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Nitrogen stable isotope ratio in the manila clam, *Ruditapes philippinarum*, reflects eutrophication levels in tidal flats

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ABSTRACT

Understanding the effects of anthropogenic eutrophication on coastal fisheries may help in the enhancement of fishery production by effective utilization of sewage effluents, as well as in the consequent reduction of eutrophication. In this study, it was revealed that the nitrogen stable isotope ratio (δ^{15}N) in the soft tissues of the manila clam, *Ruditapes philippinarum*, can be used as an indicator of anthropogenic eutrophication levels in tidal flat environments by investigation of δ^{15}N in dissolved inorganic nitrogen (DIN), particulate organic matter (POM), sedimentary organic matter (SOM) and soft tissues of the clam in five tidal flats in Japan with different levels of DIN concentration. In addition, it was found that the acid insoluble fraction of the shell organic matrix, conchiolin, can be used as a proxy for the soft tissues in δ^{15}N analyses. This will contribute in easier storage handling and the expansion of chances for sample acquisition.

1. Introduction

Human activities have led to increases in nutrients, nitrogen and phosphorus, in aquatic environments, resulting in eutrophication of coastal waters (Nixon, 1995). Anthropogenic nutrient enrichments have ecological impacts, including enhanced primary production and changes in community structure (Paerl, 1997; Sommer et al., 2002). While adverse effects of eutrophication, such as the occurrence of toxic red tides and consequent hypoxia are often emphasized, moderate eutrophication may also have positive effects upon coastal fisheries (Yamamoto, 2003). It may become unnecessary, for instance for farmers to apply fertilizers for *Porphyra* aquaculture to prevent bleaching of thalli due to depletion of nitrogenous nutrients (Nishikawa et al., 2007) if effective use of anthropogenic waste in the watersheds is achieved. Appropriate control of nutrient load to coastal waters through sewage and riverine water may lead to not only enhanced coastal fisheries in algae and animals but also the consequent effective reduction of eutrophication.

Fishery production of the manila clam, *Ruditapes philippinarum*, has markedly declined for the last two decades due to stock depletion in tidal flats in Japan. While the stock depletion is considered to be attributable to factors, such as reclamation of tidal flats, environmental deteriorations and poor fishery management (Ishii et al., 2001; Kakino, 1986; Toba, 2004), local fishermen in some parts of Japan anecdotally claim that the depletion is due partly to the diminished food supply for *R. philippinarum* by reduced nutrient loads to coastal waters. Understanding the effects of anthropogenic nutrient loads to stock conditions of *R. philippinarum* may help improve fishery management; however, there is little information available about it.

Sewage treated water contains dissolved inorganic nitrogen (DIN) with significantly high nitrogen stable isotope ratio (δ^{15}N) due to denitrification during the treatments (Macko and Ostrom, 1994). Applications of nitrogenous fertilizer to agricultural farmlands lead to an enhancement of soil denitrification and increase in δ^{15}N in groundwater (Ogawa et al., 2001). The δ^{15}N of nitrate has higher values in human and animal wastes (10–20‰) than in atmospheric deposition (2–8‰) and N fixation by cyanobacteria (−2 to 0‰) (McClelland et al., 1997; Oowada et al., 2003). Thus, groundwater with an elevated δ^{15}N appears to act as an indicator of the level of anthropogenic nitrogen loads to coastal waters (McClelland et al., 1997), and the δ^{15}N delivered to the coastal waters has a pervasive influence on nitrogen of primary producers living in the coastal waters (Costanzo et al., 2005; McClelland and Valiela, 1998).

Some benthic animals (zebra mussels, amphipods and snails) were found to preferentially assimilate sewage-derived particulate

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organic matter (DeBruyn and Rasmussen, 2002) and consequently have higher $\delta^{15}N$ values. The $\delta^{15}N$ of animals including polychaetes, bivalves, shrimps and teleost fishes are suggested to be higher in the food web originated from primary producers assimilating sewage effluent (Hadwen and Arthington, 2007; McKinney et al., 2001), and therefore, $\delta^{15}N$ of these animals may be used as an indicator of nitrogen sources to coastal waters. However, these studies did not directly compare DIN concentrations in ambient water and $\delta^{15}N$ in the animals.

In this study, concentration and $\delta^{15}N$ in DIN were measured in various tidal flats in Japan to confirm the relationship between the level of anthropogenic eutrophication and ^{15}N enrichment. The $\delta^{15}N$ of particulate organic matter in the bottom water, sedimentary organic matter and soft tissues of *R. philippinarum* were measured to study the mutual relationships to determine whether $\delta^{15}N$ in *R. philippinarum* can be used as an indicator of anthropogenic eutrophication levels in the tidal flat environment. In addition, the feasibility of using shell organic matrix was also tested as a substitute for soft tissues in *R. philippinarum* for easier storage handling and the possibility of expansion of sample acquisition.

2. Materials and methods

2.1. Sample collection

Bottom water, surface sediment and *R. philippinarum* were sampled at tidal flats in Kanzanji (34°46′N, 137°37′E), Sakume (34°47′N, 137°36′E) and Washizu (34°43′N, 137°33′E) in Lake Hamana, Shizuoka Prefecture on August 10, 2005, Wajiro (33°41′N, 130°26′E) in Hakata Bay, Fukuoka Prefecture on December 8, 2005, and Seaside Park of Yokohama (35°20′N, 139°38′E) in Tokyo Bay, Kanagawa Prefecture on December 12, 2005. These sites were selected in an attempt to obtain data from varied eutrophication levels. Lake Hamana was expected to be less eutrophied as compared to heavily populated Hakata Bay and Tokyo Bay. Sewage treatment plants were located near Washizu, Wajiro and Seaside Park of Yokohama. Kanzanji was located in the vicinity of a river mouth where household effluent water was discharged. Sakume was close to neither a sewage treatment plant nor household effluent and therefore expected to be the most oligotrophic among all the sites.

2.2. Bottom water analyses

The bottom water samples were collected with a vampire sampling suction pump (The Fluid Life Corporation) through an opening of PVC pipe that was fixed to the tidal flat with a metal plate (500 ml, $n = 5$ for each site except for Seaside Park of Yokohama where $n = 3$). A small portion of the water samples (approx 10 ml) were immediately filtered through 0.45 μm Millipore HV filters (Millipore Corporation) into acid washed polypropylene tubes and was frozen at −20 °C until laboratory analysis for concentration of dissolved inorganic nitrogen, [DIN] ($n = 5$). Concentrations of ammonia ($[NH_4^+]$), nitrate ($[NO_3^-]$) and nitrite ($[NO_2^-]$) were measured according to the standard method using a TRAACS 800 auto analyzer (Buran Lubbe Co.).

The rest of the water samples were filtered with pre-combusted Whatman GF/F filters (GE Healthcare). Particulate organic matter (POM) collected on the filter was kept overnight in a glass jar saturated with HCl fume to remove $CaCO_3$. The POM sample was then scraped off with a spatula and concealed in a tin container for $\delta^{15}N$ analysis ($n = 5$ except for Seaside Park of Yokohama where $n = 3$). The modified ammonia diffusion method (Holmes et al., 1998; Sigman et al., 1997) was employed to collect DIN from the filtered bottom water ($n = 3$ for each site). DIN was recovered in Whatman GF/D filters soaked with 25 μl 2 M H_2SO_4 and bound by a Teflon membrane by floating the filter on the sample water in a sealed container. After the DIN recovery, the membrane was removed and the filter was concealed in a tin container, and $\delta^{15}N$ was measured immediately.

2.3. Surface sediment analyses

The top 1 cm sediment of the tidal flat was collected ($n = 5$ for each site) with a core sampler made of a 50 ml injection syringe (inner diameter of 29 mm) by cutting off the needle end. The sediment sample was placed in a 50 ml centrifugal tube. Distilled water was added ad libitum to the tube and sonicated in a cold water bath to collect particulate sedimentary organic matter (SOM) that accumulated on the sample surface with a Pasteur pipette. The SOM sample was stored in a 2 ml micro test tube, decarbonated with 1 N HCl, rinsed three times with distilled water, oven-dried at 60 °C overnight, and concealed in a tin container for $\delta^{15}N$ measurement ($n = 5$ for each site).

2.4. Clam analyses

The whole soft tissue samples of *R. philippinarum* ($n = 20$ for each site) were collected by gently scraping with forceps; the samples were then lyophilized and ground to a fine powder. Since lipids can fluctuate in amount and have a lighter carbon stable isotope ratio ($\delta^{13}C$) than other tissue fractions (DeNiro and Epstein, 1981; Tieszen et al., 1983), samples for stable isotopic analyses are usually defatted. A fraction of powdered samples was defatted by the conventional Folch method (Folch et al., 1957) using methanol and chloroform. The defatted samples were then dried and concealed in a tin container for $\delta^{15}N$ and $\delta^{13}C$ measurement.

The acid insoluble fraction of shell organic matrix (i.e. conchiolin, Fremy, 1855) was obtained by decalcification of the shells. The shells were carefully cleaned of remaining soft tissues, broken into small pieces and placed in a 15 ml centrifugal tube. Concentrated HCl was added to the tube drop by drop until $CaCO_3$, the main inorganic component of the shell, was completely removed as bubbles ($CaCO_3 + 2HCl \rightarrow CaCl_2 + H_2O + CO_2\uparrow$). The decalcified samples were then collected into a 1.5 ml micro test tube, rinsed and centrifuged with distilled water three times and oven-dried at 60 °C overnight. The conchiolin obtained was concealed in a tin container for $\delta^{15}N$ and $\delta^{13}C$ measurements.

2.5. Stable isotopic analyses

The $\delta^{15}N$ and $\delta^{13}C$ of the prepared samples were analyzed using an EA-1108 elemental analyzer (Carlo Erba) coupled with an isotope ratio mass spectrometer (Finnigan Mat ConFlo II, Mat 252; Toyokawa, 2001). The isotope ratios were expressed as a per mil (‰) deviation from international standards (i.e. fossil calcium carbonate for C and air for N): $\delta^{13}C$, $\delta^{15}N = (R_{sample}/R_{standatrd} - 1) \times 1000$, where R is $^{13}C/^{12}C$ and $^{15}N/^{14}N$. Instrumental precision was 0.2‰ (Toyokawa, 2001). Atomic organic carbon: nitrogen (C/N) ratios of the soft tissues and conchiolin in *R. philippinarum* were analyzed at the same time.

The isotopic fractionation factor (α) of $\delta^{15}N$ was estimated expediently based on the results of the measurements. The values for α were calculated as the difference between two samples; for example, α for DIN to SOM was obtained as $1 + (\delta^{15}N_{SOM} - \delta^{15}N_{DIN})/1000$, assuming that total DIN is used for primary production in the SOM. The coefficient of variation (CV) was calculated as standard deviation/mean (%) for comparison of variance among the α values. In these estimations, the ratio of $[NO_3^-]$, $[NO_2^-]$ and $[NH_4^+]$ in [total DIN] and constituents of POM and SOM are ignored for simplification.