Stony Brook University

Vitamin B_{12} distribution patterns in marine sediments revealed by a new ELISA method

A Thesis Presented

by

Meichen Liu

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

Marine and Atmospheric Science

Stony Brook University

August 2013

Stony Brook University

The Graduate School

Meichen Liu

We, the thesis committee for the above candidate for the

Master of Science degree, hereby recommend

acceptance of this thesis.

Qingzhi Zhu– Thesis Advisor

Assistant Professor

School of Marine and Atmospheric Sciences

Robert C. Aller – Second Reader

Distinguished Professor

School of Marine and Atmospheric Sciences

Josephine Y. Aller – Third Reader

Professor

School of Marine and Atmospheric Sciences

This thesis is accepted by the Graduate School

Charles Taber
Interim Dean of the Graduate School

Abstract of the Thesis

Vitamin B_{12} distribution patterns in marine sediments revealed by a new ELISA method

by

Meichen Liu

Master of Science

in

Marine and Atmospheric Science

Stony Brook University

2013

Vitamin B_{12} is an organic micronutrient in the ocean, and it is required for the growth of majority of phytoplankton. Because of the ultra-low concentrations of vitamin B_{12} in the ocean and the lack of suitable analytical methods, the distribution, transport and biogeochemical cycling of vitamin B_{12} in marine ecosystem have not been essentially documented. In this thesis, vitamin B_{12} distribution and reaction patterns in water column and marine sediments were preliminary studied by using newly developed immunoassay methods.

Two new sensitive and specific enzyme-linked immunosorbent assay (ELISA) methods have been developed and compared in Chapter two, based on indirect competitive immunoassay format, to measure the concentration of vitamin B_{12} in coastal seawater and sediment porewater. Rabbit anti-vitamin B_{12} polyclonal antibody was used to specific recognize vitamin B_{12} from samples, horseradish peroxidase (HRP) was used as a labeling enzyme and tetramethylbenzidine (TMB) was as enzyme substrate. All the immunoassay conditions were optimized. Under the optimal conditions, the absorbance signal was inversely proportional to the concentration of vitamin B_{12} in samples. The dynamic range for B_{12} was 0.1 – 100 ng/ml with a detection limit of

0.05 ng/ml (3σ). Coupled with C-18 column solid-phase extraction – preconcentration, the ELISA methods were readily applicable to measure B_{12} in marine samples.

Vitamin B_{12} distribution patterns in Long Island Sound water column were studied in Chapter three. Results show that vitamin B_{12} has a higher concentration in sediment porewater than in overlying seawater, and the B_{12} concentration gradually increases with depth in water column because the phytoplankton consumption and benthic sources could influence the vertical patterns of B_{12}. The distributions of B_{12} in seawater also show a seasonal variation. Vitamin B_{12} distributions in marine sediments were also revealed by ELISA measurement, a vitamin B_{12} concentration maximum zone was observed for the first time at the depth of ~2 cm in the sediment. The concentration of B_{12} at sediment surface is close to that in the bottom water, however it increases sharply just below the water-sediment interface and reaches maximum at the oxic-anoxic boundary. Beneath the maximum, B_{12} concentration significantly decreases with depth, and reaches almost constant below 4 cm in sediment, suggesting that bacteria at or near the oxic-anoxic boundary may be involved in the generation of vitamin B_{12}. The new B_{12} profiles provide insight into the source, cycling and transport of vitamin B_{12}.

Adsorption-desorption behaviors of vitamin B_{12} on sediment particles under various conditions were studied in order to elucidate the B_{12} profiles and transport in sediments. It was found that in natural conditions over 99% vitamin B_{12} was adsorbed on particles in marine sediments, and the adsorption was likely irreversible. The physical and chemical adsorption of B_{12} on particles may dominate its distribution and transport in sediment and across sediment-water interface.

Table of Contents

List of Figures

Chapter 1 Introduction

1.1 Introduction

Phytoplankton growth is critical to the regulation of the global carbon cycle and global climate change. Nitrogen, phosphorus, silicon, and trace metal iron are generally considered to be the limiting nutrients for the growth of phytoplankton. In the past decades, some studies indicated that organic micronutrients or coenzymes, such as vitamin B_{12}, could also control phytoplankton growth (Droop 1957, Carlucci and Silbernagel 1966; Guillard 1968; Swift 1981). More recent studies have in fact, demonstrated that vitamin B_{12} is required for the growth of the majority of phytoplankton (Croft et al. 2005; Tang et al. 2010) therefore influencing marine primary production and phytoplankton community composition in the world's oceans (Bertrand et al 2007; Okbamichael and Sanudo-Wilhelmy 2005; Gobler et al. 2007; Panzeca et al. 2009; Koch et al. 2011, 2012; Sañudo-Wilhelmy et al. 2012). Based on gene data, vitamin B_{12} plays a key role in nitrogen metabolism and sulfur metabolism (Bertrand and Allen, 2012). The algal growth studies of Croft et al. (2005) showed that the red alga Porphyridium purpureum, the dinoflagellate Amphidiniuk operculatum and the freshwater euglenoid Euglena gracilis need at least 10 ng/L (~7.4 pM) vitamin B_{12} for growth. The study of Sanudo-Wilhelmy et al. (2006) showed that vitamin B_{12} could increase phytoplankton biomass and may cause community structure shifts. B_{12} additions stimulated large phytoplankton proliferation, as well as small

phytoplankton (Koch et al., 2011). On the other hand, under limited vitamin B_{12} conditions, phytoplankton growth rate and primary productivity were suppressed (King et al., 2011). In spite of its acknowledged importance in ocean ecosystem, however, the details of sources, distributions, transport mechanisms, and factors controlling production patterns of vitamin B_{12} in marine environments remain essentially undocumented because there is no generally suitable analytical methods and tools to quantify the ultra-low (ppt to sub-ppt) concentrations of B_{12} typical of seawater. Here we use a newly developed immunoassay method, ELISA, to reveal vitamin B_{12} distribution patterns in seawater and marine sediment, and show that it has intense concentration gradients around oxic-anoxic interfaces in sediments, and the gradients and B_{12} reaction patterns may be associated with biogenic structures. Our findings provide new insights into source, the cycling and transport of vitamin B_{12}, and a basis for modeling the behavior of this bioactive trace micronutrient at the seafloor.

Vitamin B_{12} (also called cobalamin) refers to a group of compounds containing cobalt ion (trivalent cobalt) and the substituted corrin ring (Figure 1-1), and is typically any substituted cobalamin derivative having similar biological activity. The substituent R includes cyanide ion, hydroxyl group, methyl group, and deoxyadenosyl group, and the corresponding vitamin B_{12} is called cyanocobalamin, hydroxocobalamin, methylcobalamin and adenosylcobalamin, respectively. Vitamin B_{12} is a water-soluble vitamin and concentrations of vitamin B_{12} have a wide range in spatial distributions. Usually concentrations of vitamin B_{12} are lower in open oceans, and relative higher in coastal seawater (Okbamichael and Sanudo-Wilhelmy, 2004; Panzeca et al., 2009). Sewage discharges and river inputs could contribute to the high level of B_{12}. Concentrations of B_{12} are closely associated with the concentration of free cobalt ions as vitamin B_{12} is a cobalt-containing organometallic compound, and low concentration of free

cobalt ion may limit B_{12} synthesis. Cobalt addition experiments showed that Co^{2+} additions could increase B_{12} concentrations by two-fold in regions with low levels of Co^{2+} and B_{12} (Panzeca et al., 2008).

It has been hypothesized that heterotrophic bacteria in the water column are the primary vitamin B_{12} producers in the ocean (Karl 2002). Several studies demonstrated that B_{12}-auxotrophic phytoplankton could grow in B_{12}-deficient media in the presence of marine bacteria such as *Halomonas sp.* which can produce and supply vitamin B_{12} to the algae. Some cyanobacteria such as *Crocosphaera watsonii* and *Synechococcus sp.*, have also been shown to produce vitamin B_{12} in culture (Bonnet et al. 2010). Eukaryotic phytoplankton are thought to be the primary vitamin B_{12} consumers, taking up free dissolved vitamin B_{12} from the water and/or through a close bitrophic symbiotic relationship with cell-surface-associated heterotrophic bacterial populations (Bertrand et al. 2007; Haines and Guillard, 1974; Croft et al. 2005). Because of these reasons, vitamin B_{12} concentrations have been found to vary seasonally and with variations in eukaryotic phytoplankton biomass. For example, the decline of B_{12} concentrations during the spring bloom in the Gulf of Maine and the Sargasso Sea have been reported (Menzel and Spaeth, 1962; Swift, 1981; Bruno et al. 1981). Vitamin B_{12} is cycled in the ocean, like other nutrients. After production by bacteria, dissolved vitamin B_{12} can be taken up by marine organisms. Particulate vitamin B_{12} and dead bodies or feces of B_{12}-containing organisms can sink to deep water and/or buried in surface sediment, and the dissolved B_{12} may be regenerated at depth. Then the B_{12} can be transported to the surface water by diffusion, upwelling and other processes (Karl, 2002). Through nutrient cycling, B_{12}-dependent algae might be tightly connected to B_{12}-producing bacteria. In some cases, B_{12}-dependent algae and B_{12}-producing bacteria can develop a symbiotic relationship (Croft et al., 2005). This means that the

bacteria use carbon source produced by algal photosynthesis, in turn, the algae consumes the vitamin B_{12} produced by the bacteria. However, the study of vitamin B_{12} cycling and sources in marine environments is in initial stages, the cycling model or reaction – distribution patterns in seawater reported by Karl and Croft is not enough to tell the whole story of vitamin B_{12}, and further study about the mechanism of vitamin B_{12} supply and consumption is required. In addition, the production and consumption rate of vitamin B_{12} in the water column, as well as the potential sources and reaction patterns in sediments, have not yet been documented.

Studying the concentration of dissolved vitamin B_{12} in seawater, sediment porewater and sediment particles is critical to reveal vitamin B_{12} fluxes cross sediment-water interface, production and consumption reaction patterns, transport and relationship to phytoplankton growth. In the past decades, only a few studies have considered the vertical distribution profiles of B_{12} in ocean. It has been observed that vitamin B_{12} concentration was depleted at the sea surface but increased along with depth and reached a maximum at the depth of 100-300 m in North Pacific Ocean (Carlucci and Silbernagel, 1966) and Sargasso Sea (Menzel and Spaeth, 1962). More recent study showed that the patterns of B_{12} profile in seawater have a large spatial variability (Sanudo-Wilhelmy, et al. 2012). Earlier studies of B_{12} profile in sediment indicated that the content of B_{12} was highest near the sediment surface and sharply decreased with depth in the top 15 cm and then was almost constant below 15 cm (Nishijima and Hata, 1988). However, either quantification of B_{12} in the water column or in sediment was performed using insensitive and nonspecific traditional methods or was done at a poor spatial resolution. Thus, further study of B_{12} distributions in seawater and marine sediments at fine scale is desirable in order to improve the understanding of B_{12} source and biogeochemical cycling.

A microbiological assay utilizing vitamin B_{12} as a growth factor for certain organisms such as *Lactobacillus leichmannii* (Ross 1950; Carlucci and Silbernagel 1966) is the earliest method for the measurement of vitamin B_{12}. This indirect method is time consuming because it requires a long period of incubation of microorganisms, for example 1-20 days (Anderson, 1964; Carlucci and Silbernagel, 1966). This method is also subject to microbial contamination which is not always specific to vitamin B_{12}. Compared with a microbiological assay, high performance liquid chromatography (HPLC) had been proposed as providing an easy, rapid and reproducible method to determine vitamin B_{12} concentration. However, HPLC with UV-VIS detection is not sensitive enough to detect the ambient low concentrations of B_{12} in seawater (Frenkel et al., 1979). In order to improve detection sensitivity, HPLC has been combined with sample preconcentration by C_{18} solid phase extraction (SPE) to measure the ambient vitamin B_{12} in seawater (Okbamichael and Sanudo-Wilhelmy, 2004). A large volume of seawater sample was run through a C_{18} column; analyte vitamin B_{12} associated with other organic matter was extracted by solid-phase C_{18}, and subsequently eluted with a very small volume methanol after the column was washed with distilled water. The detection limit of this method was significantly improved by 3 orders of magnitude by loading large volumes of sample. However, the co-extracted dissolved organic matter (DOM) can produce a very high background in HPLC UV-VIS measurements because the C_{18} sorbent retains analytes primarily by non-specific reverse-phase adsorption rather than specific molecular recognition. Additionally, our recent research has indicated that C_{18} SPE combined HPLC cannot generally be used to quantify vitamin B_{12} in sediment porewater due to high levels of co-extracted DOM from the samples (Zhu et al., 2011). In order to study B_{12} distributions in marine sediments, the development of a sensitive and specific method free from DOM interference for direct measurement of ambient B_{12} (without

preconcentration or preconcentrating small volume water sample) in sediment porewater is necessary.

Immunoassay, a technique based on specific molecular recognition between antibody and antigen (targeted analyte), has been widely applied in environmental monitoring (Knopp 2006; Hage 1999; Anderson et al. 1997). This technique is generally characterized by high specificity and high liability, and provides an alternative approach for specifically analyzing organic compounds in complex environmental and biological samples. Immunoassays of vitamin B_{12} in biological and food samples have been reported at a picomolar sensitivity level based on radioimmunoassay technique (O'Sullivan, et al. 1992). More recently, an Enzyme-Linked ImmuoSorbent Assay (ELISA) method for sensitive and specific determination of vitamin B_{12} in seawater and pore water was described by using a commercially available ELISA kit (Zhu et al., 2011). However, its sensitivity (0.2 ng/ml vitamin B_{12}) is not sufficient to directly measure ambient B_{12} in porewater, the high cost of the ELISA kit also limits its wide application. High sensitive and low cost ELISA method for B_{12} quantification is thus essential for studying vitamin B_{12} in complex marine environments. A modified ELISA method is described here and utilized to examine B_{12} patterns and controlling processes in seawater and sediments.

1.2 Hypotheses

(1) The sensitivity of our current ELISA system can be improved by using customized antibody due to its high affinity and specificity to vitamin B_{12}.

(2) The concentration of vitamin B_{12} in seawater is lower than that in marine sediment; vitamin B_{12} can be produced in the top layer of sediment and serve as a source for vitamin B_{12} in water column.

(3) Vitamin B_{12} is generally adsorbed on the particles, and this adsorption is reversible.

1.3 Objectives

The objectives of this proposal are:

(1) to develop a new ELISA method using customized rabbit anti-vitamin polyclonal B_{12} antibody,

(2) to study vitamin B_{12} spatial and temporal distribution profiles in seawater and sediment porewater and discuss the possible source of vitamin B_{12} in the seawater, and

(3) to study the transport of vitamin B_{12} in marine sediments.

References

Anderson, B. B. 1964. Investigations into the Euglena method for assay of vitamin B_{12} in serum. Journal Clin Pathol. 17 : 14-26.

Anderson, D. J., B. Guo, L. M. Ng, L. J. Kricka, K. J. Skogerboe, D. S. Hage, L. Schoeff, J. Wang, L. J. Sokoll, and D. W. Chan. 1997. Clinical chemistry. Anal. Chem. 69: 165R-229R.

Bertrand, E. M., M. A. Saito, J. M. Rose, C. R. Riesselman, M. C. Lohan, A. E. Noble, P. A. Lee, and G. R. Ditullio. 2007. Vitamin B-12 and iron colimitation of phytoplankton growth in the Ross Sea. Limnol. Oceanogr. 52: 1079-1093.

Bertrand, E.M., Allen, A.E. 2012. Influence of vitamin B auxotrophy on nitrogen metabolism in eukaryotic phytoplankton. Frontiers in microbiology. 3, article 375.

Bonnet, S., E. A. Webb, C. Panzeca, D. M. Karl, D. G. Capone, and S. A. Sanudo-Wilhelmy. 2010. Vitamin B-12 excretion by cultures of the marine cyanobacteria Crocosphaera and Synechococcus. Limnol. Oceanogr. 55: 1959-1964.

Bruno, S. F., Staker, R. D., and Curtis, L. L. 1981. Seasonal variation of vitamin B_{12}, B_{12} analogs, and phytoplankton in a long island estuary. J. Mar. Res. 39 (2): 335-352.

Carlucci, A. F., and S. B. Silbernagel. 1966. Bioassay of seawater. 3. Distribution of vitamin B_{12} in the Northeast Pacific Ocean. Limnol. Oceanogr. 11: 642-646.

Croft, M. T., A. D. Lawrence, E. Raux-Deery, M. J. Warren, and A. G. Smith. 2005. Algae acquire vitamin B-12 through a symbiotic relationship with bacteria. Nature 438: 90-93.

Droop, M.R. 1957. Vitamin B_{12} in marine ecology. Nature 180:1041-1042.

Frenkel, E. P., Kitchens, R. L., and Prough, R. 1979. High-performance liquid chromatographic separation of cobalamins. J. Chromatogr. 174: 393-400.

Gobler, C. J., C. Norman, C. Panzeca, G. T. Taylor, and S. A. Sanudo-Wilhelmy. 2007. Effect of B-vitamins (B-1, B-12) and inorganic nutrients on algal bloom dynamics in a coastal ecosystem. Aquat. Microb. Ecol. 49: 181-194.

Guillard, R. R. 1968. B_{12} specificity of marine centric diatoms. J. Phycol. 4: 59-64.

Hage, D. S. 1999. Immunoassays. Anal. Chem. 71: 294-304.

Karl, D. M. 2002. Nutrient dynamics in the deep blue sea. Trends Microbiol. 10: 410-418.

King, A. L., Sanudo-Wilhelmy, S. A., Leblanc, K., Hutchins, D.A., Fu, F. 2011. CO_2 and vitamin B_{12} interactions determine bioactive trace metal requirements of a subarctic Pacific. The ISME Journal. 5, 1388-1396.

Knopp, D. 2006. Immunoassay development for environmental analysis. Anal. Bioanal. Chem. 385: 425-427.

Koch, F., A. Marcoval, C. Panzeca, K. W. Bruland, S. A. Sanudo-Wilhelmy, and C. J. Gobler. 2011. The effects of vitamin B_{12} on phytoplankton growth and community structure in the Gulf of Alaska. Limnol. Oceanogr. 56:1023-1034.

Koch, F., T. K. Hattenrath-Lehmann, J. A. Goleski, S. A. Sanudo-Wilhelmy, N. S. Fisher, and C. J. Gobler. 2012. Vitamin B_1 and B_{12} uptake and cycling by plankton communities in coastal ecosystems. Frontiers Aquatic Microbiology. October 12, 2012 | doi: 10.3389/fmicb.2012.00363.

Menzel, D. W., and Spaeth, J. P. 1962. Occurrence of vitamin B_{12} in the Sargasso Sea. Limnol. Oceanogr. 7: 151-154.

Nishijima T, and Hata Y. 1988. Distribution of vitamin B_{12}, thiamine, and biotin in the sediment of Harima-Nada Sea. Nippon Suisan Gakkaishi. 54(3): 511-516.

Okbamichael, M., and S. A. Sanudo-Wilhelmy. 2004. A new method for the determination of Vitamin B-12 in seawater. Anal. Chim. Acta 517: 33-38.

Okbamichael, M., and S. A. Sanudo-Wilhelmy. 2005. Direct determination of vitamin B-1 in seawater by solid-phase extraction and high-performance liquid chromatography quantification. Limnol. Oceanogr. Meth. 3: 241-246.

O'Sullivan, J. J., R. J. Leemimg, S. S. Lynch, and A. Pollock. 1992. Radioimmunoassay that measures serum vitamin B_{12}. J. Clin. Pathol. 45: 328-331.

Panzeca, C., A. J. Beck, A. Tovar-Sanchez, J. Segovia-Zavala, G. T. Taylor, C. J. Gobler, and S. A. Sanudo-Wilhelmy. 2009. Distributions of dissolved vitamin B_{12} and Co in coastal and open-ocean environments. Estuar. Coast. Shelf Sci. 85: 223-230.

Panzeca, C., A.J. Beck, K. Leblanc, G.T. Taylor, D.A. Hutchins, and S.A. Sanudo-Wilhelmy. 2008. Potential cobalt limitation of vitamin B_{12} synthesis in the North Atlantic Ocean. Global biogeochemical cycles. 22: GB2029.

Ross, G. I. M. 1950. Vitamin B_{12} assay in body fluids. Nature 166: 270-271.

Santschi, P., Hohener, P., Benoit, G., Brink, B-t. M. 1990. Chemical processes at the sediment-water interface. Marine chemistry. 30, 269-315.

Sañudo-Wilhelmy, S. A., L. S. Cutter, R. Durazo, E. A. Smail, L. Gómez-Consarnau, E. A. Webb, M. G. Prokopenko, W. M. Berelson, and D. M. Karl. 2012. Multiple B-vitamin depletion in large areas of the coastal ocean. Proceedings of the National Academy of Sciences. 109(35): 14041-14045.

Sanudo-Wilhelmy, S. A., Gobler, C. J., Okbamichael, M., Taylor, G. T. 2006. Regulation of phytoplankton dynamics by vitamin B_{12}. Geophysical research letters. 33, L04604.

Swift, D. 1981. Vitamin levels in the Gulf of Maine and ecological significance of vitamin B_{12} there. J. Mar. Res. 39:375-403.

Tang, Y. Z., F. Koch, and C. J. Gobler. 2010. Most harmful algal bloom species are vitamin B-1 and B-12 auxotrophs. Proc. Natl. Acad. Sci. U. S. A. 107: 20756-20761.

Zhu, Q. Z., R. C. Aller, A. Kaushik. 2011. Analysis of vitamin B_{12} in seawater and marine sediment porewater using ELISA. Limnol. Oceanogr. Methods 9:515-523.

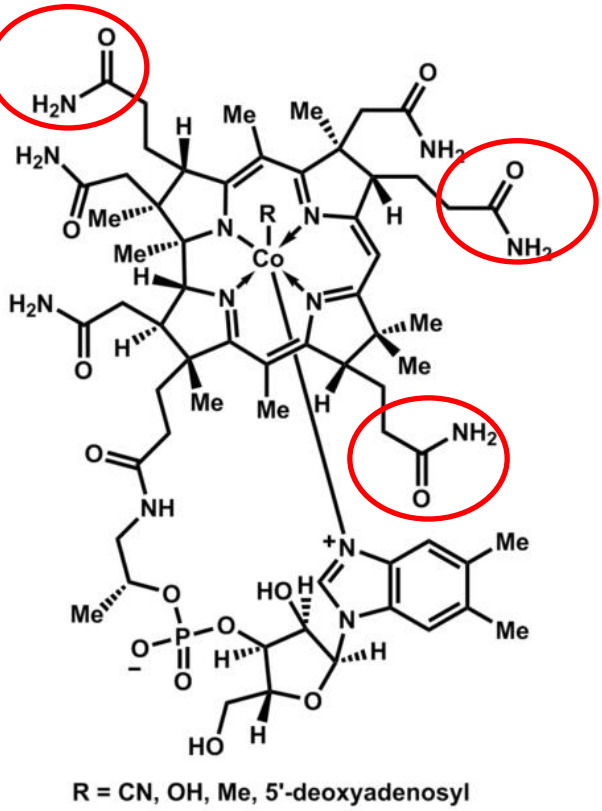


Figure 1-1. The structure of vitamin B_{12}. R = CN, OH, Me and deoxyadenosyl are substituents. The three amide groups in red circles are those can be hydrolyzed with mild acid such as 0.1 HCl.

Chapter 2

Development of a New ELISA Method for B_{12} Determination in Marine Samples

2.1 Introduction

Immunoassay is a highly specific analytical technique that utilizes the specific molecular recognition between antibody and antigen. Based on the different types of labeling and detection system used, immunoassay can be classified as radioisotope immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), fluorescence immunoassay, chemiluminescence immunoassay and non-label immunoassay. The most common immunoassay format is the ELISA, where enzymes are used as labels to detect the interaction between antibodies and antigens. ELISA has become a predominant immunoassay form in the past decades because of its high selectivity, sensitivity and reliability. This method utilizes a solid support, for example a 96-well microtiter plate, to immobilize an antibody or antigen and perform immunoassays as shown in Figure 2-1. After the bonded enzyme reacts with a substrate for a certain time, absorbance of the developed color is correlated to the antigen concentration. There are two basic types of ELISA, one is a competitive assay format, in which the unknown unlabeled analyte competes with the known enzyme-labeled analyte for binding the immobilized antibody. The signal is inversely proportional to the analyte concentration (Figure 2-1 (A), (B)). The other type is a sandwich assay format, in which the unlabeled antigen is captured by immobilized antibody on microtiter plate first, and then binds to the second enzyme-labeled antibody (Nunes et al., 1998). The signal

is proportional to the analyte concentration (Figure 2-1 (C)). ELISA has been accepted as a standard detection platform with a long tradition of use in the biological, clinical, and pharmaceutical research communities. In the past decade, it has also been applied in environmental research (Knopp 2006; Hage 1999; Nunes et al., 1998; Joos et al., 2000). Its analytical accuracy and precision have also been demonstrated by alternative laboratory techniques such as HPLC and GC/MS (Deng et al. 2003). More recently, an ELISA method for specific measurement of dissolved vitamin B_{12} in seawater and sediment porewater has been developed and applied in our laboratory with a detection limit of 0.2 ng/ml B_{12} (~150 pM) (Zhu et al. 2011). Because the ambient vitamin B_{12} concentration in seawater is often smaller than 10 pg/ml (~7.4 pM), a more sensitive ELISA method is needed.

Chemiluminescence enzyme immunoassay (CLEIA) is an alternative to ELISA, which integrates the advantages of chemiluminescence determination and immunoassay techniques such as high specificity and ultra-high sensitivity, and therefore offers distinct advantages over ELISA or immunofluorescent (IF) technologies (Zhao et al. 2009). Chemiluminescence (CL) has been known to be a powerful analytical technique with sensitivity which is often 3 – 4 orders of magnitude higher than UV-VIS absorbance methods. There are many chemiluminescence systems of which the most widely used is the luminol based systems. CLEIA has the same immunoassay principles as ELISA, except using chemiluminescence substrate to replace colorimetric substrate. When the immunoreaction is complete, chemiluminescence substrate solution is added and the intensity of chemiluminescence is subsequently measured. The advantages of chemiluminescence immunoassays include: (1) ultra-high sensitivity so that less sample may be required or diluted sample may be used, (2) ultra-low background because no light source is required, (3) fast reaction and detection, and (4) a wide linear working range

because the range of chemiluminescence photo counts is usually much greater than absorbance detection. In this thesis, a new ELISA method has been developed to measure vitamin B_{12} in seawater and sediments, and a new CLEIA method is demonstrated.

2.2 Experimental Section

2.2.1 Materials and Reagents

All reagents were of analytical grade unless specified otherwise. Vitamin B_{12} standard was purchased from MP Biomedicals, LLC. Bovine serum albumin (BSA, IgG free), horseradish peroxidase (HRP) and HRP labeled goat anti-rabbit IgG antibody (Ab-HRP) were obtained from Jackson Immuno Research Laboratories. Vitamin B_{12}-HRP conjugate (300 units/mg) was purchased from CalBioreagents. Rabbit anti-B_{12} antibody (B_{12}-Antibody, rabbit antiserum) was customized in our laboratory. BSA-B_{12} conjugated antigen was home-made with a label ratio of 2.6. 3,3',5,5'-Tetramethylbenzidine (TMB) was from Sigma-Aldrich. Luminol (4-Aminophthalyl hydrazide) was obtained from Fisher Sci. TMB substrate solution. SuperBlock blocking solution, and Pico-chemiluminescence substrate were obtained from Thermo Sci. HRP chemiluminescience Western substrate was purchased from Millipore Co. Potassium phosphate monobasic, sodium phosphate dibasic, sodium chloride, hydrogen peroxide (30%), Tween-20 and sulfuric acid were obtained from Sigma-Aldrich.

Phosphate buffered saline (PBS, 0.1 M, pH 7.4) was prepared by dissolving 0.2 g of potassium chloride, 0.27 g of potassium phosphate monobasic, 2.68 g of sodium phosphate dibasic and 8.0 g of sodium chloride in 1.0 L Milli-Q water. Coating solution carbonate buffer (pH 9.5) was prepared by dissolving 1.59 g of sodium carbonate, and 2.93 g of sodium bicarbonate in 1.0 L Milli-Q water. Washing solution (PBS, pH 7.4, with 0.1% Tween 20) was

prepared by mixing PBS with 0.1% Tween-20. Blocking solution (2% BSA in PBS, pH 7.4) was prepared by dissolving BSA in PBS solution at concentration of 2%. 96-Well microtiter plates (clear and white) were obtained from Thermo.

2.2.2 Apparatus

A POLARstar Omega multifunctional plate reader (BMG Labtech GMbH) was used for the ELISA absorbance measurement at 450 nm with reference wavelength at 620 nm. This instrument was also used to detect chemiluminescence.

2.2.3 Immunoassay Methods

ELISA Method 1

An indirect competitive immunoassay format was applied to ELISA method 1. 100 μl of certain diluted anti-vitamin B_{12} serum in coating solution was added into each well of a 96-well microtiter plate. The plate was sealed with parafilm and incubated at 4 °C overnight. Then, the plate was washed with washing solution for five times (350 μl/well each time). Binding sites that were not occupied by the coating antibody were blocked by adding 300 μl of blocking solution into each well and the plate was shaken for 1.5 hours at room temperature in dark. The plate was washed five times again, and the antibody coated plate was ready for the immunoassay. 50 μl of vitamin B_{12} standard solution or sample and 50 μl of diluted B_{12}-HRP solution (in PBS, pH 7.4) were added into each well. The plate was sealed with parafilm and shaken at room temperature for 1 hour. After washing five times with washing solution, 100 μl of HRP substrate (tetramethylbenzidine (TMB) and hydrogen peroxide mixture) was added into each well using a multi-channel pipette and the enzyme catalyzed reaction was allowed for 20 min at room

temperature in the dark. Subsequently, 100 µl of stop solution (5% H_2SO_4) was added to each well to stop the reaction and the absorbance of the solution in each well was measured at 450 nm using an ELISA plate reader with reference wavelength at 620 nm. The absorbance at 450 nm was used to construct a calibration against log [B_{12}].

ELISA Method 2

A new ELISA method for vitamin B_{12} detection was developed based on another competitive immunoassay format. A 96-well microtiter plate was coated with BSA-B_{12} coating antigen at 4 °C overnight. Then, the plate was washed with washing solution (350 µl/well) for five times and blocked with 300 µl blocking buffer solution for 3 hours at room temperature. The plate was washed again, then 50 µl of diluted primary vitamin B_{12} antibody and 50 µl B_{12} standard or sample were added into each well and the plate was incubated at room temperature for 1 hour. After the immunoreaction was completed and the plate was washed 5 times again, 100 µl of diluted HRP labeled goat anti-rabbit IgG secondary antibody (Ab-HRP) was added to bond with the primary rabbit antibody to form the immune-complex on the well wall: BSA-B_{12}-primary antibody-secondary IgG-HRP. After washing 5 times, 100 µl of HRP substrate (tetramethylbenzidine (TMB) and hydrogen peroxide mixture) was added into each well using a multi-channel pipette and the enzyme catalyzed reaction was allowed for 20 min at room temperature. Then, 100 µl of stop solution (5% H_2SO_4) was added into each well to stop the reaction and the absorbance of the solution in each well was measured at 450 nm using an ELISA plate reader with reference wavelength at 620 nm. The absorbance at 450 nm was used to make a calibration graph against log [B_{12}].

Chemiluminescence Enzyme Immunoassay* (*CLEIA) Method

A 96-well white microtiter plate was coated with 100 µl BSA-B_{12} antigen at 4 °C overnight. Then, the plate was washed with washing solution 5 times and blocked with blocking buffer solution for 3 hours at room temperature. The plate was washed as before, and then 50 µl of diluted primary vitamin B_{12} antibody and 50 µl B_{12} standard or sample were added into each well, and the plate was incubated at room temperature for 1 hour. After the immunoreaction was completed and the plate was washed 5 times again, 100 µl of diluted HRP labeled goat anti-rabbit IgG secondary antibody (Ab-HRP) was added to bond with the primary rabbit antibody on the well wall. After washing 5 times with washing solution, 50 µl Pico chemiluminescent substrate or western HRP substrate was added and the enzyme catalyzed reaction was allowed for 2 min in the dark. The luminescence of the light was measured by plate reader. The intensity of emission light was used to construct calibrations against log [B_{12}].

2.3 Results and Discussion

The most important component in the ELISA method is the antibody that is used to specifically recognize the antigen, and its affinity and specificity to antigen determine the ELISA method sensitivity and specificity. Another factor which can influence the ELISA sensitivity is the labeled enzyme, horseradish peroxidase (HRP), which reacts with colorless substrate to produce a colored substance so that the antigen (analyte) can be detected. High enzyme activity would give rise to high detection sensitivity. In addition, the immunoreaction conditions, such as the amount of immunoreagents (antibody or antigen) coated on microtiter plate, washing and blocking solutions, reaction temperature and time, etc., would also affect the ELISA sensitivity,

selectivity and working dynamic range. In order to develop a highly sensitive and specific ELISA, these assay conditions should be carefully optimized.

2.3.1 Optimization of ELISA Method 1

ELISA method 1 was developed by using the competitive ELISA method as shown in Figure 2-1 (A). The details of the process of ELISA method 1 were described in 2.2.3. In order to obtain high sensitivity and reliable analysis, various assay conditions were optimized.

First, the coated amount of polyclonal antibody on the 96-wll plate was optimized by coating the plate with various concentrations of antibody in coating solution. The anti-B_{12} serum was diluted by 1:10,000, 1:50,000, 1:100,000 and 1:200,000 using coating buffer solution (pH 9.5) to coat 96-well plates based on ELISA method 1 described above. When B_{12}-HRP conjugate (300 units/mg) was fixed at 1:5000 dilution, the effect of coated amount of B_{12}-antibody on ELISA response was shown in Figure 2-2. It can be seen that the absorbance signal was largely increased along with the coated antibody increase because more B_{12}-HRP were bonded on the well wall. The response sensitivity (slope) was also significantly increased when the antibody concentration was changed from 1:200,000 to 1:50,000, and then this increase became very small between 1:50,000 and 1:10,000 dilution, indicating the sensitivity may approach the maximum at 1:10,000 dilution. Because the higher antibody concentration (<1:10,000) was not tested, 1:10,000 of anti-vitamin B_{12} serum was selected.

When the concentration of B_{12}-antibody coating solution was fixed at 1:10,000 dilution, the effect of different concentrations of B_{12}-HRP on the ELISA sensitivity was studied. The results in Figure 2-3 showed that when the B_{12}-HRP (300 units/mg) was diluted from 1:10,000 to 1:1,000, the sensitivity went up slightly but the absorbance increased significantly. The

maximum absorbance was higher than 1 when B_{12}-HRP concentration was above 1:2,000 dilution. Additionally, the ELISA response showed a wider dynamic range, e.g. 0.1 – 100 ng/ml, when the B_{12}-HRP concentration was below 1:5,000. On the other hand, the calibration lines were clearly bent at the point of 0.5 ng/ml B_{12} when B_{12}-HRP was higher than 1:2,000 dilution. Therefore, 1:5,000 dilution of B_{12}-HRP (300 units/mg) was chosen for further work.

It is well-known that the unspecific adsorption of the enzyme labeled immunoreagents such as B_{12}-HRP onto the surface of the 96-well plate is one of the major factors which could lead to high background and low sensitivity of the ELISA. The non-specific adsorption of enzyme, antigen and antibody on the plastic well surface can be minimized or eliminated by blocking the unoccupied binding site on well surface using a suitable blocking reagent. The block solutions SuperBlock block solution from Sigma and BSA (IgG free) solutions were examined for their blocking capacity on this ELISA. The BSA blocking solutions were prepared with PBS (0.1 mol/L, pH 7.4) at concentrations of 1.0%, 2.0%, and 5.0%. It was found that both SuperBlock and BSA solutions worked well for the blocking. When BSA at a concentration of 2% was optimal, i.e., background signal was well below 10%. The effect of the temperature during blocking steps and blocking time were also examined. Blocking with 2% BSA was more efficient at room temperature than at 4 °C. When the blocking time is longer than 1.5 hour, the background signal was acceptable. Therefore, the 96-well plate was routinely coated at 4 °C overnight followed by a blocking with 2% BSA at room temperature for 1.5 hrs.

Washing steps are also critical for successfully conducting ELISA analysis. After blocking and each immunoreaction step, intensively washing each well is needed to remove the unbound immunoreagents. Our results showed that 3 to 5 times wash with washing solution after each step is appropriate to the assay.

2.3.2 Optimization of ELISA Method 2

In this approach, antigen vitamin B_{12} conjugated with BSA (BSA-B_{12}) was coated on a 96-well plate. The coated BSA-B_{12} (known) and free B_{12} (unknown concentration) compete for binding to a certain amount of primary B_{12} antibody. Then HRP labeled secondary antibody, i.e. sheep anti rabbit IgG antibody (HRP-IgG) was used to bond the primary B_{12} antibody on the well surface. The resulting signal is inversely proportional to the B_{12} concentration. The schematic of this ELISA format is shown in Figure 2-1 (B). Effects of various factors on the ELISA response such as concentration of BSA-B_{12} conjugate coating solution, concentration of B_{12} primary antibody and concentration of secondary IgG-HRP were studied to optimize the ELISA conditions.

The concentration of B_{12} primary antibody was optimized first by fixing the concentrations of coating B_{12}-BSA solution (10 ng/ml) and secondary IgG-HRP solution (1:8,000), and changing the concentration of B_{12} primary antibody from 1:100,000 to 1:400,000 dilution of anti-serum. The effect of B_{12} antibody concentration on ELISA response was summarized in Figure 2-4. It can be seen that when the B_{12} antibody concentration was lower than 1:100,000 dilution of the anti-serum, the ELISA response slightly increased and reached maximum and constant in the range of 1:150,000 – 300,000 dilution. On the other hand, the final absorbance decreased as the B_{12} primary antibody concentration decreases, but it was strong enough to be detected even at 1:400,000 dilution. Therefore, a 1:300,000 dilution of the anti-serum was selected to perform the assays.

The concentration of B_{12}-BSA coating solution was optimized first by fixing the B_{12} antibody concentrations at 1:300,000 dilution of anti-serum and secondary IgG-HRP solution at

1:8,000. The results in Figure 2-5 indicated that when the B_{12}-BSA concentration was in the range of 1 ng/ml to 4 ng/ml, the response sensitivity and absorbance signal were appropriate, so 1 ng/ml (~740 pM) of B_{12}-BSA conjugate was chosen to coat the 96-well plate. The secondary IgG-HRP concentration was also optimized, results in Figure 2-6 show that the ELISA response sensitivity was constant when the IgG-HRP was diluted from 1:4,000 to 1:10,000. So a higher dilution of IgG-HRP 1:8000 was selected.

2.3.3 Calibration Curves of ELISA Methods

The calibration curves of ELISA method 1 and 2 were constructed for the determination of vitamin B_{12} in the concentration range of 0.1 – 100 ng/ml under the optimal conditions, and the typical curves are shown in Figure 2-7. For both methods, the relative standard deviation of the measured absorbance for three replicates at each standard concentration was lower than 4%. The detection limits of the both methods were down to 0.05 ng/ml (~37 pM, 3σ) vitamin B_{12} with the similar analytical working dynamic range from 0.1 to 100 ng/ml. However, ELISA methods 2 needs two step immunoreactions and longer assay time.

2.3.4 Comparison of Different ELISA

ELISA method 1 is coated with B_{12}-Antibody, and then B_{12} standard and B_{12}-HRP were added to complete the reaction. However, ELISA method 2 uses BSA-B_{12} conjugate as the coating solution, and then B_{12} standard and B_{12}-Antibody were added to complete the first step of the reaction. After washing, Ab-HRP was added to complete the whole reaction. Therefore, ELISA method 1 uses fewer steps and saves more time compared with ELISA method 2.

2.3.5 Effects of DOM, Humic Acids, and Salinity on the ELISA

The effects of salinity, natural DOM, and humic acids on the ELISA were recently studied in our laboratory (Zhu et al, 2011) but were not repeated for the present ELISA methods 1 and 2. Zhu et al. (2011) reported that the salinity up to 35‰ did not interfere with B_{12} immunoreaction and ELISA measurement. In addition, up to 30 mg/l added reagent humic acid and DOM did not interfere with the ELISA system for analysis of 10 ng/ml vitamin B_{12}. In this work, because we used a similar B_{12} polyclonal antibody and ELISA assay format, it is expected that the organic matters and salinity do not interfere with the new ELISA methods. Lack of interferences has been demonstrated by the application of ELISA in seawater and porewater, no interferences were observed even with high DOM in samples.

2.3.6 Chemiluminescence Enzyme Immunoassay

Chemiluminescence enzyme Immunoassay (CLEIA) is potentially a more powerful method than ELISA. The immunoassay principle of CLEIA is the same as that of UV-VIS ELISA described above in ELISA methods 1 and 2, except the enzyme HRP substrate. In the ELISA method, tetramethylbenzidine (TMB) is used as a colorimetric substrate for HRP, but luminol is used as chemiluminescence HRP substrate in the CLEIA method. When the immunoreaction is complete, a solution of the mixture of luminol, hydrogen peroxide and chemiluminescence enhancer is added to the microtiter plate well. Under catalyzing by HRP, luminol quickly emits strong blue light. The light intensity is inversely proportional to the antigen B12 concentration if a competitive immunoassay format is applied.

Chemiluminescence enzyme Immunoassay was studied by using different HRP chemiluminescence substrate concentrations (Figure 2-8). The plate was coated with 0.1 ng/ml

and 1.0 ng/ml BSA-B_{12}, respectively. It can be seen that both substrates worked well with the CLEIA assay, the detection limit was improved to 0.01 ng/ml vitamin B_{12} (7.4 pM) when the plate was coated with 0.1 ng/ml BSA-B_{12}. Compared with ELISA, CLEIA has a higher sensitivity, but the signal is not as stable. At room temperature, the activity of the enzyme HRP decreases quickly (Figure 2-9). Because CLEIA is not stable, the immunoreaction and measurement conditions were not optimized. Different chemiluminescence immunoassay methods without enzyme need to be investigated in order to improve the chemiluminescence stability, for example using acridinium esters as chemiluminescence label which is stable in acidic solution but emits strong light at alkaline solution.

2.3.7 Application of the ELISAs

The developed ELISAs have been applied to measure vitamin B_{12} in coastal seawater and marine sediment porewater. Because the concentrations of vitamin B_{12} in seawater and porewater are generally in the range of 5 – 40 pM (Menzel and Spaeth 1962; Panzeca et al. 2008; Zhu et al., 2011) and lower than the detection limits of ELISAs 37 pM (0.05 ng/ml), preconcentration of vitamin B_{12} is necessary prior to ELISA measurement. Solid phase extraction (SPE) based on C_{18} column is a common method to separate and enrich analytes from complicated environmental sample matrixes and has been used to enrich and clean-up vitamin B_{12} samples (Okbamichael and Sañudo-Wilhelmy, 2004; Zhu et al. 2011). In this work, C_{18}-SPE-ELISA method was applied to measure vitamin B_{12} in the sweater and porewater samples. The condition for SPE vitamin B_{12} in this work was the same as that used by Zhu et al. (2011). Over 90% recovery of standard B_{12} can be obtained when the seawater pH was adjusted to the range of 6.1–6.7 and the SPE flow rate at 1 ml/min. Generally, 50 – 100 ml of seawater sample was chosen and run

through a C_{18} column, and then the column needs to be rinsed with distilled water to remove residual salts. The enriched analyte vitamin B_{12} in C_{18} column can be eluted by a small volume methanol. Because the organic methanol solution cannot be directly used for immunoassays, the eluent needs to be dried by a gentle stream of N_2 and the residue redissolved in 0.5 ml of 0.1 M PBS for ELISA analysis. The data measured by the C_{18}-SPE-ELISA are presented in Figures of Chapter 3. A detailed C_{18} preconcentration procedure was also presented in the Experiment Section of Chapter 3.

2.4 Conclusions

The developed ELISA methods have wide working dynamic range and a low direct detection limit (0.05 ng/ml, 3σ). When combined with SPE, ELISA has been successfully applied to the quantification of trace dissolved B_{12} in seawater and marine sediment pore water. In practice, ~50 – 100 ml of sample is needed for preconcentration depending on the range of B_{12} concentration. The method has the advantages of high specificity, easy operation, rapid measurement and low cost. By using 96 or 384-well microtiter plates and multifunctional plate readers, the ELISA method can readily measure hundreds of sample extractions within two hours. Because biologically active elements are utilized, the calibration and sample measurement should be performed at the same time. Additionally, the antibody coated microtiter plate and reagents should be stored at 4 °C (up to a year). For longer term storage, small aliquots of antibody solutions should be kept at -20 °C or below.

References

Deng, A., M. Himmelsbach, Q. Z. Zhu, S. Frey, M. Sengl, W. Buchberger, R. Niessner, and D. Knopp. 2003. Residue analysis of the pharmaceutical diclofenac in different water types using ELISA and GC-MS. Environ. Sci. Technol. 37: 3422-3429.

Hage, D. S. 1999. Immunoassays. Anal. Chem. 71: 294-304.

Joos, T. O., Monika Schrenk, Peter Hopfl, and et al. 2000. A microarray enzyme-linked immunosorbent assay for autoimmune diagnostics. Electrophoresis. 21, 2641-2650.

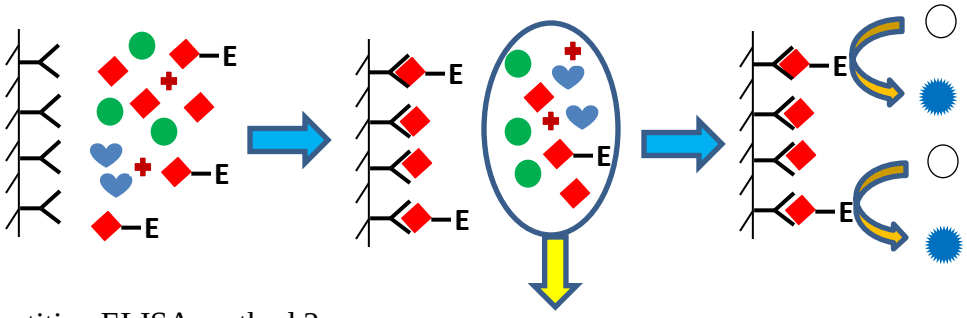
Knopp, D. 2006. Immunoassay development for environmental analysis. Anal. Bioanal. Chem. 385: 425-427.

Nunes, G. S., I. A. Toscano, and D. Barcelo. 1998. Analysis of pesticides in food and environmental samples by enzyme-linked immunosorbent assays. Trac-Trends Analytical Chemistry. 17: 79-87.

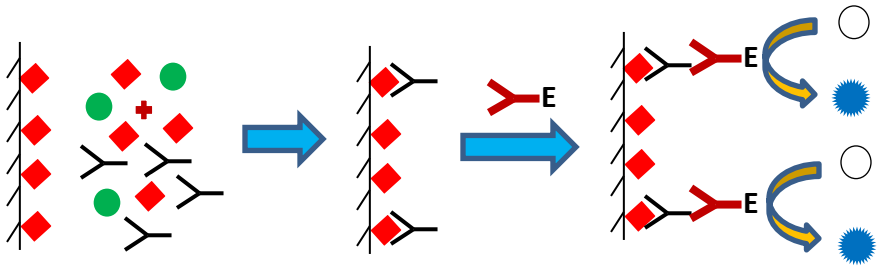
Zhao, L., Sun L., Chu X. 2009. Chemiluminescence immunoassay. Trends in Analytical Chemistry, 28 (4): 404-415.

Zhu, Q. Z., R. C. Aller, A. Kaushik. 2011. Analysis of vitamin B_{12} in seawater and marine sediment porewater using ELISA. Limnol. Oceanogr. Methods 9:515-523.

(A) Competitive ELISA method 1



(B) Competitive ELISA method 2



(C) Sandwich ELISA method

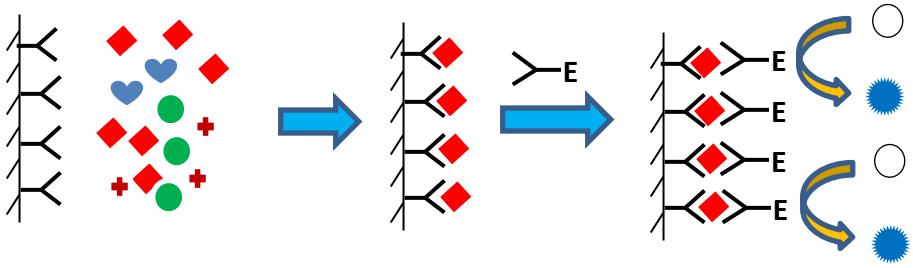


Figure 2-1. Schematic representation of ELISA. (A) Competitive assay format between the enzyme labeled (known concentration) and unlabeled antigen (analyte, unknown concentration) for binding sites on the immobilized antibody. The resulting signal is inversely proportional to the analyte concentration. (B) Competitive assay format between the coated antigen (known concentration) and free antigen (analyte, unknown concentration) for binding to the certain amount of primary antibody. The resulting signal is inversely proportional to the analyte concentration. (C) Sandwich assay format, the unlabeled antigen is determined by an enzyme-labeled antibody. The substrate is transferred to a detectible signal by the enzyme. E: enzyme.

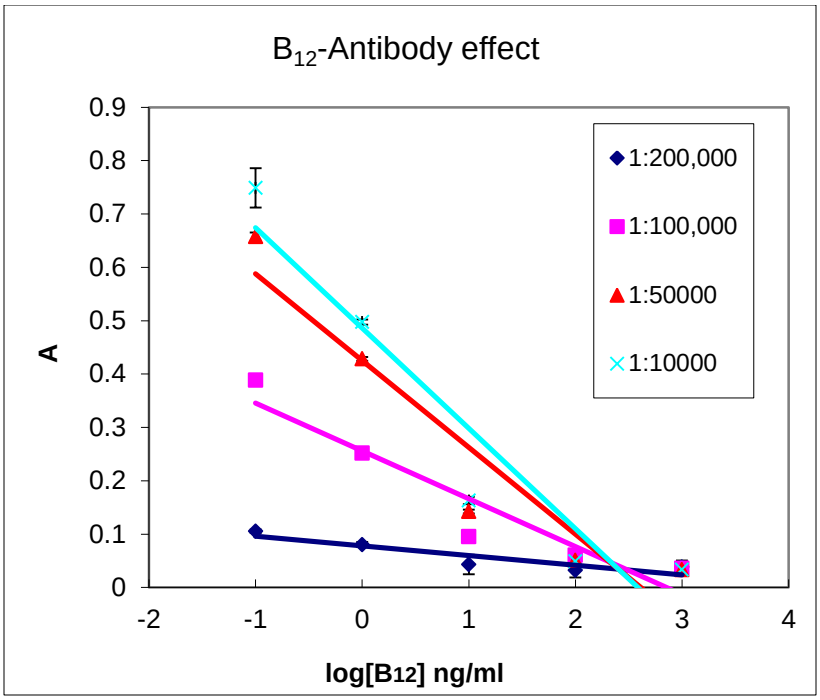


Figure 2-2. Effect of coated B_{12}-antibody amount on the response of ELISA method 1. The vitamin B_{12} anti-serum (B_{12} antibody) was diluted by 1:10,000, 1:50,000, 1:100,000 and 1:200,000 using coating buffer solution (pH 9.5), respectively. 100 μl of diluted antibody solutions were used to coat the 96-well plate using ELISA method 1.

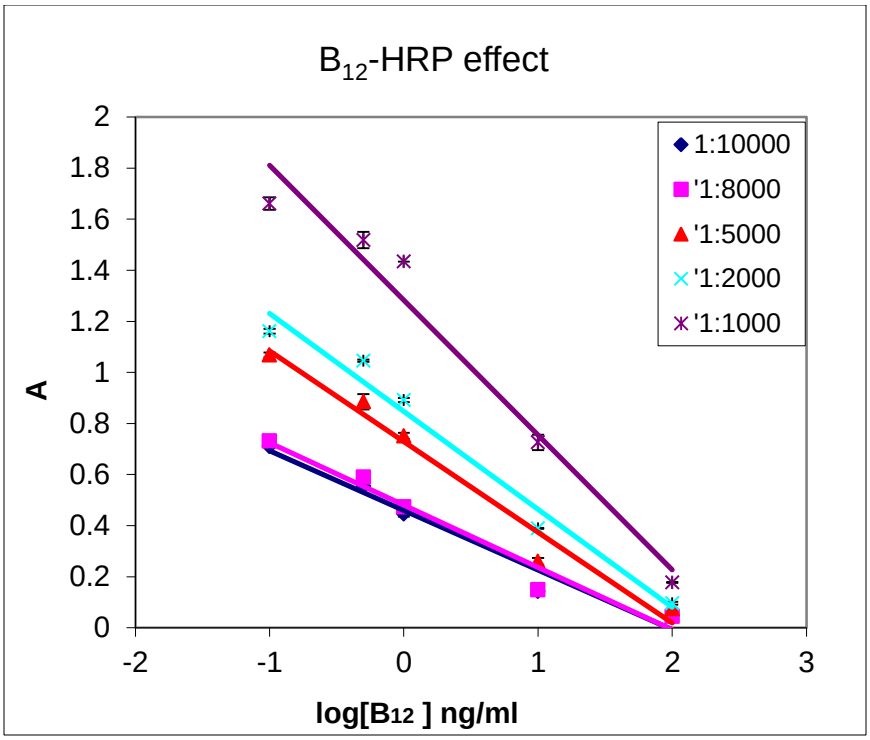


Figure 2-3. Effect of B_{12}-HRP concentration on the ELISA (method 1) response to vitamin B_{12}. 100 µl of 1:10000 diluted anti-vitamin B_{12} serum (B_{12}-Antibody) was used to coat the 96-well plate. Dilution of B_{12}-HRP (300 units/mg) was 1:10,000, 1:8000, 1:5000, 1:2000, and 1:1000 respectively. 50 µl of these dilutions and 50 µl of B_{12} standard solutions were used for the immunoreaction reaction according ELISA method 1.

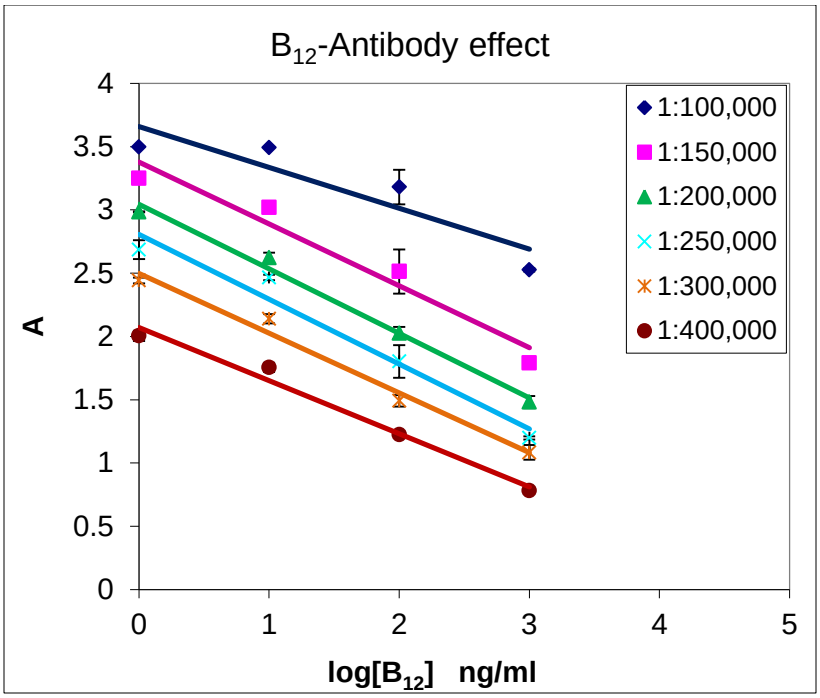


Figure 2-4. Effect of the concentration of B_{12} primary antibody on the response of ELISA method 2. A 96-well microtiter plate was coated with 10 ng/ml BSA-B_{12}. 50 µl B_{12} standard solutions and 50µl of diluted primary vitamin B_{12} antibody (rabbit anti-serum) were added into each well, the immunoreaction and measurement were carried on according to the procedure of ELISA methods 2. Dilution of anti-serum was 1:400,000, 1:300,000, 1:200,000, 1:150,000, and 1:100,000 respectively.

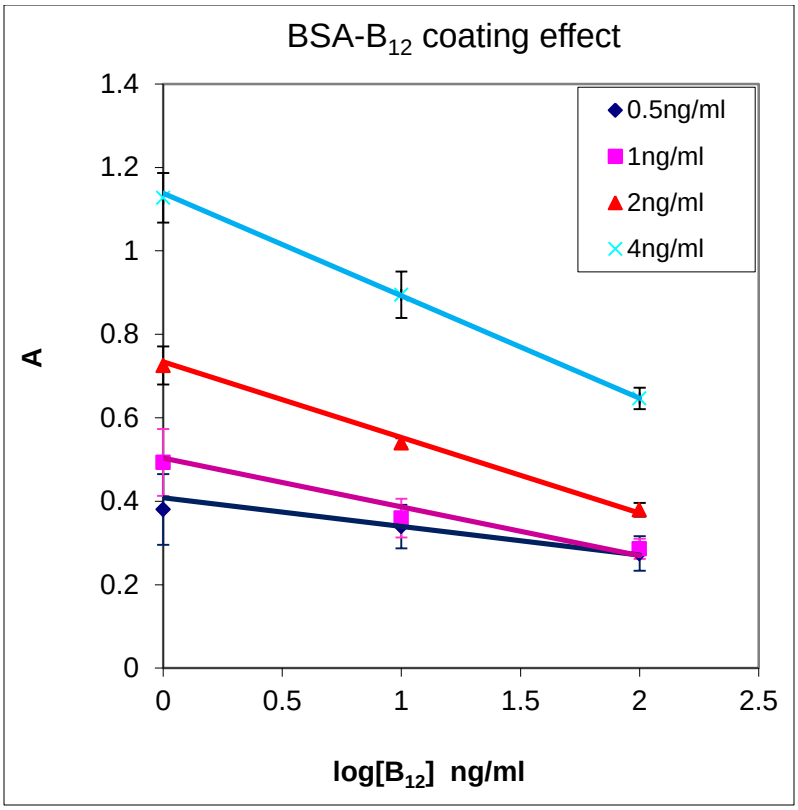


Figure 2-5. Effect of the concentration of BSA-B_{12} coating solution on ELISA method 2. Primary B_{12} antibody: 1:300,000 dilution of anti-serum; secondary IgG-HRP: 1:8000 dilution. Immunoreaction and measurement was carried out according to ELISA method 2.

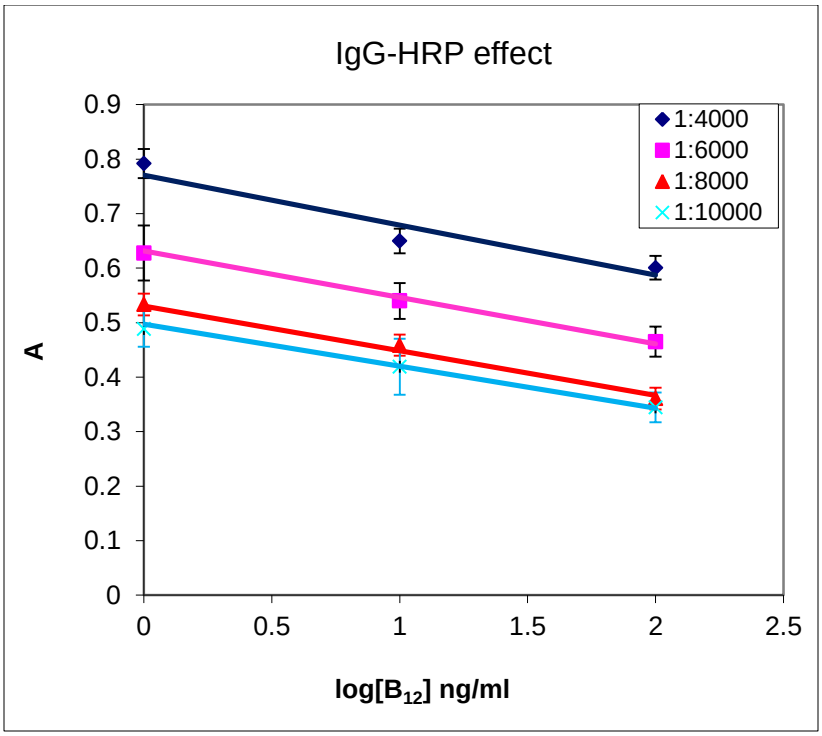


Figure 2-6. Effect of the concentration of secondary IgG-HRP on the response of ELISA method 2. Coating solution: 1ng/ml of B_{12}-BSA; primary B_{12} antibody: 1:300,000 dilution of anti-serum. Different dilution of IgG-HRP was 1:10000, 1:8000, 1:6000, and 1:4000, respectively. Immunoreaction and measurement was carried out according to ELISA method 2.

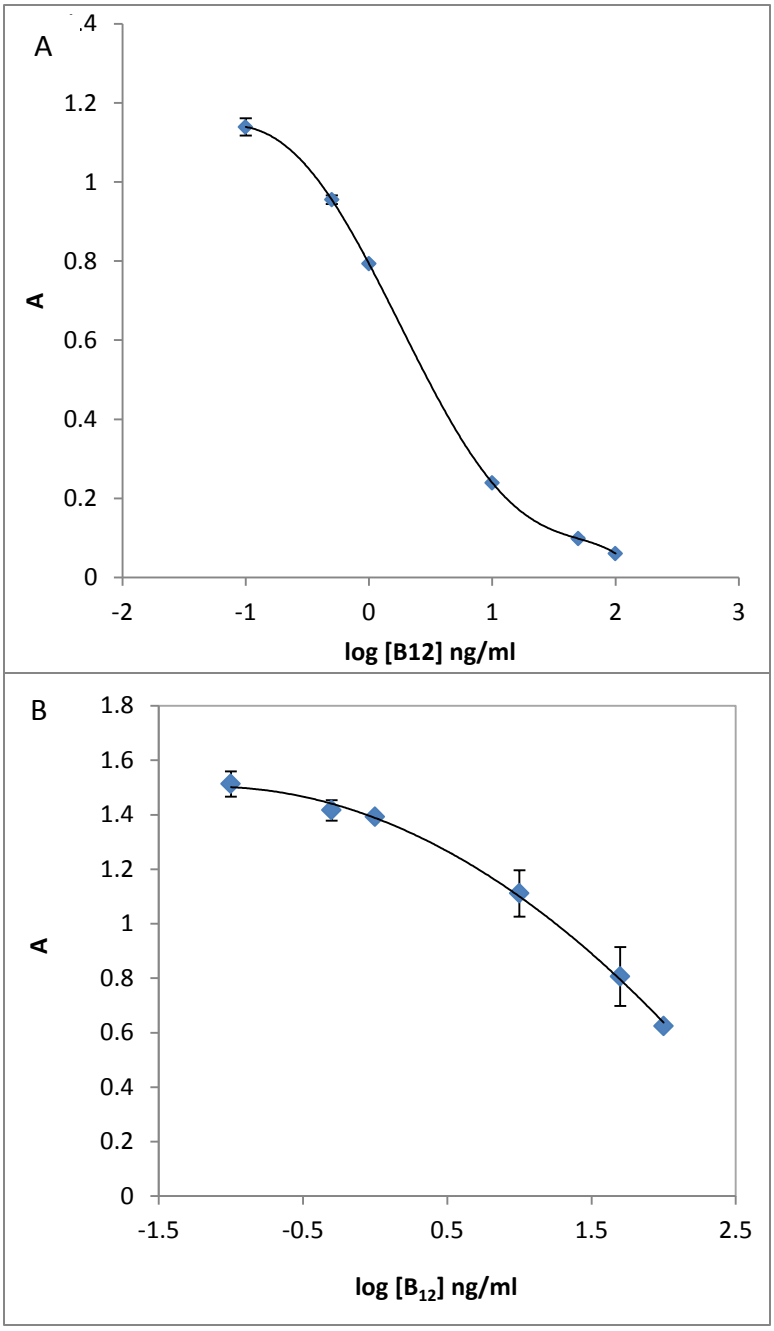


Figure 2-7. Calibration graphs for the detection of vitamin B_{12} by ELISA method 1 (A) and method 2 (B).

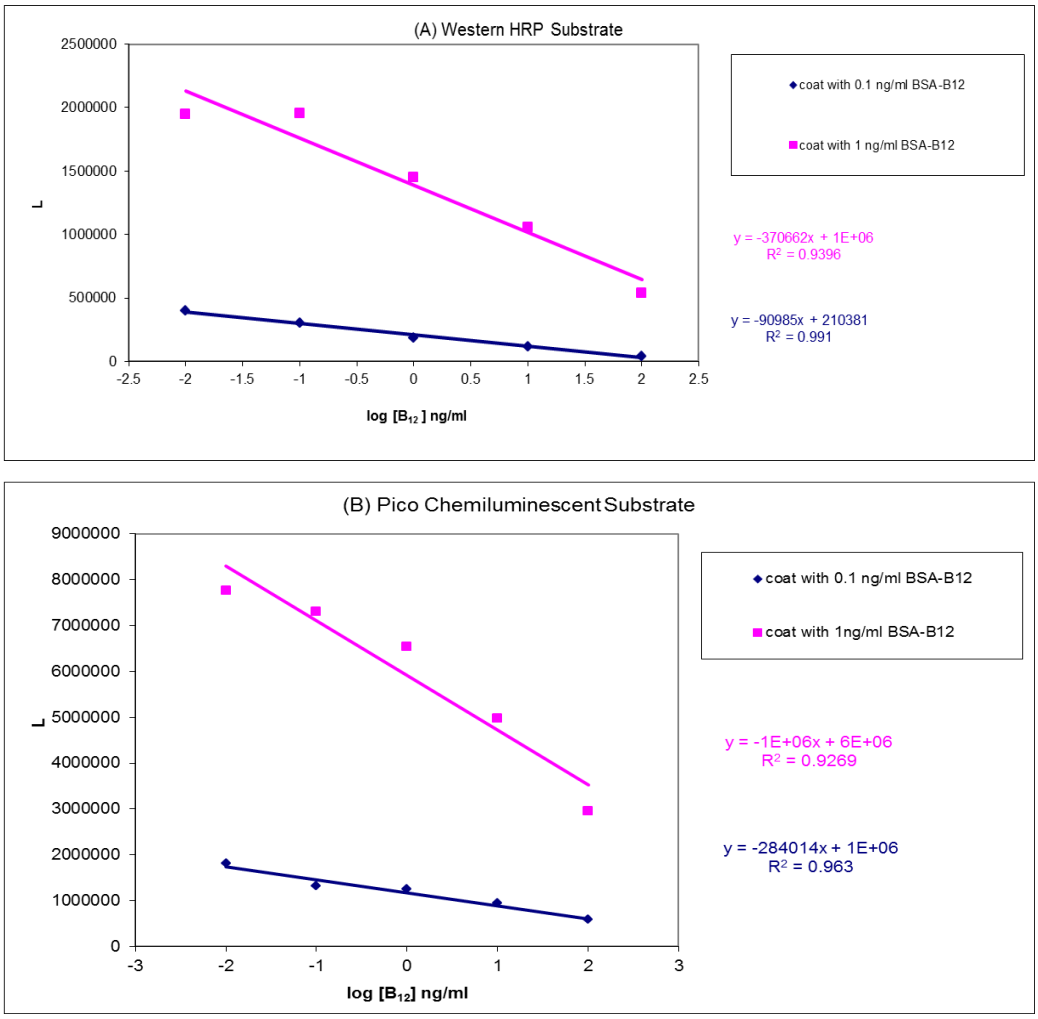


Figure 2-8. Chemiluminscence enzyme immunoassays using (A) Western HRP substrate (B) Pico chemiluminescent substrate. A 96-well white microtiter plate was coated with 100µl of 0.1 ng/ml and 1 ng/ml BSA-B_{12}. Then, the plate was washed with washing solution for three times and blocked with blocking buffer solution for 3 hours at room temperature. The plate was washed as before, and then 50 µl of 1:500,000 diluted primary vitamin B_{12} antibody and 50 µl B_{12} standard were added into each well and the plate was incubated at room temperature for 1 hour. After the immunoreaction was completed and the plate was washed 3 times again, 100 µl of 1:16000 diluted HRP labeled goat anti-rabbit IgG secondary antibody was added to bond with the primary rabbit antibody forming the immune-complex (BSA-B_{12})-primary antibody-secondary IgG-HRP. After washing 3 times, 50 µl western HRP substrate or pico chemiluminescent substrate were added and the enzyme catalyzed reaction was allowed for 2 min in dark. The luminescence of the light was measured by plate reader.

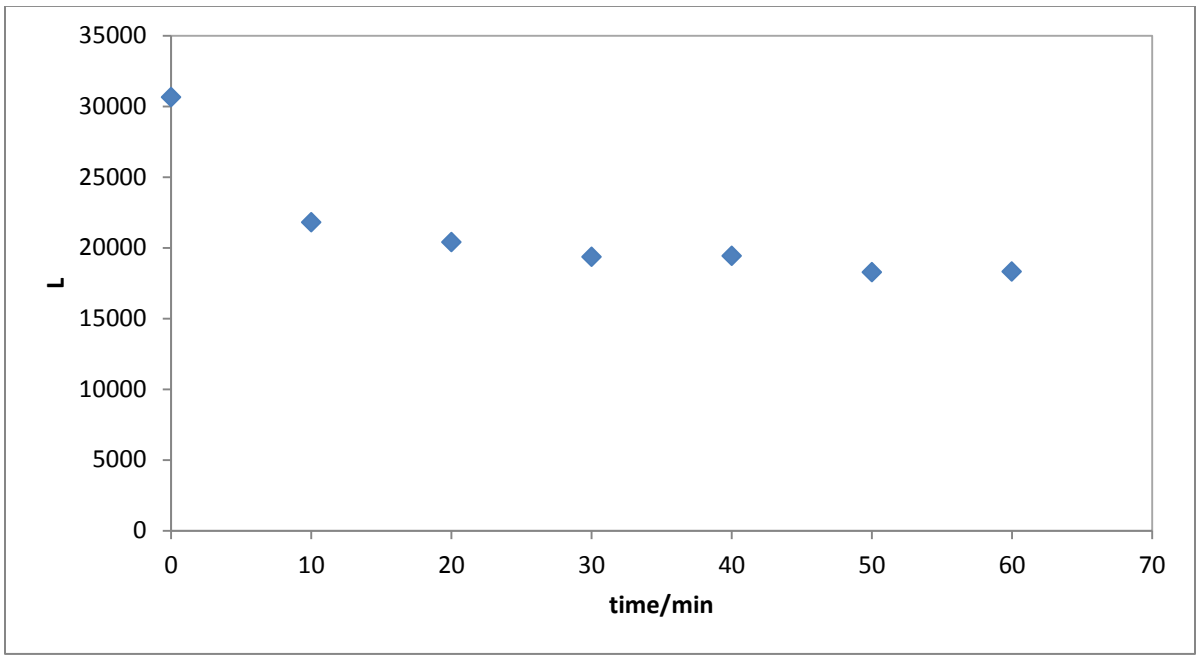


Figure 2-9. HRP activity with time in chemiluminescence enzyme immunoassay. 20 µl of Pico chemiluminescence substrate and 20 µl 1:1,000,000 diluted IgG-HRP were added into each well. One signal was detected every 10 min.

Chapter 3

B_{12} Distributions in Marine Sediments

3.1 Introduction

Vitamin B_{12}, thiamin and biotin are considered to be micrountrients to regulate phytoplankton growth in aquatic ecosystem. In the past decades, many studies of vitamin B_{12} concentrations and distributions in seawater and lake freshwater have been conducted to investigate the effect of this nutrient on phytoplankton abundance and species (Droop 1957, Carlucci and Silbernagel 1966; Guillard 1968; Swift 1981, Croft et al. 2005; Tang et al. 2010; Koch et al. 2011, 2012; Sañudo-Wilhelmy et al. 2012). However, the source of vitamin B_{12} in marine ecosystem is still not completely understood. A few studies showed that the concentration of vitamin B_{12} in the surface sediment is much higher than that in the overlying water column (Ohwada and Tage, 1969; Nishijima and Hata, 1988), implying a potential sedimentary source of vitamin B_{12} in marine systems. In this work, the distribution and transport patterns of B_{12} in sediments at fine scales were studied by using the new developed ELISA methods, a B_{12} concentration maximum zone associated with the oxic-anoxic interface in marine sediment was discovered for the first time. Seasonal variations of B_{12} distribution in seawater and marine sediments were also investigated.

3.2 Experiment Section

3.2.1 Study Sites and Sample Collection

Coastal seawater samples were collected from Long Island Sound site 5 and 6 (Fig. 3-1) at different depths in March and May 2012. The water samples were collected in brown polyethylene bottles and filtered through 0.2 µm polycarbonate membrane filters once they were delivered to the laboratory. The water salinity, temperature and dissolved oxygen were also simultaneously measured using CTD during water sample collection. Sediment cores were collected from Long Island Sound site 5 and 6 in March and May 2012 using 15.4 cm diameter corers. The sediment cores were treated as soon as possible when they were delivered to the laboratory or stored in cold room at 15˚C with air irrigation and processed within several weeks. Sediments collected in a coastal mudbank near Sinnamary, French Guiana was used to set up vitamin B_{12} absorption experiments.

3.2.2 C_{18}-SPE of B_{12} from Seawater

A carbon-18 (C_{18}) solid phase extraction column was prepared using Bondesil C_{18} resin and a 3.0 ml polypropylene column. 1.0 gram of C_{18} resin was mixed with methanol, and the C_{18} slurry was loaded into an empty column. The column was equilibrated by consecutively eluting with 10 ml methanol and 20 ml distilled water. The seawater sample was filtered through 0.2 µm polycarbonate membrane filter, its pH value was adjusted to 6.2 using 0.1 M HCl. Then 1000 ml seawater sample was passed through the C_{18} column at a flow rate of approximately 1 ml/min. The salt and other inorganic ions were removed by running 10 ml distilled water through the column. The wet C_{18} column was dried by a N_2 stream for 20 min, and the enriched vitamin B_{12} in the column was eluted with 5 ml methanol. The eluent was then dried with a gentle stream of

N_2 to eliminate methanol and the B_{12} residue was redissolved in 0.5 ml 0.01 M PBS solution. The B_{12} concentrations in the concentrated samples were then detected by ELISA.

3.2.3 Sediment Sample Treatment

The sediment core was sliced at 1 cm interval and the porewater at different depths was obtained by centrifugation of sediment at 5,000 rpm for 10 min and filtered through 0.2 μm polycarbonate membrane filter. 50-100 ml of the filtered porewater was preconcentrated by C_{18}-SPE to 0.5 ml by the procedure described in Section 3.2.2. The final 0.5 ml concentrated B_{12} sample was measured by ELISA.

3.2.4 Vitamin B_{12} Measurement

All the preconcentrated vitamin B_{12} from seawater and porewater samples were directly measured by ELISA method 1 or 2 described in Chapter 2, section 2.2.3.

3.2.5 Adsorption and Desorption of B_{12} on Particles

The adsorption-desorption behavior of trace elements is of vital importance in their transport and fate. The sediment used in this experiments was surface sediment obtained from LIS site 5 (0-2 cm; salinity = 27 - 28) and stored at 15 ˚C in a cold room. Before experiments, sediment was gently homogenized by hand mixing and sieved through 1 mm sieve (no water added). Adsorption and desorption experiments were conducted as follows.

Experiment 1: *Adsorption and desorption of B_{12} on particles*: 200 g wet sediment was added into two bottles respectively, labeled S (sample) and C (control), and then centrifuged at 5,000 rpm for 10 min. About 70 ml porewater was collected from each bottle, labeled with S1

and C1. 70 ml seawater was then added into each bottle. 100 ng vitamin B_{12} was added into S bottle, and no B_{12} was added into the control bottle C. Then the two samples were vigorously stirred 30 min to make them homogeneous. The samples were centrifuged a second time, the porewater was collected and labeled S2 and C2. 70 ml seawater was added into each bottle, and then samples were vigorously stirred 30 min. Samples were centrifuged third time, and porewater was collected, labeled S3 and C3. The process of addition of seawater and centrifugation were repeated two more times, and S4, C4, S5, and C5 were collected. Then, the collected samples were centrifuged at 7,000 rpm for 15 min, and filtered through 0.2 μm polycarbonate membrane filter. Concentrations of vitamin B_{12} in these samples were detected by ELISA.

Experiment 2: *Study of adsorption reversibility*: 200 (or 600) ng of vitamin B_{12} was added into 2 g of wet sediment, and then the sample was vigorously stirred to make it homogeneous. After shaking for 1 hour at room temperature, the porewater and particles were separated by centrifugation at 5,000 rpm for 10 min. The porewater was removed and then the same volume of seawater added to the tube and the sediment was stirred and mixed well again, shaken for 1 hour at room temperature and then separated by centrifugation. The separated seawater was collected. After successive additions of seawater and centrifugation for 5 times, a series of desorption solutions were collected. The adsorption and desorption of vitamin B_{12} on particles was evaluated by comparing the content of vitamin B_{12} in these desorbed solutions with the added vitamin B_{12}. Control experiments were conducted at the same time.

Experiment 3: *Adsorption equilibration time*: 1,000 ng B_{12} (×8) was mixed with 1 g of wet sediment in 8 separate samples. The samples were vigorously shaken at room temperature for 0, 20, 40, 60, 120, 240, 360, 480 min, respectively, then the porewater and particles were separated by centrifugation at 5,000 rpm for 10 min. The porewater was collected, filtered through 0.2 μm polycarbonate membrane filter and measured with ELISA.

3.2.6 Effect of Temperature on Adsorption

Because the activity of bacteria is strongly lowered by low temperature, in order to prove that the decreased B_{12} is caused by adsorption instead of the consumption by bacteria, adsorption experiments were conducted at different temperatures (0 ºC and room temperature). Two sets of samples were prepared: 0, 10, 100, 1000, and 10000 ng B_{12} were added into 2 g wet sediment, respectively, and 1 ml seawater was added into each sample. One set of samples were shaken at 0 ºC, and the other one was at room temperature. After 1 hour, samples were centrifuged at 5,000 rpm for 10 min at 0 ºC and room temperature, respectively. About 1.5 ml porewater was collected from each sample, and filtered through 0.2 μm polycarbonate membrane filter. Concentrations of B_{12} in samples were measured by ELISA.

3.2.7 Anoxic Sediment Incubation

Sediment collected from French Guiana was used to conduct this experiment. This sediment was anoxic (suboxic) and nonsulfidic. The sediment was homogenized by hand and then transferred into 20 centrifuge tubes. Each centrifuge tube was filled with 40 ml wet sediment and then the 20 tubes were sealed and buried in wet anoxic sediment for serial anoxic incubation. Two tubes were taken out each week to collect the porewater sample. When the tubes

were removed from the anoxic environment, they were centrifuged at 5,000 rpm for 15 min, and ~40 ml porewater was collected and filtered through a 0.2 μm polycarbonate membrane filter. The porewater was preconcentrated by C_{18} column for ELISA measurement. The anoxic sediment incubation experiment lasted for 10 weeks and 10 porewater samples were collected.

3.3 Results and Discussion

3.3.1 B_{12} Distributions in Water Column

The distribution profiles of B_{12} in coastal seawater were determined. The seawater samples were collected from Long Island Sound site 5 and 6 at different depths in March and May 2012, respectively. Site 5 is about 15 m deep and closer to the shore, site 6 is about 25 m deep (Fig. 3-1). The B_{12} distribution profiles are shown in Figure 3-2. It can be seen that vitamin B_{12} concentrations in seawater were very low for all the sites and seasons, lower than 4 pg/ml (3 pM), but they still showed spatial and temporal information. B_{12} concentrations in March were higher than those in May for both sites, implying the phytoplankton abundance was higher in May and more B_{12} was consumed. A slight B_{12} concentration minimum zone, between 5 to 10 m deep, was observed in most profiles, indicating the phytoplankton abundance in this layer was higher than that on the sea surface. The B_{12} concentrations in all the bottom water samples were higher than surface water and increased with depth, implying that the source of B_{12} may come from the bottom sediments. Also, phytoplankton uptake is decreased with depth.

3.3.2 B_{12} Distributions in Sediment Porewater

By using the specific and accurate ELISA methods, the vitamin B_{12} distribution patterns in the sediments obtained from different seasons and sites were measured, the results are

summarized in Figure 3-3 (A). B_{12} concentration in the overlying water was very small, consistent with results shown in Figure 3-2. However, B_{12} concentration significantly increased in surface sediment and reached a maximum at about 2 cm depth, and then sharply decreased with depth. B_{12} concentration within the maximum zone was about 5 – 10 times than that in seawater, but decreased to a comparable level below 4 cm depth. For the first time, significant B_{12} concentration gradients below the water-sediment interface and maxima at about 1 – 3 cm depth were found in sediment cores during the two seasons, suggesting that bacteria at or near the oxic-anoxic boundary may be involved in the generation of vitamin B_{12}. It has been hypothesized that the heterotrophic bacteria are the primary vitamin B_{12} producers in the ocean, earlier studies observed that B_{12}-auxotrophic phytoplankton could grow in B_{12}-deficient media in the presence of marine bacteria such as *Halomonas sp.* (Fogg, 1966) which has been isolated from surface deep-sea sediments (Mabinya et al., 2011; da Silva et al., 2013). The decrease of the B_{12} concentrations above and below the maximum zone may be attributed to the following reasons: 1) the bacteria have higher activities in the anoxic B_{12} maximum zone, 2) B_{12} can be significantly absorbed by the oxic FeO(OH) and MnO_2 particles. Generally, proton maximum and CO_2 maximum zones could be observed just below oxic-anoxic boundary due to the decomposition of organic matters in the surface sediments (Zhu et al., 2005, 2006a,b, 2010). Interestingly, the B_{12} maximum zone overlaps well with CO_2 maximum which may serve as C source for the marine bacteria to generate vitamin B_{12} (Croft et al., 2005). Another phenomenon should be pointed out that dissolved iron (Fe^{2+}) and manganese (Mn^{2+}) also showed concentration maxima just below oxic-anoxic interface due to the redox reactions of these reduced metal ions with dissolved oxygen, the overlap of B_{12} maximum zones with Fe^{2+} and Mn^{2+} maximum zones indicate that the dissolved form vitamin B_{12} in the oxic sediment

porewater might be precipitated (absorbed) by FeO(OH) and MnO_2 particles during the oxidation of Fe^{2+} and Mn^{2+}. Because of the high B_{12} concentration in the maximum zone, we can reasonably hypothesize that, baring total uptake by aerobic bacteria within the thin oxic zone, dissolved B_{12} can flux to overlying water through the oxic sediment zone and serve as a source of B_{12} in water column. However, the magnitude of the B_{12} flux across the oxic sediment layer and water-sediment interface not only depends on the B_{12} concentration gradients between water and sediment and uptake by aerobes, but also depends on the adsorption capacities of sediment particles to B_{12} and the adsorption properties such as reversible or irreversible absorption. Although these B_{12} profiles may provide novel insights into the source, cycling and transport of vitamin B_{12} between water and sediment, the adsorption of B_{12} on sediment particles and the distribution between sediment porewater and particles need to be studied in order to further elucidate B_{12} cycling.

The B_{12} distribution profile shown in Figure 3-3 (B) was obtained from a heavily bioturbated sediment core. The results showed that vitamin B_{12} distribution pattern was associated with networks of macrofaunal biogenic structures, and the increase of vitamin B_{12} at 4 and 7 cm depth was attributed to burrows and tubes. Because the oxygen was introduced deep anoxic sediment through macrofaunal irrigation, complicated three dimensional suboxic zones could be created in the deep sediment. Marine bacteria within these suboxic-anoxic zones presumably produced additional vitamin B_{12} in deep sediment. The B_{12} in deep sediment may flux into burrow water and be transported to overlying through worm irrigation.

3.3.3 Adsorption of B_{12} on Sediment Particles

Adsorption-desorption of vitamin B_{12} on sediment particles were studied to elucidate B_{12} reactions in sediment. Three experiments were conducted in order to study the adsorption-desorption of vitamin B_{12} on sediment particles. The first experiment was performed by adding small amount of standard vitamin B_{12} (100 ng) into large amount of wet sediment (200 g), accompanying by a control experiment, to mimic B_{12} transport in sediment. The results in Figure 3-4 indicated that the natural produced vitamin B_{12} in the 70 ml porewater extruded from 200 g homogenized sediment was about 0.68 ng. When 100 ng B_{12} was vigorously mixed with the sediment S, over 99% vitamin B_{12} was immediately absorbed on the particles. Only 0.9, 0.9, 0.7, and 0.8 ng B_{12} was removed from the particles by the 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} wash step, respectively, and there was still 96% vitamin B_{12} adsorbed on particles even after washing 4 times. These results demonstrate that over 99% of vitamin B_{12} in marine sediment is absorbed on the particles because the naturally produced vitamin B_{12} in the 200 g wet sediment is expected to be less than 100 ng, and the B_{12} adsorption appears to be irreversible under real conditions. On the other hand, when the control sediment was washed by natural seawater, the vitamin B_{12} concentration in the 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} washed solution, respective 0.0061, 0.0055, 0.0045, and 0.0028 ng/ml, is higher than the natural B_{12} concentration in seawater (Fig. 3-2). Because the B_{12} concentration in control porewater C1 is only 0.0097 ng/ml, if we assume B_{12} concentration in the last wash step (0.0028 ng/ml) is close to the natural B_{12} level in seawater according to Figure 3-2, the B_{12} extracted from sediment particles in the 1^{st}, 2^{nd} and 3^{rd} time wash step is 34%, 28% and 18% of the natural B_{12} concentration in porewater. Because the B_{12} concentration in porewater is much higher than that in seawater, we conclude that vitamin B_{12} could be removed from sediment and dissolve in seawater if the sediment particles are resuspended.

The second experiment was conducted by adding large amounts of standard vitamin B_{12} (200 and 600 ng) into small amounts of wet sediment (2 g), to study the adsorption reversibility of B_{12} on particles and mimic the conditions of B_{12} transport in B_{12} maximum zone. The results in Figure 3-5 indicated that when 200 ng vitamin B_{12} was applied to 2 g homogenized sediment, 139 ng B_{12} (70% of original addition) was immediately absorbed onto the particles. However, the wash steps could efficiently desorb B_{12} from the particles, the 5 steps subsequently wash removed 61, 43, 32, 21 and 10 ng B_{12} from the particles, respectively. After the 5 times wash (more wash was not tested), only 33 ng vitamin B_{12} remained on sediment particles, suggesting that the desorption of B_{12} from particles was easier when the ratio of B_{12} concentration to sediment amount was high. Under this condition, the B_{12} adsorption-desorption procedure was likely reversible. The adsorption capacity of sediment particle to vitamin B_{12} was tested by adding more B_{12} into 2 g wet sediment. When 600 ng B_{12} was mixed with 2 g wet sediment, about 84.5% (507 ng) of the added B_{12} could be initially adsorbed on the particles (Fig. 3-5), demonstrating the high adsorption capacity of the sediment. Similarly to 200 ng B_{12} adsorption experiment, the 507 ng adsorbed B_{12} on particles could also be efficiently desorbed by washing with seawater. After 5 times seawater washing, about 295 ng B_{12} remained on the particles.

Equilibration time for vitamin B_{12} adsorption is described in Figure 3-6. When B_{12} was added into the sediment, it was immediately adsorbed by the particles and reached the highest adsorption rate. However, along with the sample shaking at temperature, the adsorption decreased, and the adsorption-desorption equilibrium could be developed at 20 min.

The effect of temperature on B_{12} adsorption was also tested. Various amounts of vitamin B_{12} were separately added into 2 g wet sediment, the mixtures were shaken for 1 hour at 0 ºC and room temperature, respectively. The B_{12} adsorption results are summarized in Figure 3-7. It can

be seen that more B_{12} was adsorbed on the particles at lower temperature, following the general Langmuir Isotherm, i.e. an increase in adsorption with a decrease in temperature. This experiment also further demonstrated the high adsorption capacity of the sediment particles to B_{12}. When the concentration of B_{12} was low, a high percentage of B_{12} was adsorbed. The adsorbed percentage decreased with the increase of B_{12} added amount, but the absolute adsorbed B_{12} increase. For example, when 10,000 ng B_{12} mixed 2 g sediment, 4000 ng B_{12} (40% of added amount) can be adsorbed by 2 g sediment at 0 ºC. These results also further demonstrated that most of the natural produced vitamin B_{12} in marine sediment was adsorbed on the particles.

The effect of bacteria on vitamin B_{12} during adsorption experiments might be evaluated by Figure 3-7. All the adsorbed B_{12} data were calculated by measuring the B_{12} concentration in solution after centrifuging. Because vitamin B_{12} is a micronutrient, it may be consumed by microorganisms during experiment to result in an "apparent high adsorption". Generally the bacteria show higher activity at room temperature than 0ºC, so if bacteria consume B_{12}, the "apparent adsorption" of B_{12} on particles at room temperature should be higher than at 0ºC. The results in Figure 3-7 showed that the adsorption of B_{12} at room temperature is lower than that at 0 ºC, indicating the effect of bacteria on B_{12} (or the B_{12} consumption by bacteria) is minor during the experiment and that behavior is dominantly physically controlled.

Based on the B_{12} distribution profiles in Figure 3-3 and specifically the B_{12} maximum zone below oxic-anoxic boundary and B_{12} adsorption patterns in sediment, we hypothesize that vitamin B_{12} is produced by marine bacteria in anoxic condition but is almost completely removed (>99%) from porewater through particle adsorption during its flux to overlying water. In order to prove this hypothesis, anoxic sediment incubation experiment was conducted. The sediment used in this experiment was collected from French Guiana. The reason we choose this sediment is

because of the higher B_{12} and iron concentrations. The anoxic sediment incubation experiment lasted for 10 weeks and concentrations of vitamin B_{12} in sediment porewater were measured every week. The variation of vitamin B_{12} concentration along with incubation time is shown in Figure 3-8. The concentration of B_{12} in the porewater was the lowest before the anoxic incubation experiment, and it increased during the incubation. Porewater B_{12} concentration increased from 0.07 to 0.22 ng/ml in the first week and remained almost constant in the first five weeks. However, at week 6, there was a significant increase of B_{12} concentration in sediment porewater, increased to 0.35 ng/ml. After 6 weeks, concentrations of B_{12} kept a high constant value. The increased release was closely associated with a rapid increase of dissolved Fe^{2+} measured in a second incubation series (data not shown). The sharp increase of vitamin B_{12} may be because of the release of vitamin B_{12} from iron particles which were reduced to Fe^{2+} during the incubation. We assume that a critical quantity of Fe (II) was achieved in the solid FeO(OH) that allowed release of B_{12} to solution at week 6.

3.4 Conclusions

The distributions of B_{12} in seawater have a seasonal variation, as demonstrated in Long Island Sound. Phytoplankton and benthic sources can influence the vertical pattern of B_{12}. B_{12} concentrations in March were higher than those in May, implying the phytoplankton abundance was higher in May and more B_{12} was consumed. B_{12} concentrations were lower in surface water and higher in bottom water, implying that the source of B_{12} may come from the bottom sediments and that there is less photosynthetic uptake.

Vitamin B_{12} has a higher concentration in sediment porewater than in overlying seawater. Significant B_{12} concentration gradients below the water-sediment interface and maxima at about 1 – 3 cm depth are found in sediments during two seasons, suggesting that bacteria at the oxic-anoxic boundary, and in particular the suboxic zone, may be involved in the generation of vitamin B_{12}. In addition, the redox reactions of metals such as iron across oxic-anoxic boundary may also play a critical role in the B_{12} distribution pattern. Vitamin B_{12} distribution is also associated with networks of macrofaunal biogenic structures. B_{12} profiles provide insight into the source, cycling and transport of vitamin B_{12} in marine ecosystem.

The study of adsorption-desorption of vitamin B_{12} on sediment particles demonstrated that over 99% vitamin B_{12} was adsorbed on particles in marine sediments, and the adsorption was likely irreversible under real conditions. However, when the concentration of B_{12} is high in the sediment, the adsorption of B_{12} on the particles may shift toward reversible side. The adsorption and desorption processes of B_{12} can reach equilibrium at about 20 min in sediment at room temperature, and follow general Langmuir adsorption Isotherm.

3.5 Future work

The distribution patterns of vitamin B_{12} in seawater and sediment porewater has been studied in this research. Also, the adsorption-desorption behavior of vitamin B_{12} has been primarily revealed. Results in this study have shown that bacteria and physical processes could influence the distribution of B_{12}. Adsorption-desorption behavior of B_{12} on particles have demonstrated strong physical processes of B_{12} in sediment, and no direct experiments of the relationship between bacteria and vitamin B_{12} are conducted, therefore, physical processes of B_{12} in sediment are hypothesized to be the dominant factor influencing the distribution patterns of B_{12} in sediment. However, further studies need to figure out how bacteria influence the production and consumption rate of B_{12}, and how physical and chemical processes control the profile of B_{12} in sediment. Bacteria incubation experiments are required to calculate the production and consumption rate of B_{12}. B_{12} flux experiments between sediment and seawater are required to know more about the diffusion, transport and distribution of B_{12}.

References

Carlucci, A. F., and S. B. Silbernagel. 1966. Bioassay of seawater. 3. Distribution of vitamin B_{12} in the Northeast Pacific Ocean. Limnol. Oceanogr. 11: 642-646.

Croft, M. T., A. D. Lawrence, E. Raux-Deery, M. J. Warren, and A. G. Smith. 2005. Algae acquire vitamin B-12 through a symbiotic relationship with bacteria. Nature 438: 90-93.

Da Silva, M.A.C., et al. 2013. Phylogenetic identification of marine bacteria isolated from deep-sea sediments of the eastern South Atlantic Ocean. SpringerPlus 2:127.

Droop, M.R. 1957. Vitamin B_{12} in marine ecology. Nature 180:1041-1042.

Fogg, G. E. 1966. The extracellular products of algae. Oceanographic Marine Biological Annual Review 4**:** 195-212.

Guillard, R. R. 1968. B_{12} specificity of marine centric diatoms. J. Phycol. 4: 59-64.

Koch, F., A. Marcoval, C. Panzeca, K. W. Bruland, S. A. Sanudo-Wilhelmy, and C. J. Gobler. 2011. The effects of vitamin B_{12} on phytoplankton growth and community structure in the Gulf of Alaska. Limnol. Oceanogr. 56:1023-1034.

Koch, F., T. K. Hattenrath-Lehmann, J. A. Goleski, S. A. Sanudo-Wilhelmy, N. S. Fisher, and C. J. Gobler. 2012. Vitamin B_1 and B_{12} uptake and cycling by plankton communities in coastal ecosystems. Frontiers Aquatic Microbiology. October 12, 2012 | doi: 10.3389/fmicb.2012.00363.

Mabinya. L.V., Cosa, S. Mkwetshana, N. and Okoh, A. I. 2011. *Halomonas* sp. OKOH—A Marine Bacterium Isolated from the Bottom Sediment of Algoa Bay—Produces a Polysaccharide Bioflocculant: Partial Characterization and Biochemical Analysis of Its Properties. Molecules. 16:4358-4370.

Nishijima T, and Hata Y. 1988. Distribution of vitamin B_{12}, thiamine, and biotin in the sediment of Harima-Nada Sea. Nippon Suisan Gakkaishi. 54(3): 511-516.

Ohwada, K. and Taga, N. 1969. Distribution of vitamin B_{12}, thiamine and biotin in marine sediments. J. Oceanogr. Soc. Japan. 25:123-136.

Sañudo-Wilhelmy, S. A., L. S. Cutter, R. Durazo, E. A. Smail, L. Gómez-Consarnau, E. A. Webb, M. G. Prokopenko, W. M. Berelson, and D. M. Karl. 2012. Multiple B-vitamin depletion in large areas of the coastal ocean. Proceedings of the National Academy of Sciences. 109(35): 14041-14045.

Swift, D. 1981. Vitamin levels in the Gulf of Maine and ecological significance of vitamin B_{12} there. J. Mar. Res. 39:375-403.

Tang, Y. Z., F. Koch, and C. J. Gobler. 2010. Most harmful algal bloom species are vitamin B-1 and B-12 auxotrophs. Proc. Natl. Acad. Sci. U. S. A. 107: 20756-20761.

Zhu, Q. Z., R. C. Aller, and Y. Fan 2005. High-performance planar pH fluorosensor for twodimensional pH measurements in marine sediment and water. Environ. Sci. Technol., 39, 8906-8911.

Zhu, Q. Z., R. C. Aller, and Y. Fan. 2006a. Two dimensional pH distributions and dynamics in bioturbated marine sediments. Geochim. Cosmochim. Acta, 70, 4933-3939.

Zhu, Q. Z., R. C. Aller, and Y. Fan. 2006b. A new ratiometric, planar fluorosensor for measuringhigh resolution, two-dimensional pCO_2 distributions in marine sediments. Mar. Chem. 101, 40-53.

Zhu, Q. Z. and R. C. Aller. 2010. A Rapid Response Planar Fluorosensor for Measuring Two-Dimensional pCO_2 Distribution and transient Dynamics in Marine Sediments. Limnol. Oceanogra. Methods. 8: 326-336.

Zhu, Q. Z. and R. C. Aller. 2012. Two-dimensional dissolved ferrous iron distributions in marine sediments as revealed by a novel planar optical sensor. Mar. Chem. 136-137: 14-23.

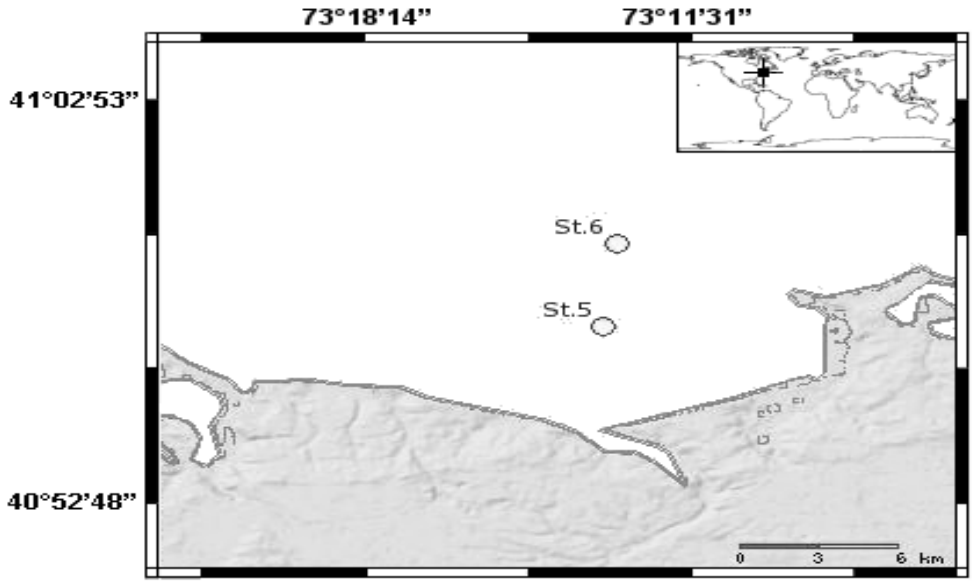


Figure 3-1. Location of sampling sites in Long Island Sound (Smithtown Bay).

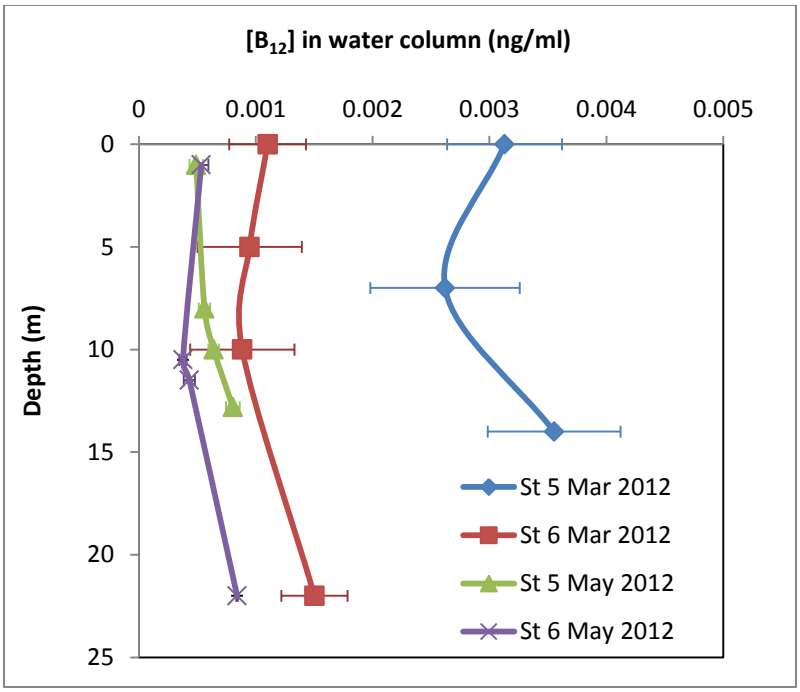


Figure 3-2. Vitamin B_{12} distribution profiles in LIS water column in different seasons. Seawater samples were obtained from Long Island Sound Site 5 and 6 in March and May 2012, respectively.

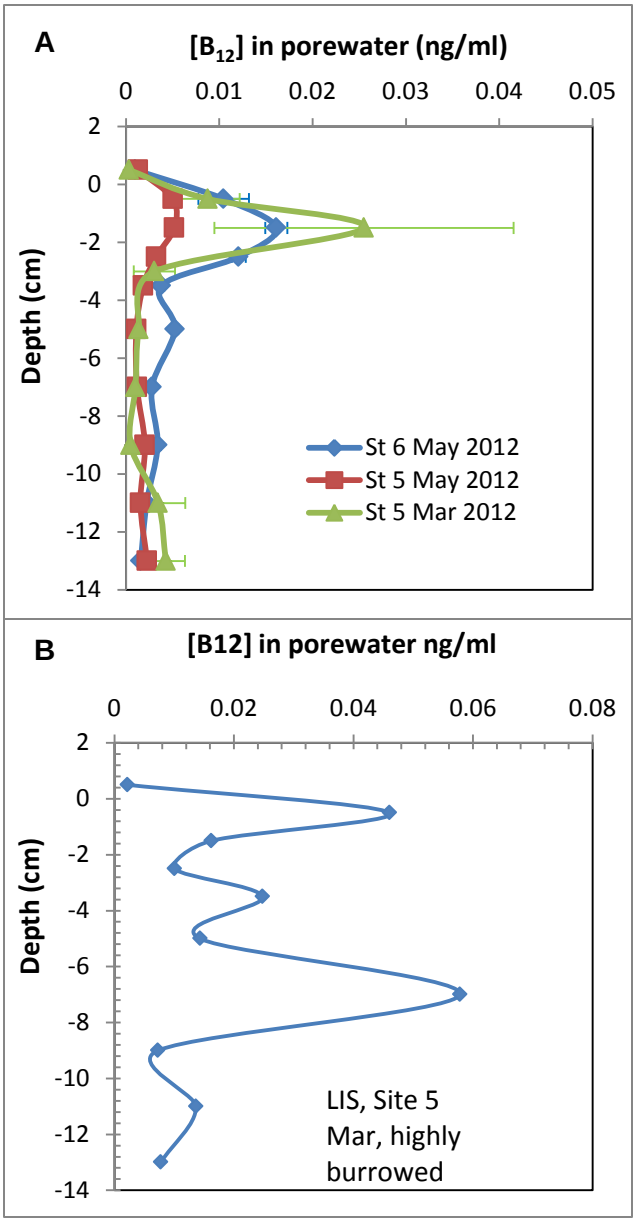


Figure 3-3. (A) Vitamin B_{12} vertical profiles in marine sediments which were obtained from Long Island Sound site 5 and 6. Maximum zone of vitamin B_{12} between 1 and 2 cm can be clearly seen from all profiles. (B) Vitamin B_{12} profiles in bioturbated sediment from Long Island Sound site 5.

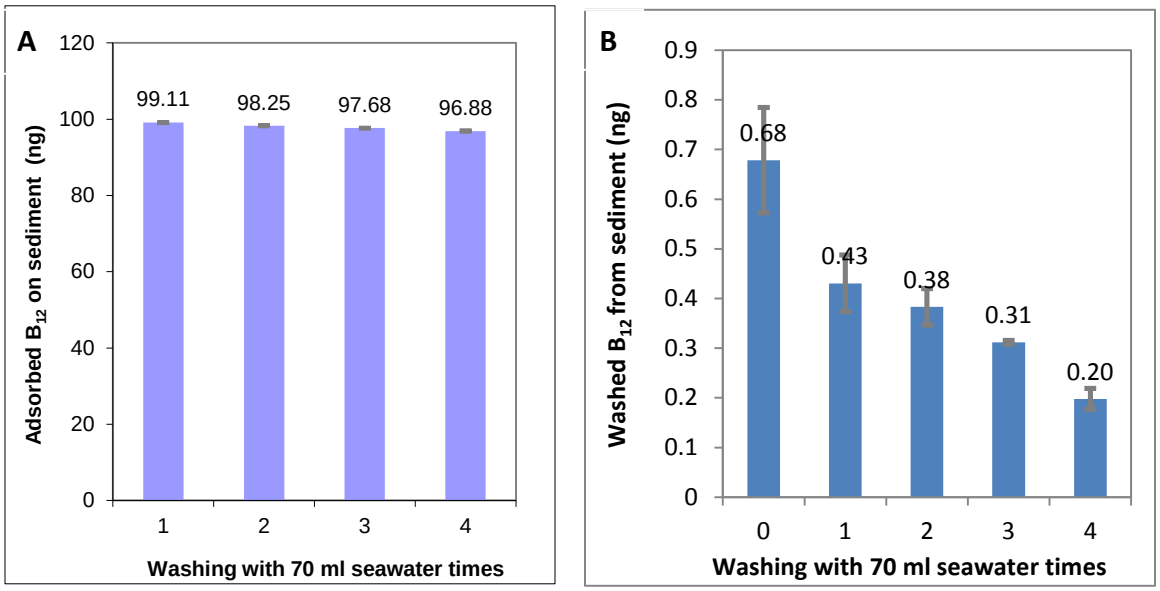


Figure 3-4. Adsorption and desorption of B_{12} on large amount of sediment particles. 200 g wet sediment was added into sample bottle (A) and control bottle (B), respectively, and then centrifuged at 5000 rpm for 10 min. About 70 ml porewater was collected from each bottle, labeled S1 and C1. 70 ml seawater was then added into each bottle. 100 ng vitamin B_{12} was added into sample bottle, and the control one was without B_{12}. Then the two samples were vigorously stirred 30 min to make them homogeneous. Then the samples were centrifuged second time, and the porewater was collected, labeled S2 and C2. 70 ml seawater was added into each bottle, and then samples were vigorously stirred 30 min. Samples were centrifuged third time, and porewater was collected, labeled S3 and C3. The process of addition of seawater and centrifugation were repeated two more times, and S4, C4, S5, and C5 were collected. Then, the collected samples (S1 to S5, and C1 to C5) were centrifuged at 7000 rpm for 15 min, and filtered through 0.2 μm polycarbonate membrane filter. Concentrations of vitamin B_{12} in these samples were detected by ELISA.

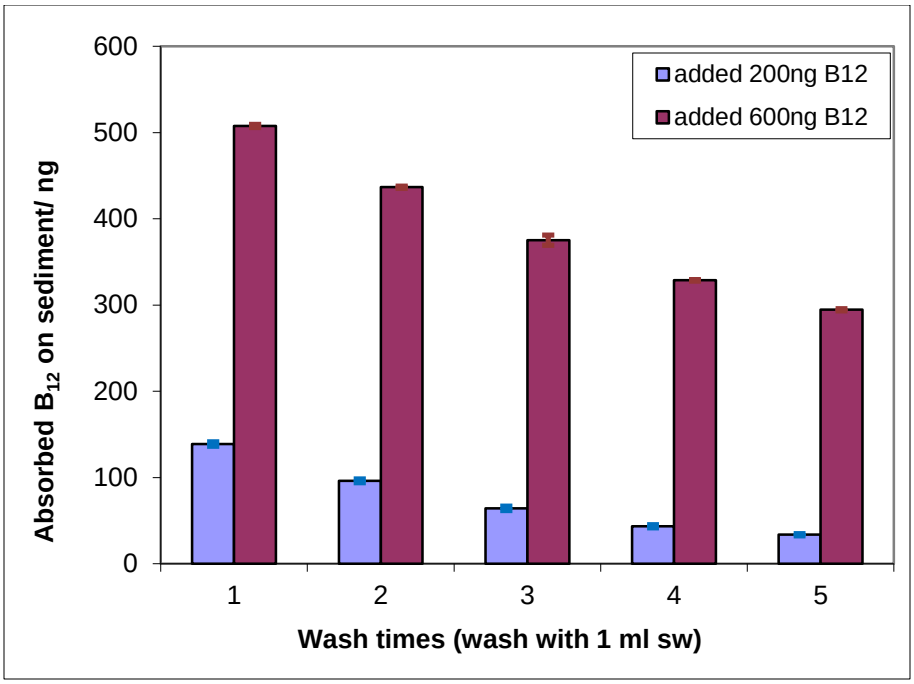


Figure 3-5. The Adsorption and desorption of vitamin B_{12} on small amount of sediment particles. 200 ng (or 600 ng) B_{12} mixed with 2 g of wet sediment. After shaking for 1 hour at room temperature, the porewater and particles were separated by centrifugation at 5000 rpm for 10 min. The porewater was removed and then 1 ml seawater was added to the tube and the sediment was stirred and mixed well again, shaken for 1 hour at room temperature and then separated by centrifugation. After successive additions of seawater and centrifugation for 5 times, a series of desorption solutions were collected.

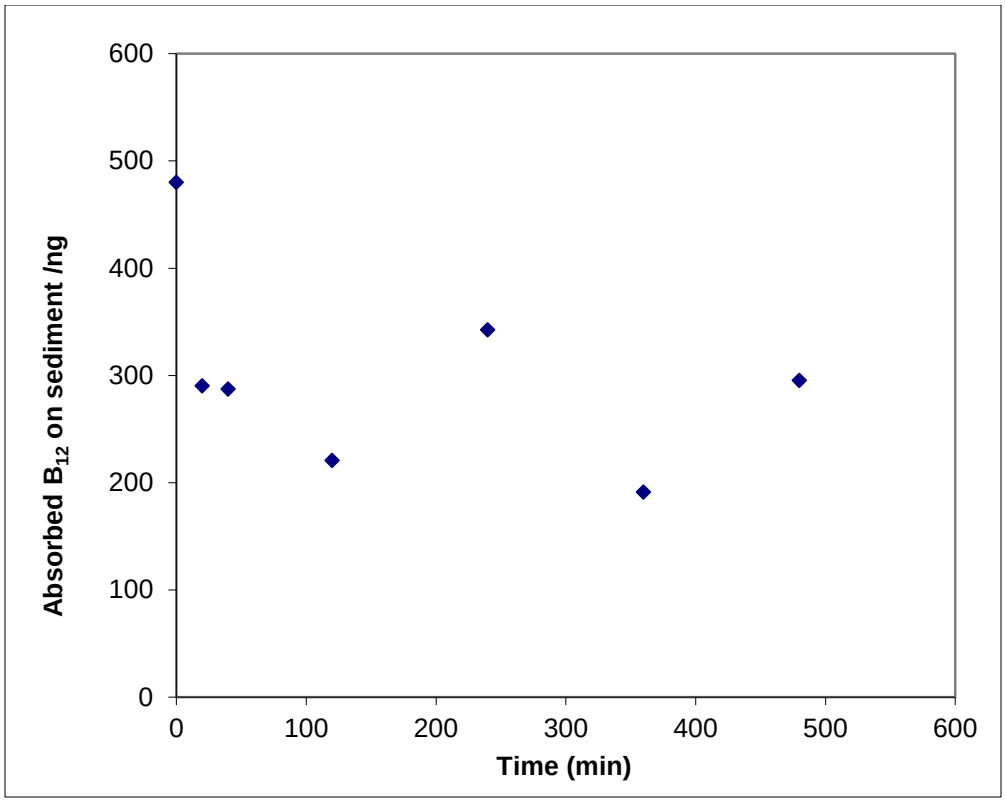


Figure 3-6. Adsorption equilibrium time of vitamin B_{12} on sediment particles. Mix 1000 ng B_{12} (×8) with 1 g of wet sediment (×8). The samples are vigorously shaken at room temperature for 0, 20, 40, 60, 120, 240, 360, 480 min, respectively.

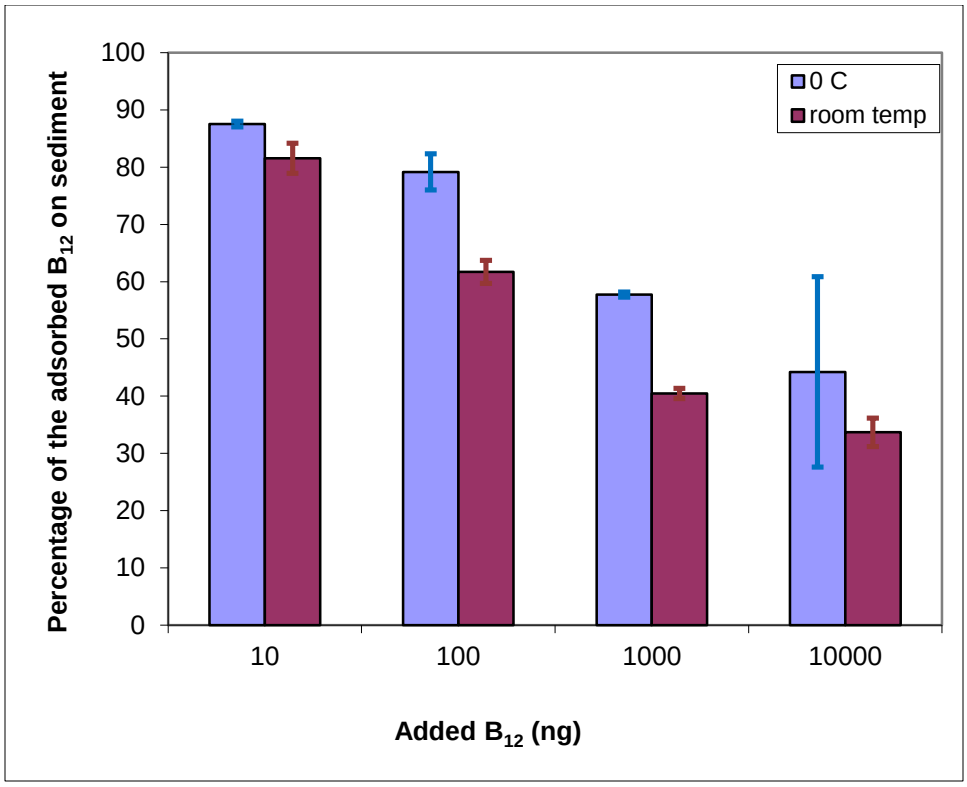


Figure 3-7. Effect of temperature on B_{12} adsorption. Two sets of samples were prepared: 0, 10, 100, 1000, and 10000 ng B_{12} were added into 2 g wet sediment, respectively, and 1 ml seawater was added into each sample. One set of samples were shaken at 0 ºC, and the other one was at room temperature. After 1 hour, samples were centrifuged at 5000 rpm for 10 min at 0 ºC and room temperature, respectively. About 1.5 ml porewater was collected from each sample, and filtered through 0.2 μm polycarbonate membrane filter. Concentrations of B_{12} were measured by ELISA.

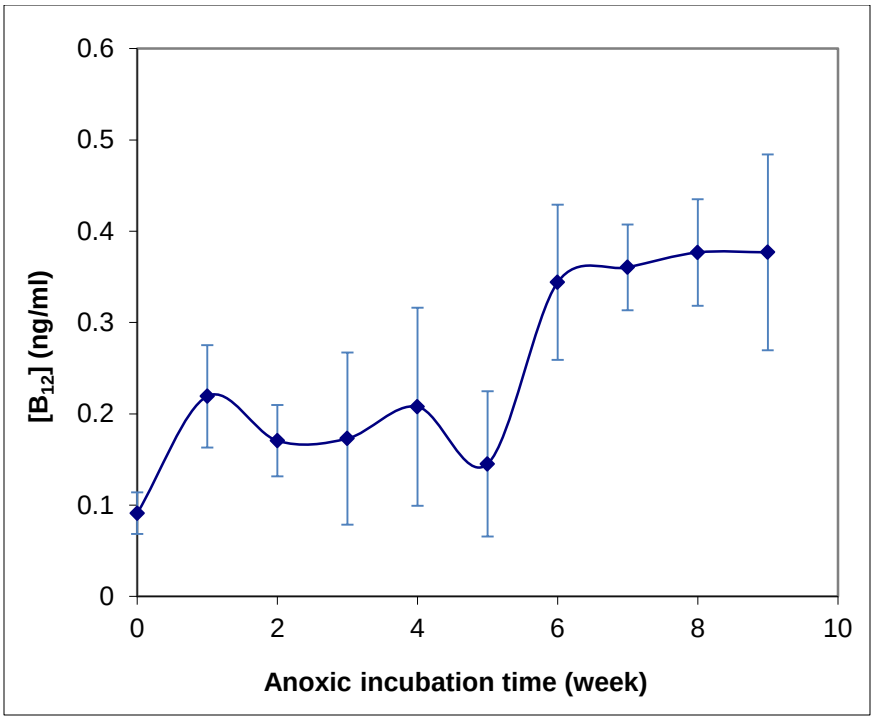


Figure 3-8. Anoxic sediment incubation experiment. 20 centrifuge tubes were filled with 40 ml wet sediment, respectively, and then the tubes were sealed and covered in sediment for anoxic incubation. Two tubes were taken out every week to make the porewater sample. When the tubes were taken out from the anoxic environment, they were centrifuged at 5000 rpm for 15 min, and about 40 ml porewater was collected in total and filtered through 0.2 μm polycarbonate membrane filter. The porewater was treated and preconcentrated for the measurement by ELISA. The anoxic sediment incubation experiment lasted for 10 weeks and 10 porewater samples were collected.

Bibliography

Anderson, B. B. 1964. Investigations into the Euglena method for assay of vitamin B_{12} in serum. Journal Clin Pathol. 17 : 14-26.

Anderson, D. J., B. Guo, L. M. Ng, L. J. Kricka, K. J. Skogerboe, D. S. Hage, L. Schoeff, J. Wang, L. J. Sokoll, and D. W. Chan. 1997. Clinical chemistry. Anal. Chem. 69: 165R-229R.

Bertrand, E. M., M. A. Saito, J. M. Rose, C. R. Riesselman, M. C. Lohan, A. E. Noble, P. A. Lee, and G. R. Ditullio. 2007. Vitamin B-12 and iron colimitation of phytoplankton growth in the Ross Sea. Limnol. Oceanogr. 52: 1079-1093.

Bertrand, E. M., Allen, A. E. 2012. Influence of vitamin B auxotrophy on nitrogen metabolism in eukaryotic phytoplankton. Frontiers in microbiology. 3, article 375.

Bonnet, S., E. A. Webb, C. Panzeca, D. M. Karl, D. G. Capone, and S. A. Sanudo-Wilhelmy. 2010. Vitamin B-12 excretion by cultures of the marine cyanobacteria Crocosphaera and Synechococcus. Limnol. Oceanogr. 55: 1959-1964.

Bruno, S. F., Staker, R. D., and Curtis, L. L. 1981. Seasonal variation of vitamin B_{12}, B_{12} analogs, and phytoplankton in a long island estuary. J. Mar. Res. 39 (2): 335-352.

Carlucci, A. F., and S. B. Silbernagel. 1966. Bioassay of seawater. 3. Distribution of vitamin B_{12} in the Northeast Pacific Ocean. Limnol. Oceanogr. 11: 642-646.

Croft, M. T., A. D. Lawrence, E. Raux-Deery, M. J. Warren, and A. G. Smith. 2005. Algae acquire vitamin B-12 through a symbiotic relationship with bacteria. Nature 438: 90-93.

Deng, A., M. Himmelsbach, Q. Z. Zhu, S. Frey, M. Sengl, W. Buchberger, R. Niessner, and D. Knopp. 2003. Residue analysis of the pharmaceutical diclofenac in different water types using ELISA and GC-MS. Environ. Sci. Technol. 37: 3422-3429.

Da Silva, M. A. C., et al. 2013. Phylogenetic identification of marine bacteria isolated from deep-sea sediments of the eastern South Atlantic Ocean. SpringerPlus 2:127.

Droop, M. R. 1957. Vitamin B_{12} in marine ecology. Nature 180:1041-1042.

Fogg, G. E. 1966. The extracellular products of algae. Oceanographic Marine Biological Annual Review 4**:** 195-212.

Frenkel, E. P., Kitchens, R. L., and Prough, R. 1979. High-performance liquid chromatographic separation of cobalamins. J. Chromatogr. 174: 393-400.

Gobler, C. J., C. Norman, C. Panzeca, G. T. Taylor, and S. A. Sanudo-Wilhelmy. 2007. Effect of B-vitamins (B-1, B-12) and inorganic nutrients on algal bloom dynamics in a coastal ecosystem. Aquat. Microb. Ecol. 49: 181-194.

Guillard, R. R. 1968. B_{12} specificity of marine centric diatoms. J. Phycol. 4: 59-64.

Hage, D. S. 1999. Immunoassays. Anal. Chem. 71: 294-304.

Joos, T. O., Monika Schrenk, Peter Hopfl, and et al. 2000. A microarray enzyme-linked immunosorbent assay for autoimmune diagnostics. Electrophoresis. 21, 2641-2650.

Karl, D. M. 2002. Nutrient dynamics in the deep blue sea. Trends Microbiol. 10: 410-418.

King, A. L., Sanudo-Wilhelmy,S.A., Leblanc, K., Hutchins, D. A., Fu, F. 2011. CO_2 and vitamin B_{12} interactions determine bioactive trace metal requirements of a subarctic Pacific. The ISME Journal. 5, 1388-1396.

Knopp, D. 2006. Immunoassay development for environmental analysis. Anal. Bioanal. Chem. 385: 425-427.

Koch, F., A. Marcoval, C. Panzeca, K. W. Bruland, S. A. Sanudo-Wilhelmy, and C. J. Gobler. 2011. The effects of vitamin B_{12} on phytoplankton growth and community structure in the Gulf of Alaska. Limnol. Oceanogr. 56:1023-1034.

Koch, F., T. K. Hattenrath-Lehmann, J. A. Goleski, S. A. Sanudo-Wilhelmy, N. S. Fisher, and C. J. Gobler. 2012. Vitamin B_1 and B_{12} uptake and cycling by plankton communities in coastal ecosystems. Frontiers Aquatic Microbiology. October 12, 2012 | doi: 10.3389/fmicb.2012.00363.

Mabinya. L.V., Cosa, S. Mkwetshana, N. and Okoh, A. I. 2011. *Halomonas* sp. OKOH—A Marine Bacterium Isolated from the Bottom Sediment of Algoa Bay—Produces a Polysaccharide Bioflocculant: Partial Characterization and Biochemical Analysis of Its Properties. Molecules. 16:4358-4370.

Menzel, D. W., and Spaeth, J. P. 1962. Occurrence of vitamin B_{12} in the Sargasso Sea. Limnol. Oceanogr. 7: 151-154.

Nishijima T, and Hata Y. 1988. Distribution of vitamin B_{12}, thiamine, and biotin in the sediment of Harima-Nada Sea. Nippon Suisan Gakkaishi. 54(3): 511-516.

Nunes, G. S., I. A. Toscano, and D. Barcelo. 1998. Analysis of pesticides in food and environmental samples by enzyme-linked immunosorbent assays. Trac-Trends Analytical Chemistry. 17: 79-87.

Ohwada, K. and Taga, N. 1969. Distribution of vitamin B_{12}, thiamine and biotin in marine sediments. J. Oceanogr. Soc. Japan. 25:123-136.

Okbamichael, M., and S. A. Sanudo-Wilhelmy. 2004. A new method for the determination of Vitamin B-12 in seawater. Anal. Chim. Acta 517: 33-38.

Okbamichael, M., and S. A. Sanudo-Wilhelmy. 2005. Direct determination of vitamin B-1 in seawater by solid-phase extraction and high-performance liquid chromatography quantification. Limnol. Oceanogr. Meth. 3: 241-246.

O'Sullivan, J. J., R. J. Leemimg, S. S. Lynch, and A. Pollock. 1992. Radioimmunoassay that measures serum vitamin B_{12}. J. Clin. Pathol. 45: 328-331.

Panzeca, C., A. J. Beck, K. Leblanc, G. T. Taylor, D. A. Hutchins, and S. A. Sanudo-Wilhelmy. 2008. Potential cobalt limitation of vitamin B_{12} synthesis in the North Atlantic Ocean. Global biogeochemical cycles. 22: GB2029.

Panzeca, C., A. J. Beck, A. Tovar-Sanchez, J. Segovia-Zavala, G. T. Taylor, C. J. Gobler, and S. A. Sanudo-Wilhelmy. 2009. Distributions of dissolved vitamin B_{12} and Co in coastal and open-ocean environments. Estuar. Coast. Shelf Sci. 85: 223-230.

Ross, G. I. M. 1950. Vitamin B_{12} assay in body fluids. Nature 166: 270-271.

Santschi, P., Hohener, P., Benoit, G., Brink, B-t. M. 1990. Chemical processes at the sediment-water interface. Marine chemistry. 30, 269-315.

Sañudo-Wilhelmy, S. A., L. S. Cutter, R. Durazo, E. A. Smail, L. Gómez-Consarnau, E. A. Webb, M. G. Prokopenko, W. M. Berelson, and D. M. Karl. 2012. Multiple B-vitamin depletion in large areas of the coastal ocean. Proceedings of the National Academy of Sciences. 109(35): 14041-14045.

Sanudo-Wilhelmy, S. A., Gobler, C. J., Okbamichael, M., Taylor, G.T. 2006. Regulation of phytoplankton dynamics by vitamin B_{12}. Geophysical research letters. 33, L04604.

Swift, D. 1981. Vitamin levels in the Gulf of Maine and ecological significance of vitamin B_{12} there. J. Mar. Res. 39:375-403.

Tang, Y. Z., F. Koch, and C. J. Gobler. 2010. Most harmful algal bloom species are vitamin B-1 and B-12 auxotrophs. Proc. Natl. Acad. Sci. U. S. A. 107: 20756-20761.

Zhao, L., Sun L., Chu X. 2009. Chemiluminescence immunoassay. Trends in Analytical Chemistry, 28 (4): 404-415.

Zhu, Q. Z., R. C. Aller, and Y. Fan 2005. High-performance planar pH fluorosensor for twodimensional pH measurements in marine sediment and water. Environ. Sci. Technol., 39, 8906-8911.

Zhu, Q. Z., R. C. Aller, and Y. Fan. 2006a. Two dimensional pH distributions and dynamics in bioturbated marine sediments. Geochim. Cosmochim. Acta, 70, 4933-3939.

Zhu, Q. Z., R. C. Aller, and Y. Fan. 2006b. A new ratiometric, planar fluorosensor for measuringhigh resolution, two-dimensional pCO_2 distributions in marine sediments. Mar. Chem. 101, 40-53.

Zhu, Q. Z. and R. C. Aller. 2010. A Rapid Response Planar Fluorosensor for Measuring Two-Dimensional pCO_2 Distribution and transient Dynamics in Marine Sediments. Limnol. Oceanogra. Methods. 8: 326-336.

Zhu, Q. Z., R. C. Aller, A. Kaushik. 2011. Analysis of vitamin B_{12} in seawater and marine sediment porewater using ELISA. Limnol. Oceanogr. Methods 9:515-523.

Zhu, Q. Z. and R. C. Aller. 2012. Two-dimensional dissolved ferrous iron distributions in marine sediments as revealed by a novel planar optical sensor. Mar. Chem. 136-137: 14-23.