Proceedings of the 11th International Coral Reef Symposium, Ft. Lauderdale, Florida, 7-11 July 2008
Session number 19

New approach to study the coral symbiotic complex: Application to vitamin B12

S. Agostini [1*], Y. Suzuki [1], B.E. Casareto [1,2], Y. Nakano [3], M.F.M. Fairoz [1], K. Shiroma [1], A. Irikawa [1], K. Daigo [1]

1) Shizuoka University, 836 Oya Suruga ku Shizuoka Japan
2) LASC Co. Ltd, Meishin, Kamiikedai 1-14-1, Ota ku, Tokyo 145-0064 Japan
3) University of the Ryukyu, 1 Senbaru,Nishihara Okinawa Japan 903-0213

Abstract. Coral symbiotic complex was investigated with a new approach for sampling and measurements of vitamin B12 and bacteria abundances in the coelenteric fluid. Sampling procedure consisted in the introduction of a glass capillary using a micromanipulator into the mouths of the coral polyps, *Galaxea fascicularis,* under anesthesia and aspiration of the fluid using a vacuum pump. 0.5 to 1 ml of coelenteric fluid could be sampled by aspiration of the fluid of around 20 polyps. Flowcytometry for the measurement of bacteria abundances and radioassay with ^{57}Co labeled vitamin B12 for the measurement vitamin B12 with a minimum volume, were used. Detection limit of the radioassay was 35 pmol l^{-1} with a volume of sample of 0.4 ml. High abundances of bacteria with order of magnitude of 10^7 cell ml^{-1} and high concentrations in vitamin B12 with ranging 100 to 700 pmol l^{-1} were found in the coelenteric fluid, compared with those in surrounding water. Such approach provides a new view on the internal cycling of chemical and biological components in coral reefs. Our study it suggested that coelenteric bacteria produce vitamin B12 and that inner coral forms a semi-closed system.

Key words: Coelenteric Fluid, Radioassay, *Galaxea fascicularis*, Vitamin B12.

Introduction

Coral is described as a symbiosis among an animal, *Cnidarian*, a photosynthetic organisms, the dinoflagellate *Symbiodinium* also known as zooxanthellae, and more recently, the associated prokaryotes. The two first components of this symbiotic complex are widely accepted however the last one is now under focus. Thus coral and zooxanthellae interactions have been studied for a long time. The different methods applied for these studies included the use of carbon isotopes as tracer and separation of host cells and zooxanthellae by detaching the tissue from the skeleton and centrifugation (Muscatine 1990). These classic techniques however totally omitted the associated prokaryotes. Therefore even if associated prokaryotes were the subject of several publications most remain descriptive (Ducklow and Mitchell 1979; Rohwer et al. 2001; Rohwer et al. 2002; Kellogg 2004; Klaus et al. 2004; Bourne and Munn 2005; Koren and Rosenberg 2006; Lampert et al. 2006) and few described the mechanisms of the interactions among prokaryotes, the coral and the zooxanthellae (Herndl and Velimirov 1986; Shashar et al. 1994; Reshef et al. 2006; Rosenberg et al. 2007; Siboni et al. 2008). This lack of knowledge is partly due to the lack of methodology and to the only recent gain in interest.

Corals belong to the taxum Coelenterata and are characterized by the presence of a coelenteron which can be compare to a stomach cavity with only one aperture (i.e. the mouth). Moreover all polyps of one colony are connected together through tubes running through the coenosarc (Gladfelter 1983; Blackstone 1996). The coenosarc is composed of three tissue layers: the epidermis, the mesoglea and the endodermis. The last one is separated from the skeleton by a thin calicoblastic tissue (Goldberg 2002). Therefore coelenterons of polyps from the same colony seems to be connected together. The fluid inside the coelenteron contains, named here as "coelenteric fluid" could be shared by all polyps and is in contact with the surrounding water throughout the mouth. These observations lead to the concept that the coelenteron, the inner coral, could be considered as a semi-closed system. Furthermore zooxanthellae are known to be principally distributed in the endodermis, close to the coelenteric fluid. After all this consideration it is easy to understand the importance of studying the processes occurring in the coelenteric fluid.

In order to study coelenterate nutrition Porter (Porter 1978) described a technique to sample the coelenteric fluid of corals. On another hand Herndl et al. (Herndl et al. 1985)following Porter's method

sampled the coelenteric fluid of a giant anemone and reported for the first time the presence of bacteria living in the coelenteron. However the technique used in these two papers is not applicable directly to corals bearing small polyps as it was designed for *Montastrea cavernosa* and later applied to Giant anemone.

Here we focused our studies on the methodology required to investigate the role of the coelenteric bacteria. In order to consider as strictly as possible the processes occurring in the coelenteron we modified Porter's method to sample coelenteric fluid of small polyps corals. Also we chose vitamin B12 to investigate the linkage between the coral and the bacteria. Vitamin B12 is known to be only produced by prokaryotes. Moreover vitamin B12 is known to be required by all animals and by the majority of phytoplankton. The requirement of zooxanthellae for vitamin B12 was checked (Agostini et al., in press) and it was found that zooxanthellae were vitamin B12 dependent. For this study, measurements of vitamin B12 required the development of a new methodology with high sensitivity and requiring a low volume of sample. Determination of coelenteric bacteria abundances were done using flow-cytometry as it can be used on low volume samples. The different techniques developed and applied here are presented.

Material and Methods

Tests organisms

The coelenteric sampling technique was tested on different coral species in order to select the most suitable one. All coral colonies were taken from the reef in front of Sesoko Station, Ryukyu University, Okinawa Japan, and kept at the station in an aquarium with continuous flow of sea water. The different species tested were: *Galaxea fascicularis* (Linnaeus 1767), *Fungi fungites* (Linnaeus 1758), *Favites chinensis* (Verill 1866), and *Montastrea curta* (Dana 1864).

Coelenteric sampling

A specimen was transferred to a smaller aquarium at the time of sampling. To avoid retraction of the polyps during sampling, some corals were anesthetized using menthol (Moore, 1989). Anesthesia was done by scattering ground crystals of menthol on the surface and putting the aquarium in dark for about 45 min (depending on the species and size of the specimen and aquarium). Coral could be kept insensitive for 3 hours, until water was changed. After water change, the corals recovered quickly and therefore several anesthesia could be done in one day without any harm. Coelenteric fluid was sampled using a glass capillary mounted on a micromanipulator (Sutter Instrument model MM-33) under zoom stereomicroscope. Capillary used for the sampling was made from glass pipe and its external diameter was of less than 1 mm. The diameter should be adjusted to the polyp size. After introduction of the capillary into the mouth, the gastric content is gently sucked using a vacuum pump (max vacuum of 0.09 Bar). The fluid was collected into an HCl washed sampling tube kept in the dark during the time of sampling. Tubing connecting the capillary to the sampling tube were silicon tubes HCl washed before setup of the apparatus and between consecutive sample the tubing was washed by aspiration of MilliQ water. The volume collected by this method was of 0.5 to 1 ml after anesthesia and by sampling around 20 polyps of the same colony.

Radioassay of vitamin B_{12}

It was commonly used for determination of the level of vitamin B12 in human blood (Houts and Carney, 1981) and an attempt to use it on sea water was done by using another binder to decrease the detection limit (Sahni et al. 2001). The method used in this study was based on competitive binding radioassay using the binder and tracer from the commercial kit Simultrac-SNB (MP Biomedicals). It was optimized for seawater and required less than 0.5 ml (measurement in duplicates) of sample. Standards were done in vitamin B12 free seawater. Some samples were boiled for 30 min to check that no endogenic binder interfere with the assay. After optimization the following protocol was chosen: samples (200 µl) were incubated with 50 µl of [^{57}Co] labeled cyanocobalamin and 750 µl of Binder (purified porcine intrinsic factor bound on a solid support) for 2 hours at constant temperature and gently shaken. After centrifugation at 1000 g for 10 min, the supernatant was discarded and the pellet radioactivity was counted during 5 min using a gamma counter (Aloca, ARC-380, Tokyo Japan) set up for [^{57}Co] with a window of 100-180 KeV. Concentrations were read against standards, made with pure cyanocobalamin (Sigma Aldrich) in vitamin B12 free seawater, plotted with a logit-log scale.

Affinity of the binder in seawater was investigated by saturating aliquots of the binder with increasing amount of labeled cyanocobalamin (MP Biomedicals). Kinetics of the reaction was also investigated to determine the optimum reaction time and conditions. Incubation was done in two different conditions: at room temperature, not shaken and in a water bath at 25°C gently shaken.

Bacteria abundance determination.

In order to determine the abundances of bacteria in the coelenteric fluid flowcytometry was used (Monger and Landry, 1993). Bacteria were stained with SYBR-Green and then counted using a Beckman Coulter flow Cytometer. Picocyanobacteria were counted

using the same flowcytometer using their natural fluorescence due to main pigments, phycoerythrine and chlorophyll. Relative size of cells and their fluorescence allowed distinguishing populations of both *Prochlorococcus* and *Synechococcus*.

Results

Coelenteric sampling

Not all species were successfully sampled. Following detailed results by species may help for future applications to other species and further modifications of the technique. Mainly the results depended on the anatomy, size of the polyp.

Fungi fungites: This was the biggest polyp tested. As the polyp did not retract no anesthesia was used. Mouth of the specimen tested was more then 1 cm long and few millimeters wide. After introduction of the capillary the coral opened its mouth and secreted abundant mucus. Therefore the samples collected were largely contaminated in surrounding and freshly secreted mucus.

Favites chinensis: Size of the polyps were of the centimeter order and the mouth was almost as large as the polyp. However the sampling was not successful. First this species expands its polyps only during night time and during retraction the mouth is not accessible. Furthermore it was very sensitive: a light movement and the polyps retracted, so anesthesia in expanded state was not possible.

Montastrea curta: The size of the polyps were of the cm order and the mouth was more than 1mm. The polyp was not sensitive and introduction of the capillary was possible without anesthesia. However, this species secreted a lot of mucus and aspiration was very slow due to the high viscosity.

Galaxea fascicularis: this species was finally chosen for this study. Polyps were around 1 cm large and, depending one the specimen, could be 1 cm high. The mouth was smaller than 1 mm. The polyp was calcified until the oral disk giving it rigidity. Retraction was only of the oral disk and the tentacles which then covered the mouth. To avoid this the coral was anesthetized. The anesthesia time was of 45 min and should be done in the dark to obtain a maximum of expansion. Capillary could be inserted quite deep but it was blocked by internal calcified septum. The volume of coelenteric fluid collected by this technique was of 0.5 to 1 ml, by sampling 20 to 50 polyps in a maximum of 3 hours. Depending on the vacuum used, mesenteric filaments may be aspirated. To avoid this, pressure must be decreased but the volume and speed of collection might decrease. As a rule we limited the pressure to -0.09 Bar, usually collecting at -0.05 Bar and increasing just when necessary.

Radioassay of the vitamin B_{12}

Affinity of the binder in seawater.

Total saturation was not reached with the amount of tracer added, therefore the affinity constant was calculated using the linearisation of Hanes Woolf : the ratio between bound and added labeled cyanocobalamin was plotted against the bound vitamin (Zettner, 1973). Added vitamin was measured before centrifugation and bound in the pellet after centrifugation:

$$\frac{Vit_b}{Vit_a} = Vit_a \times \frac{1}{V_m} \square \frac{K_d}{V_m} \quad (1)$$

with Vit_b being the vitamin bound (in the pellet), Vit_a the vitamin added, V_m the maximum velocity (here the saturation), K_d the dissociation constant. For calculation of the affinity constant cpm were converted to mol l^{-1} using a specific activity of 9.02 10^{17} cpm mol^{-1}. The resulting constant of dissociation was 4.41 10^{-12} mol l^{-1}, thus the affinity was of 10^{11} l mol^{-1} which allow measurement of the picomolar order (Zettner, 1973).

Radioassay detection limit, when defined as 3 times the SD of the trace binding was of 35 pmol l^{-1} which was in agreement with the affinity constant found. The optimum conditions for the incubation were determined to be 2 hours at 25 °C gently shaken.

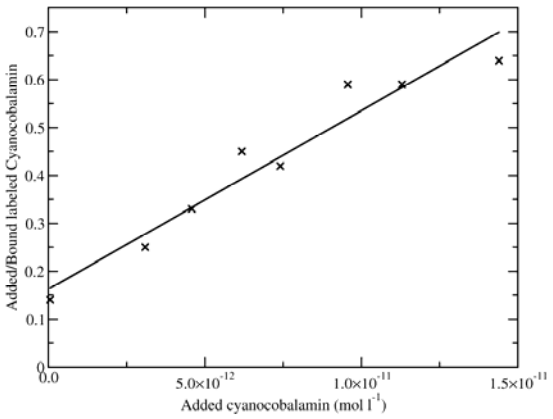

Figure 1: Linearisation of Hanes Woolf for determination of the affinity of the binder in seawater. R^2 =0.9389, slope is the inverse of the saturation and y-intercept is the ratio between Saturation and the dissociation constant

Coelenteric Fluid Analysis

Bacteria abundances

Flowcytometry was useful for the determination of coelenteric bacteria abundances. It gave reproducible results and required only 30 µl for each measurement. Abundances were always found higher in the coelenteric fluid with 7.5 x 10^6 to 4.6 x 10^7 cell ml^{-1} than those in the surrounding water: 3.54 x 10^5 cell

ml^{-1} for the aquarium water and 3.9 x 10^5 cell ml^{-1} as an average value for the natural surface water. Variations in abundances were observed but no trend, such as diurnal variation, could be observed. Picocyanobacteria (*Synechococcus* spp and *Prochlorococcus* spp) abundances were also measured one time and had a concentration similar to the aquarium water: *Synechococcus* spp : 8.9 x 10^2 cell ml^{-1} (aquarium) and 6.1 x 10^2 cell ml^{-1} (coelenteric); *Prochlorococcus* spp 4.8 x 10^2 cell ml^{-1} (aquarium) and 6.1 x 10^2 cell ml^{-1} (coelenteric).

Vitamin B_{12} concentrations

In the coelenteric fluid concentrations of vitamin B12 ranged from 108 pmol l^{-1} to 704 pmol l^{-1} . Surface water and aquarium water had no detectable level of vitamin B12 by radioassay. The difference of concentrations between coelenteric fluid and surrounding water is of 1-2 orders of magnitude (data not shown).

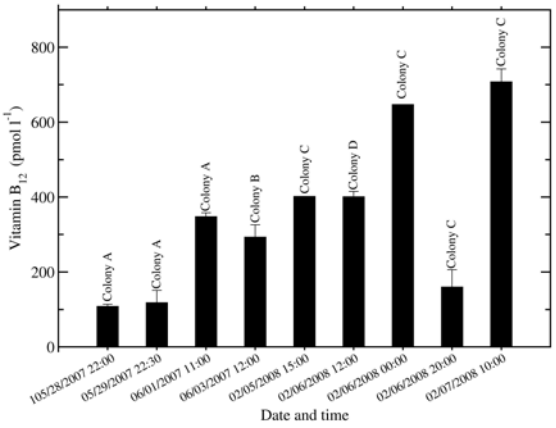

Figure 2: Coelenteric vitamin B12 found in the coelenteric fluid of Galaxea fascicularis. Colony A, B, C and D represent the 4 different colonies used. Error bars represent the standard deviation between 3 measurements.

Discussion

Radioassay allowed the measurement of vitamin B12 in the coelenteric fluid using 400 µl and the concentrations found were high and could be detected. Although the present detection limit was 30 pmol l^{-1}, by using pre concentration of the samples, lower concentrations would be detected but it will require a higher volume. Such pre concentration may be useful for the measure of vitamin B12 in the surrounding water. The affinity found of intrinsic factor, the binder used here, in saline solution (35 g l^{-1} NaCl) do not differs significantly from the one reported for blood analysis (Sahni et al. 2001). Therefore the detection limit was still comparable with the one reported for blood analysis. The affinity of intrinsic factor was reported to be maximum at pH 9 which is closed to seawater pH. Standards should be done in vitamin B12 free seawater and not in MilliQ water as affinity may be different in the two solutions leading to inaccurate concentrations. We considered that the chemical characteristics (especially pH, ionic strength and protein content) of coelenteric fluid and seawater were relatively close and therefore that differences in the affinity of the binder for vitamin B12 between standards and samples were negligible. Affinity may also change with long storage of the binder. The binder was stored in dark at 2-8°C and its stability was of around 3 months. To avoid inaccuracy, a standard curve was realized for each measurement. The original protocol provided by the maker include a step of chemical extraction. Such extraction is required for blood analysis, as in blood the vitamin B12 is bound to its transporter (Houts and Carney, 1981). Moreover the blood endogenic binder may interfere with the assay. In our case no endogenic binder were expected and in order to check their absence some samples were boiled. Such boiling would denature all proteic binder (Rothenberg, 1963). No difference between before and after boiling were noticed suggesting the absence of endogenic binder in our samples. The radioassay methods was also compared to an HPLC methods (Okbamichael and Sañudo-Wilhelmy 2004) and no significant differences for the measure of vitamin B12 concentration in seawater were found (Agostini et al., *in press*).

The sampling of coelenteric fluid of the coral *Galaxea fascicularis* was successful. No contamination from surrounding water seemed to occur as vitamin B12 concentrations and bacteria abundances differed widely. To ensure that no contamination occurred, the capillary was inserted as deep as possible and if deep insertion was not possible, aspiration was not done. Suspect samples, i.e. high volume of fluid aspirated in short time, were discarded. Vacuum pressure should be kept as low as possible to avoid aspiration of mesenteric filament and so lesion of coral tissue. However a too low pressure did not allow the collection of a sufficient volume in a reasonable time and the pressure was sometimes increase to a maximum of 0.09 Bar. Some samples required as much as 3 hours for their collection. Three hours seemed to be the maximum time for which we could keep the coral in the same water and under anesthesia. A longer time of sampling had several consequences: the coral started to show change of the color of the tissue, stability of the samples at room temperature may not be good, production or consumption by coelenteric bacteria of vitamin B12 may occur in the sampling tube. Finally a too long sampling time decrease the time resolution of the technique. In this report all time of sampling indicated the time at which the coral colony was transferred to a smaller aquarium for sampling. A shorter time of sampling may be

obtained by selecting a coral species with bigger polyps but the risk of contamination from surrounding water would increased as it happened when the coral *Fungia fungites* was tested.

Coelenteric high abundances in bacteria and high concentrations in vitamin B12 lead to 2 conclusions: (1) the coelenteric bacteria seem to produce the vitamin B12 required for the growth of the coral and symbiotic zooxanthellae. (2) the coral forms a semi-closed system enabling high concentration of essential nutrients that are scarce in the surrounding water. The concept that coral forms a semi closed system, as it was suggested by their anatomy, was confirmed by the difference between internal (inside the coelenteron) and external (in the surrounding water) vitamin B12 and bacteria abundances. Further discussion on this concept will be done elsewhere (Agostini et al*.,* in preparation).

The radioassay as described in this report provided an easy, accurate and fast way to measure vitamin B12 in a minimum of volume. Sampling of coelenteric fluid of alive coral followed by chemical and biological analysis allowed the study of the processes occurring in the coelenteron. Here we show the application of radioassay for the measure of vitamin B12 and of flowcytometry for the measure of bacteria abundances but an exhaustive description of the coelenteric fluid, biological and chemical characteristics may be required for further understanding of the relations among the different component of the coral symbiotic complex.

Acknowledgement
This work was supported by grants from Mitsubishi corporation, from the ministry of Education, Science, Sports and Culture of Japan, and by the scholarship for foreign students of Honda Benjiro.

References

Agostini S, Suzuki Y, Casareto B E, Y Nakano, M Hidaka & N Badrun (2008) Coral symbiotic complex: Hypothesis through vitamin B12 for a new evaluation. Galaxea Journal of Coral Reef Studies, *in press*

Blackstone NW (1996) Gastrovascular flow and colony development in two colonial hydroids. Biol Bull 190: pp. 56-68

Bourne D, Munn C (2005) Diversity of bacteria associated with the coral pocillopora damicornis. Environ Microbiol 7: pp. 1162-1174

Ducklow H, Mitchell R (1979) Bacterial populations and adaptations in the mucus layer of living corals. Limnol Oceanogr 24: pp. 715-725

Gladfelter EH (1983) Circulation of fluids in the gastrovascular system of the reef coral acropora cervicornis. Biol Bull 165: pp. 619-636

Goldberg WM (2002) Gastrodermal structure and feeding responses in the scleractinian mycetophyllia reesi, a coral with novel digestive filaments. Tissue and Cell 34: pp. 246-261

Herndl G, Velimirov B. Role of bacteria in the gastral cavity of athozoa. In *Ifremer actes de colloques:deuxieme coloque international de bacteriologie marine 1984*. 1986.

Herndl G, Velimirov B, Krauss R (1985) Heterotrophic nutrition and control of bacteria density in the coelenteron of giant sea anemone stoichactis giganteum. Mar Ecol Prog Ser 22: pp. 101-105

Houts TM, Carney JA (1981) Radioassay for cobalamin (vitamin B12) requiring no pretreatment of serum. Clin Chem 27: pp. 263-267

Kellogg CA (2004) Tropical archaea: diversity associated with the surface microlayer of corals. Mar Ecol Prog Ser 273: pp. 81-88

Klaus JS, Frias-Lopez ÆJ, Bonheyo GT, Jeffrey Æ, Heikoop M, Fouke BW (2004) Bacterial communities inhabiting the healthy tissues of two caribbeanreef corals: interspecific and spatial variation. Coral Reefs 24: p. 129–137

Koren O, Rosenberg E (2006) Bacteria associated with mucus and tissues of the coral oculina patagonica in summer and winter. Appl Environ Microbiol 72: pp. 5254-5259

Lampert Y, Kelman D, Dubinsky Z, Nitzan Y, Hill RT (2006) Diversity of culturable bacteria in themucus of the redsea coral fungia scutaria. FEMS Microbiol Lett :

Monger BC, Landry MR (1993) Flow cytometric analysis of marine bacteria with hoechst 33342. Appl Environ Microbiol 59: pp. 905-911

Moore S (1989) Narcotising sea anemones. J Mar Biolog Assoc UK 69: pp. 803-811

Muscatine L (1990) Flux of photosynthetic fixed carbon in corals. In *Ecosytem of the world. 25 coral reefs*. Z. Dubinsky (Ed.). 1990. pp. pp. 265-289.

Okbamichael M, Sañudo-Wilhelmy S (2004) A new method for the determination of vitamin B12 in seawater. Anal Chim Acta 517: pp. 33-38

Porter J. Coral feeding on zooplankton. Stoddart D & Johannes R (Eds.). UNESCO, 1978.

Reshef L, Koren O, Loya Y, Zilber-Rosenberg I, Rosenberg E (2006) The coral probiotic hypothesis. Environ Microbiol 8: p. 2068–2073

Rohwer F, Breitbart M, Jara J, Azam F, Knowlton N (2001) Diversity of bacteria associated with the caribbean coral montastrea fransk. Coral Reefs 20: pp. 85-91

Rohwer F, Seguritan V, Azam F, Knowlton N (2002) Diversity and distribution of coral-associated bacteria. Mar Ecol Prog Ser 243: pp. 1-10

Rosenberg E, Koren O, Reshef L, Efrony R, Zilber-Rosenberg I (2007) The role of microorganisms in coral health, disease and evolution. Nature Reviews : microbiology 5: pp. 355-362

Rothenberg SP (1963) Radioassay of serum vitamin B12 by quantitating the competition between [^{57}Co]B12 and unlabeled B12 for the binding sites of intrinsic factor. J Clin Invest 42: pp. 1391-1398

Sahni MK, Spanos S, Wahrman MZ, Sharma GM (2001) Marine corrinoid-binding proteins for the direct determination of vitamin B12 by radioassay. Anal Biochem 289: pp. 68-76

Shashar N, Cohen Y, Loya Y, Sar N (1994) Nitrogen fixation (acetylene reduction) in stony corals: evidence for coral-bacteria interactions. Mar Ecol Prog Ser 111: pp. 259-264

Siboni N, Ben-Dov E, Sivan A, Kushmaro A (2008) Global distribution and diversity of coral-associated archaea and their possible role in the coral holobiont nitrogen cycle. Environ Microbiol 10: pp. 2979-2990

Zettner A (1973) Principles of competitive binding assays (saturation analyses). 1 equilibrium techniques. Clini Chem 19: pp. 699-705