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Plasma lead, silicon and titanium concentrations are considerably higher in green sea turtle from the suburban coast than in those from the rural coast in Okinawa, Japan

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ABSTRACT. The purpose of this study was to compare the concentration of trace elements in the plasma of sea turtles that inhabited the suburban (Okinawa Main Island, n=8) and the rural coast (Yaeyama Island, n=57) in Okinawa, Japan. Particle induced X-ray emission allowed detection of 20 trace and major elements. The wild sea turtles in the suburban coast in Okinawa were found to have high concentrations of Pb, Si and Ti in the plasma when compared to the rural area but there were no significant changes in the Al, As and Hg concentrations. These results may help to suggest the status of some elements in a marine environment. Further, monitoring the plasma trace and major element status in sea turtles can be used as a bio-monitoring approach by which specific types of elements found here could indicate effects that are related to human activities.

KEY WORDS: marine pollution, Okinawa Main Island, particle induced X-ray emission, sea turtle, Yaeyama Island

Trace and major element pollutants resulting from negative human activities are found in aquatic ecosystems. This remains a problem in marine environments and is an ongoing subject of research. High urbanization and industrialization rates, as well as the rapidly increasing population growth rates over the last few decades have been responsible for the production of huge quantities of wastewater, often disposed of in the environment without any treatment. Excessive amounts of heavy metals are introduced to estuarine and coastal environments through rivers, runoffs, and land-based point sources [12]. De Carvalho *et al*. [7] reported that 39% of the total number of turtles that they studied had ingested marine debris such as soft plastic, hard plastic, metal, polyethylene terephthalate bottle caps, human hair, tampons, and latex condoms. It is possible that anthropogenic activities in suburban areas pose one of the worst environmental problems in marine ecosystems, acting as sources of contaminants in aquatic systems. Pollution of the ocean due to anthropogenic environmental contaminants has been linked to the emergence of diseases and syndromes in individuals, populations, and ecosystems [1]. In particular, exposure to heavy metals has been linked to the rapid degradation of coastal habitats of sea turtles [3, 13, 14]. In consequence, knowledge about the accumulation of heavy metals and elements in sea turtles is an important focal point to assess the potential impact of these pollutants on this endangered organism.

The western region of the North Pacific including the Coast of Okinawa Main Island can be expected to have a large influence on the distribution of metals in the Pacific Ocean because the rate of deposition of Asian dust is much larger than that in the central or eastern North Pacific region [15].

In most studies on pollution in marine ecosystems using sea turtles, liver, kidney, muscle, heart, and shell tissue samples are obtained during autopsy of animals collected from the beach or caught for commercial purposes [11, 18]. Several researchers [6, 22, 31, 32] have indicated that using blood samples is an excellent method and a relatively non-invasive way to measure

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baseline values of heavy metals in sea turtles. Furthermore, the blood concentration of total Hg was proved to be an effective predictor, which is stored in the muscle and the spinal cord [6].

The purpose of this study was to compare the concentrations of trace and major elements in the plasma of wild sea turtles that inhabit the suburban coastal (Okinawa Main Island) and the rural coastal (Yaeyama Island) areas in Okinawa, Japan.

Fifty-seven green sea turtles that were captured for research purposes in Yaeyama Islands (122–124°E, 24°N) from February 2014 to September 2015 were enrolled in this trial as the rural coast group. Of these, 27 were captured in February 2014 or February 2015 (winter group), and 30 were captured in September 2015 (summer group). Eight wild green sea turtles were also captured for research purpose in the Coast of Yomitan Son, Okinawa Main Island, Japan (127°E, 27°N) in September 2015 and represented the suburban coast group. The Okinawa region can be categorized as the Okinawa Main Island, a densely populated area, and the islands including the Yaeyama Islands where the natural environment is conserved. From publicly available data, it was noted that in 2016, the population and density of the Yaeyama Islands (53,405 people, 91.0/km^2) was markedly lower than that of the Okinawa Main Island (1.29 million people, 1,068.8/km^2). There are few rivers in the main island of Okinawa, especially in the central and southern regions where the population is concentrated. Although the amount of precipitation is not small, most of the water received flows out into the sea because of the small scale of rivers. The coast is crowded with cement factories and crude oil storage facilities. The seacoast of the Yaeyama Islands is less affected by environmental pollution caused by human activities compared with Okinawa Main Island because of the low number of factories and population density.

Further, eight healthy and mature green sea turtles that were maintained in an outdoor sea pool at ambient temperature at the Okinawa Ocean Expo Research Center (OERC) were included in the study as a captive group in September 2015. The animals in this captive group were fed on kibinago (*Spratelloides gracilis*), capelin (*Mallotus villosus*), and spear squid (*Loligo bleekeri*), and had free access to cabbage, Chinese cabbage, and lettuce. This is a standard diet for sea turtles at the OERC, where all animals involved in the study have been living for at least 14 years. The mean carapace length (CL) of study individuals in the rural coast, suburban coast, and captured groups were 551.3 ± 121.9, 787.0 ± 232.6 and 963.2 ± 35.4 mm, respectively. There were differences in CL among groups.

Our previous study [31] conducted to assess correlations between carapace parameters and trace and major element status in the plasma using captive adult sea turtles weighing more than 27.9 kg, suggested that CL is a suitable indicator to estimate the body weight (BW) of sea turtles and that plasma trace elements in captive sea turtles showed almost no variation with BW and CL [31]. These findings may be relevant in pollution and marine ecosystem studies where physiological aging is not considered. Therefore, monitoring the increase in the concentration of trace and major elements in the plasma in wild sea turtles might be useful in monitoring for possible accumulation caused by marine pollution.

Blood samples, measuring 10 m*l*, were taken from the dorsal cervical sinus using a sterile plastic disposable syringe and needle, which were immediately placed in a heparinized tube. The samples were then centrifuged at 3,000 g for 10 min at 4°C to harvest the plasma. The plasma samples were stored at −80°C until it was assayed. Wild sea turtles were released to sea after blood samples were collected and measurements noted.

The mean concentrations of total of each element of interest in the plasma were measured by the particle induced X-ray emission (PIXE) method [29–32]. Briefly, 100 μl supernatant was placed on a subtlety Myler membrane and desiccated. The supernatants were directly irradiated with proton beams. A small (baby) cyclotron used for positron nuclear medicine at the Nishina Memorial Cyclotron Center (Iwate, Japan) provided a 2.9 MeV-proton beam on a target after passing through a graphite beam collimator. A Si (Li) detector (0.0254 mm Be window) with 300 and 1,000 μm thick Mylar absorbers was used to select X-rays with energy higher than that of K-K alpha. For lower-energy X-rays, another Si (Li) detector (0.008 mm Be) was used without an absorber.

There are many studies that evaluate the relationships between trace element concentrations in biological samples such as liver [10, 16, 33], bone [9], plasma [5, 31, 32], and serum [10, 19, 20, 33] measured by the PIXE method and the pathophysiological status and in particular, the pathological status. For example, the relationship between trace element concentrations in blood samples measured by the PIXE method and peripartum cardiomyopathy [5], and chronic hepatitis C [16] have been studied.

Analytical precision was confirmed by comparing the results with those obtained from ICP-MS, Neutron Activation Analysis, etc. [29, 30]. The PIXE System at the Nishina Memorial Cyclotron Center was maintained in the same conditions as when initially setting it [29, 30]. Moreover, precisions and accuracy of PIXE has been confirmed by using standard materials such as bovine liver, tomato leaves, city ash, and human serum (National Institute of Standards and Technology, U.S. Department of Commerce, Gaithersburg, MD, U.S.A.) at regular intervals in accordance to the guideline of the facility.

Regarding the accuracy and precision of the PIXE method, a spike and recovery test using certified reference materials was conducted in addition to the regular maintenance performed at the Nishina Memorial Foundation. Certified multi-element standard (ICP multi-element standard VIII, Merck KGaA, Darmstadt, Germany), Silicon standard solution (Si 1000, Wako Pure Chemical Ind., Ltd., Osaka, Japan), and Titanium standard solution (Ti 1000, Wako Pure Chemical Ind., Ltd.) were added to the pooled sea turtle plasma to get a final concentration of 10 μg/m*l*. Analysis of the plasma specimens before and after addition of the standard solutions were repeated six times by the PIXE method to measure the respective trace elements. The recovery ratio and coefficient of variation (CV) were calculated for 17 elements (Al, Ca, Cd, Co, Cr, Cu, Fe, Ga, Mg, Mn, Ni, Pb, Se, Sr, Ti and Zn). The values detected by PIXE analysis are summarized in Table 1. Except for Mg, the spike recovery values and CV for other elements were considered valid. However, since the recovery value and CV of plasma Mg were high, Mg was not evaluated in this study.

The data are shown as mean ± standard deviation (SD). Statistical analyses were performed using a commercial software package from IBM, SPSS Statistics, v.21 (IBM Co., Somers, NY, U.S.A.). For normally distributed data, the mean values for each dependent variable were compared between the summer and winter sub-groups within the rural group using the Student's t-test, and were compared among groups using the post-ANOVA (with F-test) Tukey's HSD test. The significance level was set at $P<0.05$.

Table 1. Results of spike and recovery test for Particle Induced X-ray Emission methods

Elements		Pre-Spike (μg/ml)		Certified value[a)] (mg/l)	Spike	Post-Spike (μg/ml)		Recovery (%)	
		mean ± SD	CV			mean ± SD	CV	mean ± SD	CV
Aluminum	Al	2.130 ± 1.313	0.617	98 ± 3	9.8	12.452 ± 2.054	0.165	96.086 ± 4.030	0.042
Calcium	Ca	63.484 ± 3.914	0.062	102 ± 3	10.2	73.483 ± 0.808	0.011	98.025 ± 7.918	0.081
Cadmium	Cd	ND		100 ± 3	10	10.096 ± 1.202	0.119	104.406 ± 4.840	0.046
Cobalt	Co	0.043 ± 0.021	0.490	99 ± 3	9.9	9.148 ± 0.151	0.017	91.965 ± 1.527	0.017
Chromium	Cr	ND		99 ± 3	9.9	9.747 ± 0.243	0.025	98.452 ± 2.457	0.025
Copper	Cu	0.156 ± 0.016	0.103	98 ± 3	9.8	9.657 ± 0.167	0.017	96.942 ± 1.709	0.018
Iron	Fe	0.980 ± 0.089	0.091	100 ± 3	10	10.933 ± 0.394	0.036	99.526 ± 3.938	0.040
Gallium	Ga	0.036 ± 0.033	0.916	100 ± 3	10	9.509 ± 0.175	0.018	94.724 ± 1.747	0.018
Magnesium	Mg	22.321 ± 2.511	0.113	98 ± 3	9.8	66.251 ± 34.161	0.516	448.262 ± 348.583	0.778
Manganese	Mn	ND		100 ± 3	10	10.181 ± 0.446	0.044	101.813 ± 4.455	0.044
Nickel	Ni	0.021 ± 0.018	0.891	100 ± 3	10	9.719 ± 0.149	0.015	96.986 ± 1.493	0.015
Lead	Pb	0.798 ± 0.086	0.108	98 ± 3	9.8	10.227 ± 0.134	0.013	97.204 ± 1.379	0.014
Selenium	Se	0.117 ± 0.062	0.534	101 ± 3	10.1	9.308 ± 0.300	0.032	91.004 ± 2.974	0.033
Silicon	Si	0.806 ± 0.009	0.011	90 ± 3	9	9.663 ± 1.614	0.167	98.413 ± 17.933	0.182
Strontium	Sr	ND		100 ± 3	10	9.896 ± 0.302	0.031	98.961 ± 3.024	0.031
Titanium	Ti	0.003 ± 0.001	0.381	90 ± 3	9	9.382 ± 0.923	0.098	100.500 ± 5.337	0.053
Zinc	Zn	0.722 ± 0.082	0.113	100 ± 3	10	10.308 ± 0.229	0.022	95.862 ± 2.293	0.024

a) Certified value by manufacturer. Uncertainty in the manufacturer guideline was ≤3%.

Averages and standard deviations (SD) of the trace and major element concentrations in plasma of wild green sea turtles are summarized in Table 2. The analytical method allowed detection of twenty trace and major elements (Al, As, Br, Ca, Cr, Cu, Fe, Hg, Mn, Mo, Ni, P, Pb, S, Se, Si, Sr, Ti, Y and Zn). In the rural coastal group, data obtained from all green sea turtles, irrespective of when they were captured, were treated as one dataset for statistical analysis, because there were no significant seasonal difference in the plasma trace and major element concentrations. There were significant differences in the plasma levels of Ca, P, Pb, S, Se, Si, Ti and Zn between the suburban and rural coastal groups whereas the other elements showed no significant differences.

The plasma Ca, P, S and Zn concentrations in the captive group were significantly higher than those in wild sea turtles from both the suburban and rural coastal groups. The specific accumulation of Ca, P, S, and Zn found in captive green sea turtles might be attributable to their feeding habits, which depends on their living environment. Immature green sea turtles are omnivorous and occupy open ocean pelagic habitats [2, 3]; however, after this life stage, they enter benthic foraging areas to be herbivores [4], thus exhibiting more selective feeding patterns.

All sea turtles kept at OERC consumed not only vegetables, but also fish and squid, so their plasma Ca, P, S and Zn concentrations were higher compared to the wild varieties. These results are in agreement with previous studies that compare captive and wild sea turtles in Okinawa Main Island [31, 32].

On comparing individuals from the suburban and rural coastal areas, differences in Pb, Se, Si and Ti concentrations in the plasma were observed. Plasma Pb, Si and Ti concentrations in the green sea turtle inhabiting the suburban areas were significantly higher than those in the captive and rural coast groups ($P<0.01$). These results suggest marine pollution by Pb from fossil materials and from paints and are in agreement with studies using tissue samples obtained from autopsy where high Pb accumulation [3, 27] in wild sea turtles has been reported. In Japan, the use of leaded gasoline for automobiles has been banned since the 1970s, and the particulate matter containing lead compounds from the exhaust of automobiles, before this ban, may have been deposited on the sediments in the coastal area of Okinawa. The sediment concentrations of heavy metals such as Cu, Pb and Zn are reflected in the concentration of the respective heavy metals in eelgrass [23], indicating that a possible source of lead in sea turtles is via the grass that it grazed on.

Titanium dioxide (TiO_2) is used for many household products such as toothpastes, soaps, and detergents; the usage of such products results in the TiO_2 entering the wastewater effluents and eventually reaching the coastal open water. Among the TiO_2 containing compounds, nano particulate-titanium dioxide (nano-TiO_2) is one of the most universally engineered nano-material. Many chemicals of emerging concern (CECs) are frequently used in cosmetic formulations and other products, and these can potentially reach the marine environment to increase concentrations that may harm the marine ecosystem. Since the nano-TiO_2 is reported to cause oxidative stress mediated phototoxic effect due to its photoactive nature, it can cause cytotoxic effects in phytoplankton. Thus, the increased concentration of nano-TiO_2 in marine ecosystems may have negative consequences [25]. Skin care products have the highest quantities of CECs, with titanium dioxide and zinc oxide nano-materials being dominant potential contaminants [8]. They are widely used and present in the aquatic environment [26]. The large production and growing use of nano-TiO_2 has resulted in their continuous release into aquatic systems, which may cause harmful effects to marine organisms either directly or due to their potential interaction with conventional toxic contaminants, which represents a growing concern for biota [28]. The plasma Ti concentration in the sea turtles in the suburban coast was higher than that in the rural coastal area, which is most likely related to the influence of residential and industrial wastewater. Therefore, measurement of the plasma Ti concentration in sea turtles might be a useful bio-monitoring method to estimate the marine pollution level due to Ti, which can

Table 2. The mean concentrations of trace and major elements in plasma from Green turtles that live in different waters

Elements (μg/m*l*)	Suburban coast group (Okinawa Main Island)		Rural coast group (Yaeyama Island)		
	Captive (n=8)	Suburban coast (n=8)	Total (n=57)	Winter (n=27)	Summer (n=30)
Al	8.743 ± 13.416	9.586 ± 8.916	5.814 ± 3.758	5.915 ± 3.409	5.680 ± 4.049
As	0.065 ± 0.075	0.279 ± 0.172	0.207 ± 0.348	0.170 ± 0.437	0.233 ± 0.241
Br	13.348 ± 1.617	15.312 ± 3.580	12.196 ± 1.898	12.114 ± 2.073	12.172 ± 1.807
Ca	137.114 ± 14.994	72.255 ± 13.089[a]	49.734 ± 11.272[A]	50.733 ± 11.585	48.308 ± 11.236
Cr	0.096 ± 0.093	0.059 ± 0.064	0.127 ± 0.079	0.127 ± 0.072	0.127 ± 0.084
Cu	0.344 ± 0.135	0.283 ± 0.143	0.433 ± 0.291	0.342 ± 0.297	0.507 ± 0.266
Fe	1.163 ± 0.347	1.261 ± 0.869	0.863 ± 0.915	0.918 ± 1.006	0.803 ± 0.822
Hg	0.060 ± 0.095	0.094 ± 0.055	0.055 ± 0.080	0.056 ± 0.079	0.053 ± 0.080
Mn	0.006 ± 0.009	0.041 ± 0.040	0.011 ± 0.035	0.002 ± 0.008	0.019 ± 0.046
Mo	0.050 ± 0.069	0.081 ± 0.097	0.041 ± 0.075	0.028 ± 0.056	0.052 ± 0.087
Ni	0.013 ± 0.022	0.036 ± 0.036	0.017 ± 0.025	0.014 ± 0.021	0.020 ± 0.027
P	211.087 ± 215.732	88.142 ± 38.948[a]	54.823 ± 19.565[A]	48.503 ± 19.094	59.936 ± 18.577
Pb	0.202 ± 0.104	0.959 ± 0.579A	0.197 ± 0.141[B]	0.151 ± 0.110	0.237 ± 0.152
S	562.184 ± 227.093	432.758 ± 114.049[a]	315.772 ± 90.123[A]	307.660 ± 90.830	320.694 ± 89.982
Se	0.179 ± 0.081	0.120 ± 0.077[a]	0.192 ± 0.074[b]	0.189 ± 0.074	0.197 ± 0.076
Si	1.173 ± 3.026	6.438 ± 5.553[a]	1.876 ± 2.751[b]	1.885 ± 2.740	1.806 ± 2.782
Sr	1.788 ± 2.412	1.041 ± 0.630	0.763 ± 0.204	0.736 ± 0.160	0.777 ± 0.243
Ti	0.154 ± 0.230	0.450 ± 0.377[A]	0.026 ± 0.090[B]	0.010 ± 0.046	0.040 ± 0.114
Y	0.010 ± 0.019	0.013 ± 0.030	0.014 ± 0.027	0.009 ± 0.021	0.017 ± 0.030
Zn	2.045 ± 1.828	1.355 ± 0.452[a]	0.900 ± 0.278[A]	0.889 ± 0.330	0.904 ± 0.222

UNIT: μg/m*l*. a: P<0.05 vs captive group, A: P<0.01 vs captive group, b: P<0.05 vs suburban coast group and B: P<0.01 vs suburban coast group, respectively.

change with changing human activities and the type of industrial processes present.

Labrada-Martagón *et al*. [21] demonstrated that wild green sea turtles from suburban coastal areas showed higher Si concentrations, but lower antioxidant enzyme activities than individuals from rural coastal area in the Baja California Peninsula. Si is an essential element in human-dominated landscapes where it is used in constructions, electronic equipment, cosmetics, and medical materials. Human impacts on Si cycling are only recently being explored [25]. The majority of Si entering the world's oceans is from rivers in the form of dissolved Si that originates from rinsing-water and waste-water from various human activities. Maguire and Fulweiler [24] highlight the influences of urban environments in altering the flux of Si from land to sea. For marine vegetation such as a standing stock of eelgrass, *Zostera marina*, Si levels in the leaves has been shown to be strongly correlated to the dissolved silicon concentration in the water column [17]. Since the eelgrass is commonly found in Okinawa area and is known to be grazed by green sea turtles, this could have contributed, along with various human activities, to a significantly higher concentration of Si in the suburban coastal group.

Se is an element related to glutathione peroxidase, which is an antioxidant. It is known to detoxify Hg, a known neurotoxin with no known essential function, in the body. However, Se can become toxic at elevated concentrations. In this study, plasma Se concentration in the green sea turtles inhabiting the suburban coast was 0.120 ± 0.077 μg/g, which was significantly lower than those in the captive (0.179 ± 0.081 μg/g, P<0.05) and rural coastal groups (0.192 ± 0.074 μg/g, P<0.05). Therefore, our results for the concentration of Pb, Si, Se and Ti for plasma samples collected from the two different areas indicates that the concentrations are related to the industrial activities in the urban regions of Okinawa.

Since mature green sea turtles in this area remain in the coastal regions instead of migrating to other oceanic regions, the concentration of trace and major elements in their body is a direct reflection of the pollution in these coastal waters. The results of this study confirm that the concentrations of some elements are higher in green sea turtles from the Okinawa Main Island, which is probably related to the high degree of anthropogenic effluent pressure in this coastal basin. Thus, these individuals are more likely to experience adverse effects related to contaminants when compared to individuals from the Yaeyama Island. Data such as those presented in this study are useful to assess marine pollution levels and to motivate local and regional authorities to continue to monitor the level of multiple elements in green turtles. This also is a potential early warning system to avoid adverse exposure, not only for sea turtles but also other marine organisms, to some trace and major elements associated with the suburban areas of Okinawa.

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