm, 5 μm) from Shimadzu. High-purity water obtained through a Milli-Q purification system was used in all procedures. HPLC grade methanol and ammonium acetate were obtained from Fisher Scientific. Vitamin B₁ standard was obtained from Alexis Biochemicals. Polypropylene columns with porous polyethylene support used in the solid-phase extractions were available from Bio-Rad Lab and the 17% High Capacity C₁₈ resin (HF Bondesil; 120 μm) was obtained from Varian Inc.

Solid-phase extraction protocol—Vitamin B₁ was extracted from seawater following the procedures developed for the solid-phase extraction of vitamin B₁₂ (Okbamichael and Sañudo-Wilhelmy 2004). A preconcentration system was set up using a peristaltic pump with rotor heads (Cole-Parmer, Masterflex, Model 7553-12, 1-100 rpm), polypropylene preconcentration columns and Teflon tubing that extended from the head of the column into the bottom of a 2 L polycarbonate bottle reservoir. The set-up was designed to process four samples simultaneously, using four peristaltic heads per pump. About 5 mL of the C₁₈ resin was cleaned and conditioned by immersing it in 10 mL methanol (100%) that formed a cloudy suspension (slurry). The slurry of C₁₈ resin was loaded into the column, left standing for about 1 h to elute the methanol and then washed with 20 mL of Milli-Q water. The pH of the filtered (<0.2 μm) seawater samples to be analyzed for B₁ was adjusted to values between 5 to 8 by adding appropriate amounts of HCl (1 M). The sample was passed through the resin at various flow rates of 1 to 5 mL min⁻¹. The resin was then rinsed with 20 mL of Milli-Q water to eliminate any salt. Sample elution was carried out by passing three to five aliquots of 5 mL methanol each through the resin in the column. Each 5-mL aliquot of eluted vitamin was collected in a separate beaker that was pre-washed with methanol and rinsed with Milli-Q water.

HPLC determination of vitamin B₁—To eliminate any interference of hydrophobic organic compounds that were co-eluted with the methanol, a 1-mL aliquot of each 5-mL eluent was transferred into a 1.5 mL HPLC vial and evaporated by purging with nitrogen. The residue was redissolved in 1 mL Milli-Q water for HPLC quantification. The detection of B₁ was carried out using reversed phase HPLC, following the chromatographic conditions described for vitamin B₁₂ determinations (Okbamichael and Sañudo-Wilhelmy 2004). The mobile phase of the HPLC system consisted of 0.05 M ammonium acetate (solvent A) and 100% methanol (solvent B). A pressure gradient mode with proportions of A:B equal to 92.5:7.5 in the range 0 to 1.5 min, 84:16 at 1.6 min, and 70:30 at 15 min was used during the analysis of B₁. The total flow rate of the mobile phase was 1 mL min⁻¹ and the sample volume injected was 40 μL. Vitamin B₁ was detected at 270 nm using the UV-Vis detector. Purging of the line and auto-sampler was carried out using a 1:1 mixture of Milli-Q water and HPLC grade methanol at the end of sample analyses each day. The HPLC column and flow cell of the UV-Vis detector were also cleaned with Milli-Q and methanol for about 15 min each, biweekly.

Sample collections—Once the optimum conditions for maximum recovery were established, the method was applied to the determination of vitamin B₁ in filtered coastal seawater, porewater, and river water samples. Seawater samples were collected in January and April 2004 from the tidal channel connecting Stony Brook Harbor with Long Island Sound. Porewaters and surface sediment (the top 1 cm) were obtained from Flax Pond, New York, in April 2004. River samples were collected in the Peconic River in April and June 2004. Additional samples were also collected from 7 sites along a salinity gradient in the Peconic River estuary in June 2004. All of the sampling locations are located in Long Island, New York.

The samples were filtered with acid-washed polypropylene filters (0.2 μm) and collected in acid-washed, dark 2-L polyethylene bottles. Replicate or triplicate samples from these sites were analyzed with and without spiking with known amounts of B₁. Water samples were also extracted, dried by purging with nitrogen, stored frozen in the dark, and analyzed after 60 d to evaluate the effect of storage on vitamin levels.

Assessment

Method development—Vitamin B₁ was detected at an HPLC retention time of about 4 min (Fig. 1A), with minor variations on daily basis because of temperature fluctuations in the laboratory (Moreno and Salvado 2000). The B₁ peak in the chromatograph was confirmed by spiking filtered seawater, which contained 0.18 nM of the vitamin, with different levels of B₁ (0.074, 0.15, and 0.30 nM). As shown in Fig. 1B-D, the retention time of B₁ was the same in all of the spiked samples, and the signal was proportional to the amount of B₁ in each sample. Although these results strongly suggest that the peak detected at 4 min is vitamin B₁, additional experiments using other independent analytical approaches (e.g., LC-MS) are needed to definitively confirm the identity of that peak. The calibration curve for B₁ was linear within the concentration range of 0 to 0.60 μM (Fig. 2). This concentration range is within the same order of magnitude of the samples measured in this study, after preconcentration onto the C₁₈ resin. The detection limit (defined as 3 times the standard deviation of the procedural blank) was about 0.083 pM. The reproducibility of the analysis was excellent, as the coefficient of variation for triplicate measurements was less than 3%.

Although it is likely that other organic compounds are being co-eluted with B₁ during the solid-phase extraction, any interference from those unknown compounds was minimized by (1) purging the methanol and redissolving the residue in Milli-Q water to remove any interferences from hydrophobic compounds such as peptides and aromatics (Jeffrey et al. 1999), (2) selecting a specific wavelength, 270 nm for B₁, that precludes any interference from micro-algal pigments and CDOM that absorb between 280-380 nm (Jeffrey et al. 1999), and (3) increasing the specificity for B₁ during the gradient mode of the HPLC detection (Moreno and Salvado 2000).

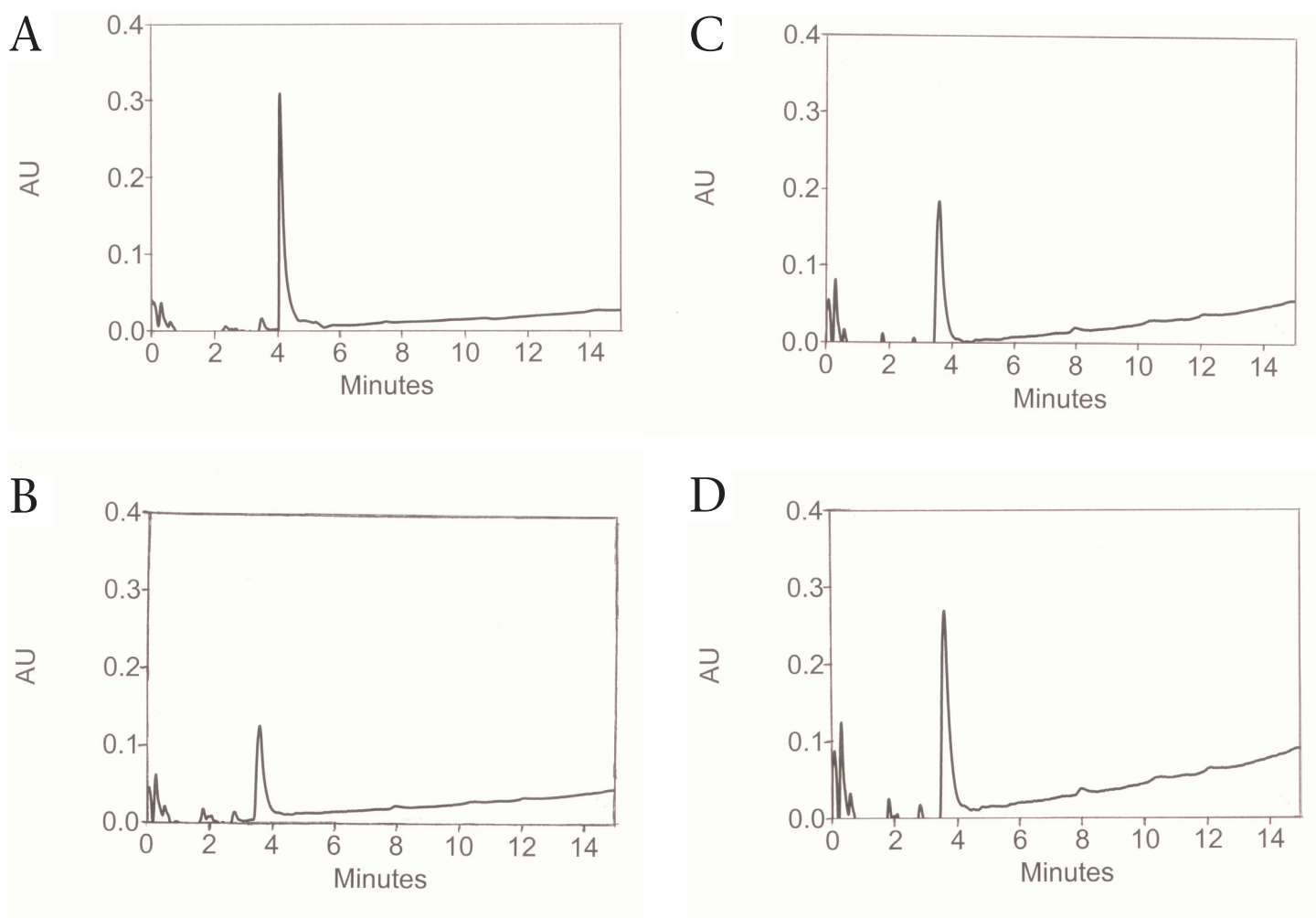


Fig. 1. HPLC chromatograms of vitamin B₁ ($\lambda = 270$ nm; retention time ~ 4 min). (A) B₁ standard in Milli-Q water (0.59 nM) and (B-D) in seawater containing 0.18 nM of the vitamin and spiked with different levels of B₁ (0.074, 0.15, and 0.30 nM, respectively). As shown in Fig. 1B-D, the retention time remained the same for all the spiked samples and the instrument's signal was linear with respect to concentration (AU = 0.14, 0.18, and 0.27 for B₁ levels of 0.25, 0.33, and 0.47 nM, respectively). As confirmed by analyzing seawater devoid of B₁ (rendered vitamin free by passing it through the C₁₈ resin), the baseline drift observed in the chromatograms was caused by seawater background absorbance and, hence, it was accounted for in peak height estimations.

During the solid phase extraction, the maximum recovery of B₁ (96% to 98%) was obtained within a pH range between 6.1 and 6.7, and a sample flow rate of 1 mL min⁻¹. The effect of the pH on the recovery of B₁ may be attributed to solvent-exchange reactions in the seawater sample, the C₁₈ resin, and the methanol, as pH affects the ionic strength of the medium (Stumm and Morgan 1996) and the surface characteristics of the C₁₈ resin. The effect of the flow rate on the extraction yield suggests that the lower the flow rate, the longer the contact time that is available for the exchange reaction between the vitamin and the resin. More than 99% of B₁ was recovered with the first three 5-mL aliquots of methanol during the elution of the solid-phase extraction.

As there is no standard reference material for vitamin B₁ in seawater, spike experiments were carried out to test the recovery

of the current method. Different concentrations of B₁ (0.074, 0.15, and 0.30 nM) were added to filtered seawater samples with known initial B₁ levels from Stony Brook Harbor channel and the Peconic River. The recovery of B₁ in those spiked samples ranged from 93% to 98% (Table 1). Furthermore, a 96% \pm 1% recovery was obtained in dried methanol aliquots stored frozen for 2 mo and redissolved in 1 mL of Milli-Q water, whereas only 76% \pm 2% was recovered when the dried aliquots were stored refrigerated (Table 1). This is crucial for the measurement of B₁ in the open ocean, where samples are collected during oceanographic cruises that may last several months, and where it is impractical to store large numbers of frozen samples. Because B₁ is stable from pH 2 to 4 (Kawasaki and Sanemori 1985), samples may be preserved by acidification. However, further studies need to evaluate that preservation option.

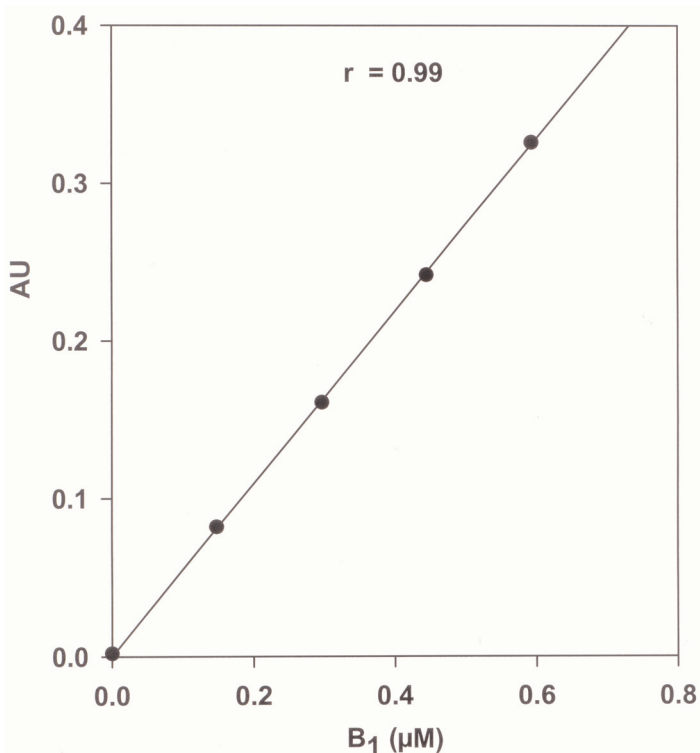


Fig. 2. Calibration curve for vitamin B₁. Linearity was established using the least squares method in standard concentration ranges of 0 to 0.60 μM.

The general protocol for the analysis of B₁ in seawater can be summarized as follows: collection of filtered water, pH adjustment of the water sample to be analyzed (between 6.1 and 6.7), loading of the sample onto 5 mL C₁₈ resin at a flow rate of 1 mL min⁻¹, rinsing of the C₁₈ resin before sample elution with 20 mL of Milli-Q water, B₁ elution with three aliquots of 5 mL methanol each, volatilizing 1 mL of each 5 mL eluent to dryness with nitrogen, redissolving the B₁ extracted in 1 mL Milli-Q water, and HPLC detection at 270 nm. Determination of ambient B₁ at concentrations lower than those measured here could presumably be attained by preconcentrating higher volumes of seawater on the C₁₈ resin. Therefore, the preconcentration using solid phase extraction renders the method applicable to detect almost any levels of B₁ in natural waters. Additional concentration factors could also be attained by redissolving the dried methanol aliquots in less than 1 mL Milli-Q water, as only 40 μL is injected into the HPLC, or by drying more than 1 mL of the eluent. Similarly, lower sample volumes could be used in environments where vitamin levels are expected to be higher. For example, we measured 0.31 nM of B₁ in a 1-L sample collected from the Peconic River and 0.75 nM in 100 mL porewater extracted from Flax Pond, New York.

Application of the method to natural waters—Concentrations of vitamin B₁ in coastal and river water samples ranged from 0.23 to 0.31 nM (Table 1). The higher concentrations of B₁ were measured in samples from the Peconic River (0.31 ± 0.01 nM; mean ± 1 standard deviation) and seawater from Stony Brook

Table 1. Concentrations of vitamins B₁ and B₁₂ in surface waters collected in Stony Brook Harbor channel in January (SBHC-Jan) and April 2004 (SBHC-Apr), and in the Peconic River (PeconicR-Apr) in April 2004

Sample	B ₁ (nM)	B ₁₂ (nM)	Recovery (%)
SBHC-Jan	0.294 ± 0.009	0.048 ± 0.001	95-98
SBHC-Jan + 100	0.579 ± 0.010	0.119 ± 0.003	
SBHC-Apr	0.233 ± 0.005	0.050 ± 0.002	93-98
SBHC-Apr + 50	0.382 ± 0.010	0.084 ± 0.002	
PeconicR-Apr	0.308 ± 0.009	0.053 ± 0.001	93-97
PeconicR-Apr + 25	0.367 ± 0.004	0.071 ± 0.002	
Frozen	0.285 ± 0.004	0.073 ± 0.001	93-97
Refrigerated	0.227 ± 0.008	0.056 ± 0.002	74-78

They were spiked with 0.074 (PeconicR-Apr + 25), 0.15 (SBHC-Apr + 50), and 0.30 nM (SBHC-Jan + 100). The recovery of the simultaneous analysis of vitamins B₁ and B₁₂ in the spiked samples ranged from 93% to 98%. Included are concentration of vitamin B₁ and B₁₂ in seawater from Stony Brook Harbor channel analyzed after being dried and stored for 60 d refrigerated and frozen. The similar levels of B₁ and B₁₂ measured before and after freezing (93%-97%) indicated that water samples can be preserved by drying and freezing for 2 mo without significant losses of B₁ and B₁₂, whereas refrigeration yields lower recoveries (74%-78%).

Harbor channel collected in January 2004 (0.29 ± 0.01 nM). Lower levels were detected at the tidal channel of Stony Brook Harbor during April 2004 (0.23 ± 0.01 nM). These results were consistent with the characteristics of the sampling sites and the sampling time. For example, the high concentration of B₁ in the shallow (0.5 m), stagnant Peconic River can likely be attributed to the high bacterial activity observed in that environment (Breuer et al. 1999). This is consistent with the bacterial production of vitamin B₁ (Burkholder and Burkholder 1956; Provasoli 1963). Furthermore, our sediment resuspension experiments indicated that sediments are an important source of B₁ in shallow environments (see following section).

The concentration of vitamin B₁ measured at the channel of Stony Brook Harbor in January was higher than that measured in April (Table 1). This could be the result of the higher biological uptake of this vitamin by phytoplankton during the spring bloom. This temporal trend is consistent with decreasing vitamin B₁ concentrations from winter to spring reported for the Gulf of Maine (Swift 1981).

Stability of vitamin B₁ in seawater—We carried out a series of laboratory incubation experiments to establish the stability of B₁ in seawater, as this vitamin is unstable at alkaline pH (Jansen 1972). In these laboratory experiments, levels of B₁ were measured at the beginning of the experiment and at 6, 12, 24, and 48 h in seawater of slightly alkaline pH (7.5) collected at Stony Brook Channel and incubated at room temperature (Fig. 3). Those results showed that about 70% of the initial B₁ concentration was hydrolyzed within 6 h. By 12 h, most of the vitamin (90%) was decomposed. As the incubated seawater was not filtered, biological uptake of B₁ could not be

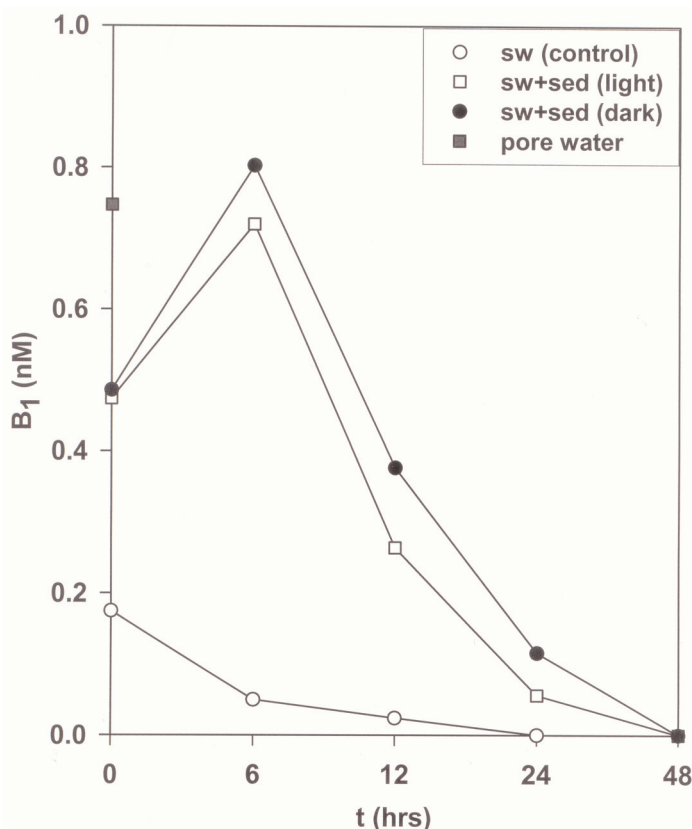


Fig. 3. Concentrations of B₁ in porewater, surface seawater, and sediment-overlying seawater under light and dark conditions. In each treatment (light and dark conditions), about 300 g of freshly collected sediments were added to polycarbonate containers filled with 0.2 μm filtered seawater and incubated for 48 h at room temperature. Sub-samples of 500 mL each were collected at the beginning of the experiment and at 6, 12, 24, and 48 h from each treatment. At each time of subsampling, sediments were resuspended by manual shaking and the water sample was filtered. The results of these experiments were compared against seawater controls. Porewater from the sediments used in the laboratory incubations was also extracted by centrifugation and analyzed for B₁ using our new protocol.

ruled out. However, these results are consistent with those reported by Gold et al. (1966) showing that about 80% of B₁ in seawater was destroyed within 12 h.

The lability of B₁ is also suggested by our laboratory results of seawater (pH ~7.8) spiked with Flax Pond sediments and incubated under light and dark conditions (Fig. 3). In those experiments, between 85% and 92% of the vitamin was also removed within 12 h, most likely due to alkaline hydrolysis and/or biological uptake. The slight difference in the levels of B₁ (10%–15%) measured in samples incubated under light and dark conditions suggests that the effect of light on the decomposition of this vitamin may not be highly significant (Fig. 3).

The importance of sediment resuspension on B₁ levels in coastal environments was also studied by measuring dissolved B₁ in our sediment addition treatments (Fig. 3). The concentration gradient between porewater (0.75 nM) and seawater (0.18 nM)

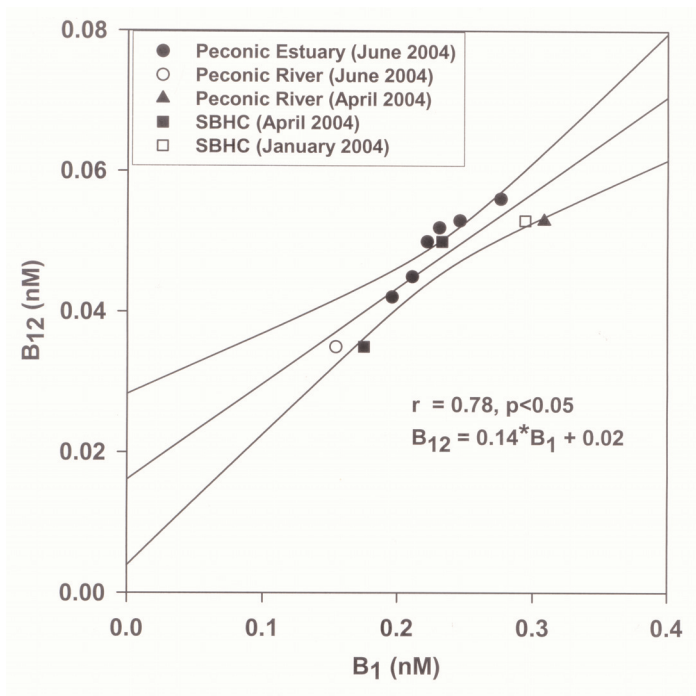


Fig. 4. Simultaneous determination of vitamins B₁ and B₁₂ in surface waters of the Peconic river estuary and Stony Brook Harbor.

suggests a strong diffusive flux from the sediments to the water column. Furthermore, the significant increase in B₁ levels (50% to 60%) after shaking the incubation chambers containing sediments suggests that sediment resuspension events are likely to influence the level of this vitamin in shallow waters.

Simultaneous determination of vitamins B₁ and B₁₂ in seawater—Dissolved water samples collected in the Peconic River estuary and Stony Brook Harbor were analyzed for vitamins B₁ and B₁₂ (Fig. 4). The simultaneous determination of both vitamins is feasible because the sampling, storage, and pre-concentration protocols for B₁ and B₁₂ are the same; the only difference is that B₁ quantification in the HPLC is carried out at a wavelength of 270 nm while B₁₂ is detected at 362 nm (Okbamichael and Sañudo-Wilhelmy 2004).

The recovery of B₁ and B₁₂ in spiked seawater from Stony Brook Harbor and the Peconic River analyzed simultaneously was excellent (93% to 98%; Table 1). The significant linear correlation between B₁ and B₁₂ levels ($r^2 = 0.78$, $P < 0.05$) observed in the different environments sampled during this study suggests a similar biogeochemical behavior of both vitamins in the aquatic environment. The non-zero intercept is consistent with the higher stability of B₁₂ with respect to B₁ at the ambient pH in the aquatic environment ranging from 5 to 8 (Kawasaki and Sanemori 1985; Lindemans and Abels 1985).

In summary, we have developed a direct method for the determination of vitamin B₁ in seawater. This new protocol involves a solid-phase extraction technique for separation and pre-concentration of the vitamin. Gradient elution with UV-Vis

detection using reversed phase HPLC was used to quantify vitamin B₁ in seawater. This method is sensitive, simple, and accurate and provides the first direct quantification of B₁ in seawater. The technique was successfully applied to the determination of B₁ along with vitamin B₁₂ in coastal seawater, porewater, and river water samples from around Long Island, New York. This new method can be employed as a routine analytical tool for the accurate determination of dissolved vitamins B₁ and B₁₂ in seawater, which is essential for understanding the cycling and ecological roles of those vitamins in the marine environment.

References

- Breuer, E., S. A. Sañudo-Wilhelmy, and R. C. Aller. 1999. Trace metals and dissolved organic carbon in an estuary with restricted river flow and brown tide bloom. *Estuaries* 22: 603-615.
- Burkholder, P. R., and L. M. Burkholder. 1956. Vitamin B₁₂ in suspended solids and marsh muds collected along the coast of Georgia. *Limnol. Oceanogr.* 1:202-208.
- Carlucci, A. F. 1966. Bioassay of seawater. II. Methods for the determination of vitamin B₁ in seawater. *Can. J. Microbiol.* 12:1079-1089.
- and R. L. Cuhel. 1975. Vitamins in South Polar Seas. I. Distribution and significance of dissolved and particulate vitamin B₁₂, thiamine and biotin in the Southern Indian Ocean, p. 115-128. *In* G. A. Llano [Ed.], *Adaptations within Antarctic ecosystems: Proceedings of the third SCAR symposium on Antarctic Biology*. Gulf Printing Co.
- and S. B. Silbernagel. 1969. Effect of vitamin concentrations on growth and development of vitamin-requiring algae. *J. Phycol.* 5:64-67.
- and P. M. Bowes. 1970. Production of vitamin B₁₂, thiamine, and biotin by phytoplankton. *J. Phycol.* 6:351-357.
- Gold, K., O. A. Roels, and H. Bank. 1966. Temperature dependent destruction of thiamine in seawater. *Limnol. Oceanogr.* 12:410-413.
- Gregg, W. W., P. Ginoux, P. S. Schopf, and N. W. Casey. 2003. Phytoplankton and iron: validation of a global three-dimensional ocean biogeochemical model. *Deep Sea Res. II* 50:3143-3169.
- Jeffrey, S. W., H. S. MacTavish, W. C. Dunlap, M. Vesik, and K. Groenewoud. 1999. Occurrence of UVA- and UVB-absorbing compounds in 152 species (206 strains) of marine microalgae. *Mar. Ecol. Prog. Ser.* 189:35-51.
- Ivanovic, D., A. Popovic, D. Radulovic, and M. Medenica. 1999. Reversed-phase ion-pair HPLC determination of some water-soluble vitamins in pharmaceuticals. *J. Pharm. Biomed. Anal.* 18:999-1004.
- Jansen, B. C. P. 1972. Thiamine, p. 99. *In* W. H. Sebrell and R. S. Harris [Eds.], *The vitamins*, Vol. 5, Academic Press.
- Kawasaki, T. and H. Sanemori. 1985. Vitamin B₁: Thiamines, p. 385-411. *In* A. P. De Leenheer, W. E. Lambert, and M. G. M. De Ruyter [Eds.], *Modern chromatographic analysis of the vitamins*. Marcel Dekker Inc.
- Lindemans, J., and J. Abels. 1985. Vitamin B₁₂ and related corrinoids, p. 497-539. *In* A. P. De Leenheer, W. E. Lambert, M. G. M. De Ruyter [Eds.], *Modern chromatographic analysis of the vitamins*. Marcel Dekker Inc.
- Lynch, P. L., and I. S. Young. 2000. Determination of thiamine by high performance liquid chromatography. *J. Chromatogr. A* 881:267-284.
- Markopoulou, C. K., K. A. Kagkadis, and J. E. Koundourellis. 2002. An optimized method for the simultaneous determination of vitamins B₁, B₆, B₁₂ in multivitamin tablets by high performance liquid chromatography. *J. Pharm. Biomed. Anal.* 30:1403-1410.
- Moreno, P., and V. Salvado. 2000. Determination of eight water and fat soluble vitamins in multi-vitamin pharmaceutical formulations by HPLC. *J. Chromatogr. A* 870:207-215.
- Natarajan, K. V. 1968. Distribution of thiamine, biotin, and niacin in the sea. *Appl. Microbiol.* 16:366-369.
- . 1970. Distribution and significance of vitamin B₁₂ and thiamine in the Subarctic Pacific Ocean. *Limnol. Oceanogr.* 15:655-659.
- and R. C. Dugdale. 1966. Bioassay and distribution of thiamine in the sea. *Limnol. Oceanogr.* 11: 621-629.
- Ohwada, K., and N. Taga. 1969. Distribution of vitamin B₁₂, thiamine and biotin in marine sediments. *J. Oceanogr. Soc. Japan* 25:123-136.
- and ———. 1972. Distribution and seasonal variation of vitamin B₁₂, thiamine and biotin in the sea. *Mar. Chem.* 1:61-73.
- Okbamichael, M., and S. A. Sañudo-Wilhelmy. 2004. New method for the determination of vitamin B₁₂ in seawater. *Anal. Chim. Acta* 517:33-38.
- Provasoli, L. 1963. Organic regulation of phytoplankton fertility, p. 165-219. *In* M. N. Hill [Ed.], *The sea*, Vol. 2, Interscience.
- and A. F. Carlucci. 1974. Vitamins and growth regulators, p. 741-787. *In* W. D. P. Stewart [Ed.], *Algal physiology and biochemistry*, Blackwell Scientific.
- Stumm, W., and J. J. Morgan. 1996. *Aquatic chemistry: Chemical equilibria and rates in natural waters* (3rd ed.), Wiley.
- Swift, D. G. 1981. Vitamin levels in the Gulf of Maine and ecological significance of vitamin B₁₂ there. *J. Mar. Res.* 39: 375-403.
- Turner, M. F. 1979. Nutrition of some marine micro algae with special reference to vitamin requirements and utilization of nitrogen and carbon sources. *J. Mar. Biol. Assoc. U.K.* 59: 535-552.
- Vishniac, H. S., and G. A. Riley. 1961. Cobalamin and thiamine in Long Island Sound: patterns of distribution and ecological significance. *Limnol. Oceanogr.* 6:36-41.

Submitted 31 July 2004

Revised 18 February 2005

Accepted 20 April 2005