Understanding environmental influences on microchemistry and morphology of fish otoliths

(魚類耳石の微量元素組成と形態に対する環境影響に関する研究)

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Abstract

The rapidly changing environment of marine systems around the world has drawn attention from multiple perspectives, and the most concerned problems must include hypoxia and ocean acidification (OA). Fish otoliths have been long considered as a useful tool to study the living conditions of fish. Thus, this study mainly focused on microchemistry and morphology of otoliths to understand the correlation between fish and environmental problems they experienced.

To examine the current conditions of Tokyo Bay, marbled flounder captured from four locations inside and outside the Bay area were analyzed by Laser Ablation Inductively Coupled Mass Spectrometry (LA ICPMS) on otolith chemistry. Results showed that $[Mn/Ca]_{otolith}$ was different among locations, especially at young age. Fish in age 0 suggested the highest $[Mn/Ca]_{otolith}$ occurred in the inner Bay area, then the mouth areas of the Bay, and the lowest value was measured outside of the Bay. This result perfectly matched the expectation of hypoxic conditions in Tokyo Bay areas. In addition, a seasonal trend of increasing in $[Mn/Ca]_{otolith}$ from opaque zones to translucent zones in otoliths also supported the summer-occurring hypoxia in Tokyo Bay, since translucent zones are usually formed under high temperature. No significant difference was found in other elements, possibly indicating that other factors, such as temperature and salinity, were not much different among the Tokyo Bay areas.

Because the mechanism of the elevated $[Mn/Ca]_{otolith}$ under hypoxia remained unclear, and one explanation was the elevated $[Mn/Ca]_{water}$ caused by redox of Mn under hypoxia, we examined the correlation of Mn concentrations between water and otoliths. Juvenile marbled flounder were reared under constant Mn concentrations for two months, and chemistry of both water and otoliths were analyzed by ICPMS. Significant difference could be found between control and the chemical treatments, but no correlation was found between $[Mn/Ca]_{otolith}$ and $[Mn/Ca]_{water}$. In addition, water sample collected from Tokyo Bay had a similar level of $[Mn/Ca]_{water}$ as the control at laboratory, but $[Mn/Ca]_{otolith}$ of Tokyo Bay was much higher than any treatment. An incremental Mn experiment was conducted with weekly increasing Mn concentrations for 11 weeks, and the result also suggested $[Mn/Ca]_{otolith}$ was not positively connected to $[Mn/Ca]_{water}$. No positive correlation was found in olive flounder either. Based on these results, the increase in $[Mn/Ca]_{otolith}$ found under hypoxia seemed not be solely caused by water Mn, and the mechanism needs further examinations.

The impacts of OA on otolith morphology were studied too. Juvenile marbled flounder were reared under three pH groups: control pH 7.8, Group pH 7.5, and Group pH 6.8 for two months. Photos of the whole otoliths were taken, and analyzed by ImageJ on area, perimeter, solidity, length, width, and thickness. Area and thickness showed to be increasing as pH declined, and solidity revealed to be smoother under elevated H^+. Significant difference found between control and

treatments in area, thickness, and solidity suggested the possibility of using these measurements to study OA.

Our results found difference in $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ among different locations around Tokyo Bay, potentially indicating the more severe hypoxic conditions of the Bay than the outside. However, the mechanism of hypoxia causing $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ to increase seemed not to be water Mn. In addition, larger sized otoliths were formed under elevated H^+, together with a smoother surface which could be examined through solidity.

Keywords: marbled flounder, otolith, dissolved oxygen (DO), ocean acidification (OA), hypoxia, water chemistry, solidity

Chapter 1

Introduction

1.1 Hypoxia and ocean acidification (OA)

Environmental changes happened in oceans have drawn attentions from ecologists recent years. Problems such as global warming, pollution, hypoxia and ocean acidification increase the concerns to marine conditions and creatures. Based on predictions of different emissions scenarios, by the end of the 21st century, surface temperature of oceans could rise 1.5-4.8 °C relative to 1850-1900 (IPCC AR6 WGI, 2021). Studies have shown that multiple species like trout, cod, salmon and bass seemed to receive stress from elevated temperature, responding as increase of cortisol release (Alfonso et al. 2020). Chemical pollution has been discussed since heavy use of chemical fertilizer. High values of NO_3^-, NH_4^+, NH^+, Fe, K, Na, and chemicals commonly seen in agricultural activities have been observed in Lake Ziway, Ethiopia, and likely to affect ecosystem negatively (Merga et al. 2020). In addition, the famous ocean pollution case, the Deepwater Horison oil spill happened in 2010 in the Gulf of Mexico, is still watched by researchers. Juvenile southern flounder, *Paralichthys lethostigma* showed a smaller body size and higher rate of gill abnormalities after a four-week exposure to polycyclic aromatic hydrocarbon contaminated sediment

(Brown-Peterson et al. 2016). Other than problems above, hypoxia and OA also catch ecologists' attention increasingly.

Hypoxia is a term borrowed from medical field to describe the situation, when low DO negatively alters aquatic organisms' behaviors or physiology (Rabalais et al. 2010). There is no universal definition of hypoxia due to the complexity of habitat and species difference, but 2 mg/L seems to be the threshold of sublethal and lethal DO for half of the species tested by Vaquer-Sunyer and Duarte (2008), thus the value becomes commonly accepted in most hypoxic studies. In nature, hypoxia could occur due to consumption (eg. Microbial respiration), lack of re-supply of DO (eg. Climate models like El Nino shift), or advection of low-oxygen water layers, but human activities seem to enhance the impact temporally and spatially (Rabalais et al. 2010). One would be eutrophication, usually caused by indiscriminate use of fertilizers, which increases the growth and respiratory decomposition of many microbes (Grall and Chauvaud 2002). Another one could be temperature, affecting river discharge, suggested by Jenny et al. (2014) through statistical modeling. Water bodies around the world have shown a dramatically increased area of hypoxia during the past few decades, such as Baltic Sea, Gulf of Mexico, East China Sea, Northern Adriatic Sea, and Chesapeake Bay, most likely due to the nutrient flux from the nearby agriculture (Rabalais et al. 2010).

The growing hypoxia around the world has caused problems in fishery and ecology. Habitat for blue crab (*Callinectes sapidus*) experienced a 27% loss due to low oxygen in 1997 in the Neuse River estuary, USA (Selberg et al. 2001). When oxygen level stayed below 1 mg / L in Lake Võrtsjärv, Estonia, the mean catch per unit effort (CPUE) of the year dropped to 1/3rd of the CPUE in normal years (Järvalt et al. 2004). On ecological level, the whole ecosystem responded to hypoxia physiologically or behaviorally, from plankton to fish; and interactions such as habitat selection, predation, and production were affected in Gulf of Mexico, USA (Rabalais et al. 2002). A low-oxygen area of approximately 432 km^3 occurred in East China Sea, China led to a dramatic density gradient in summer, 2003 (Chen et al. 2007).

The longer and larger hypoxia affects marine organisms in many ways. Sipunculida seemed to be influenced on metabolism when exposed under anoxia for less than 48 h; oligochaetes and polychaetes would die even with similar or less time; molluscs and echinoderms showed a reduced growth, while crustaceans were affected on metabolism and reproduction when DO was between 0.5 and 3.4 mg/L; and hypoxic conditions were kept on fish for relatively short time (less than 24 h), showing a more diverse impacts of metabolism, growth, reproduction, activity, and feeding (Gray et al. 2002). For more detailed reacts from fish, Prince et al. (2010) found that both sailfish, *Istiophorus platypterus* and blue marlin, *Makaira nigricans* spent longer time near the surface in low DO areas, possibly affecting catch rates. Other species, such as Bombay duck, *Harpadon nehereus*, showed a rise abundance due to its high tolerant to hypoxia, which could influence the community structure and marine ecosystem (Kang et al. 2021).

OA is describing the decline of pH in ocean water, usually caused by increasing uptake of CO_2 from atmosphere. Impacts of pH to marine organisms seem to draw attentions from early 20th century, but a study in 1957 revealed that anthropogenic CO_2 dissolved into oceans affected buffering capacity negatively, together with another 1966 study suggesting that the concentration of CO_3^{2-}, which plays a role as buffer, had impacts on community calcification, seems to be the start of recent studies on OA (Gattuso and Hansson 2011). The industrial era has induced a dramatically doubled atmospheric CO_2 by 2009, and the rate of increase kept growing higher, from 1.0% per year in the 1990s to approximately 3.4% per year in the 2000s, which leads pH in total scale to decline from 8.2 in 1766 to 7.79 in 2100 by prediction (Gattuso and Hansson 2011).

Due to these findings, more and more researchers choose to fully tap potential impacts of OA on multiple organisms. A reduction in coral reef diversity was observed when pH declined below 7.8 (Fabricius et al. 2011). Pteropods were found to be already severely affected in California, with more than half of onshore individuals experiencing corrosive waters and occurred damage (Bednarsek et al. 2014). Settlement success of juveniles seemed to be negative affected by OA in green sea urchin, *Strongylocentrotus droebachiensis*, and survival might decrease if the exposure started from the larval stage (Dupont et al. 2013). In addition, the impacts of OA seemed to be passed down to the next generation in some organisms (Parker et al. 2012). Without out-structures that would be dissolved by OA, fish still got affected by

OA from multiple perspectives. Larvae of orange clownfish (*Amphiprion percula*) lost their ability to distinguish predators through olfactory cues (Dixson et al. 2010), thus might decrease survival rate of the species. On the other hand, a behavior change in prey-selection was observed in predator species under elevated CO_2 conditions (Ferrari et al. 2011), possibly due to the olfactory impacts too (Cripps et al. 2011). Besides the predator-prey relationship, growth of fish (Ou et al. 2015), organ functions (Maulvault et al. 2018), swimming behavior (Pimentel et al. 2014), respiratory gas transport (Esbaugh et al. 2012), maximal O_2 uptake (Ou et al. 2015), metabolism (Pementel et al. 2014), acid-base balance in blood (Esbaugh et al. 2012), and immune related enzymes and gene expressions (Wang et al. 2016) were also found to be affected by OA, usually in a negative way.

Both hypoxia and OA have become the environmental problems which ecologists concern the most in recent decades, and this has drawn our attention to the potential impacts hypoxia and OA have on organisms living closer to us.

1.2 Fish otoliths

Otoliths are paired structures secreted in endolymphatic fluid beneath the encephalocoele of all teleost fishes. The composition of otoliths is calcium carbonate matrix. For example, 96.2% by weight of calcium carbonate, 0.73% non-organic trace elements, and 3.1% organic matrix were revealed from otoliths of Atlantic croaker, *Micropogonias ungulatus* (Campana et al. 1997). Through an observation of discontinuous zones formed over 24 h in otoliths of goldfish, *Carassius auratus* (Mugiya et al. 1981), Campana and Neilson (1985) further developed the idea of daily increment, and from here the ability for otoliths to represent daily environment started to be commonly accepted. Thus, otoliths have been used in multiple studies, especially connected to changes in environments around the fish.

The first utilization is study on identifying movement during a specific period of life of fish or distinguishing different locations, such as migration or nursery characteristics (Smith and Kwak 2014, Elsdon and Gillanders 2003, Reis-Santos et al. 2008). The discrimination of nurseries could reach 100% accuracy in some cases (Reis-Santos et al. 2008). Chemical components seemed to continuously reflect different conditions fish experienced, thus, life history of fish could be studied through otolith chemistry change as well (Hermann et al. 2016). Elements which are not commonly seen in natural environment, such as Se, has been used to examine pollution happened in water systems (Limburg et al. 2010). In addition, otolith microchemistry has been connected to physiological impacts like fishing pressure through its influenced microchemical signals (Catalan et al. 2018). Besides chemistry, otolith morphology also plays an important role on numerous levels. The contour and shape of otoliths had been used to reveal diversity and assemblages of fish (Tuset et al. 2016), and for identifying and distinguishing different species (Tuset et al. 2012). Altered morphometry could affect acoustic functionality of fish, such as sensitivity and locating the source of sounds (Lychakov and Rebana 2005). Of course, hypoxia and OA researches could go further by means of otoliths too, like the utilization in this study.

1.3 Study area and fish model

Tokyo Bay, as one of the most important fishery site of Japan, has been suffering from environmental changes nowadays. Chiba Prefecture has recorded the hypoxic condition of Tokyo Bay during summers for decades, indicating an increased trend in vertical area after 1960s (Ishii 2021). Hypoxia seemed to affect the settlement of dragonet (*Repomucenus valenciennei*), leading to an absence of spring cohort in Tokyo Bay (Ikejima and Shimizu 1999). Migration has been found in mantis shrimp (*Oratosquilla oratoria*) from northern hypoxic area to southern normoxic area, and the larval settlement of early cohort of the shrimp species was no longer occurred (Kodama and Horiguchi 2011). OA has not been shown to influence Tokyo Bay severely yet, but results from some studies indicate the possibility. Aragonite saturation state in the inner Tokyo Bay region was found to reach 1.55 in September, and this could lead aragonite to undersaturation earlier (Yamamoto-Kawai et al. 2015). In addtion, this aragonite undersaturation in Tokyo Bay was revealed to be happening in summer if atmospheric CO_2 exceeds 650 ppm (Yamamoto-Kawai et al. 2021), which is highly possibly reached by the end of this century (Richard et al. 2009).

Based on above, the conditions of Tokyo Bay needs attention and further examinations.

Marbled flounder (*Pseudopleuronectes yokohamae*) is a common flatfish species, which is widely distributed in the northwestern Pacific and can be found in the Sea of Japan, Yellow Sea and East China Sea (Fishbase). The spawning season seems to vary among places; it is estimated to be around January and February in Tokyo Bay and later (from February to April) in Hokkaido. As marbled flounders can be found in almost all of the waterbodies surrounding Japan, they play a very important role in Japanese fisheries.

1.4 Objectives and hypotheses

To better understand the environmental influences on fish otoliths in Tokyo Bay, our research mainly focuses on the following objectives:

Objective 1: to examine the environmental changes happened in Tokyo Bay through otolith microchemistry, especially the hypoxic condition around the Bay area though Mn. Otolith Mn, Mg, Ba, and Sr of fish collected from inside and outside the Bay were analyzed and compared.

Hypothesis 1: otolith Mn will follow a pattern from highest to lowest as samples collected from the inner Bay to the outside of the Bay due to our assumption of the hypoxic conditions among the Bay and surrounding areas. And difference of other elements should suggest changes in other environmental factors.

Objective 2: to reveal the mechanism of changing of otolith Mn under hypoxia, otolith Mn of fish reared under different water Mn concentrations were compared, and the correlation between water and otolith Mn concentration was analyzed.

Hypothesis 2: otolith Mn will follow a clear correlation (e.g. linear) to water Mn change.

Objective 3: to examine the association between OA and otolith morphology, otolith size and shape were analyzed and compared.

Hypothesis 3: otoliths under OA will be larger in size and smoother in shape.

Chapter 2

Otolith microchemistry in Tokyo Bay

2.1 Introduction

Among the hypoxic areas around the world, Tokyo Bay, located in Chiba Prefecture, is one of the most important fishing grounds in Japan. However, the vimineous mouth of the Bay makes it prone to hypoxia during the summer, especially late July to early September (Chiba Prefecture, 2021). Spreading spatially and temporally recent years (Ishii et al., 2008; Kodama and Horiguchi, 2011), hypoxia in Tokyo Bay has become a widely concern to Japanese government and ecologist (Chiba Prefecture). The main causes were revealed to be an overflow of nutrients from nearby regions and a lack of tides due to reclamation (Kodama and Horiguchi, 2011).

A commonly used way to explore hypoxia is otolith Mn. By comparing Atlantic cod (*Gadus morhua*) otoliths of ancient ages (Neolithic and Iron Age) to the present, an increase in $[Mn/Ca]_{otolith}$ was found following the increase in hypoxia in cod nursery areas over thousands of years (Limburg et al., 2011). Element ions were believed to be taken into otoliths though three ways: substitution for calcium, inclusion in the interstitial spaces, and association with organic matrix (Campana

1999). No accurate results found in mechanism of uptake, but Mn seemed to be a substitution for calcium into otoliths (Hüssy et al. 2021). Higher $[Mn/Ca]_{otolith}$ was also found in yellow perch (*Perca flavescens*), Baltic flounder (*Platichthys solemdali*), and Atlantic croaker (*Micropogonias undulatus*) in hypoxic regions compared to normoxic regions (Limburg et al. 2015). Thus, $[Mn/Ca]_{otolith}$ is considered to be an indicator to hypoxia.

Other than Mn, researchers show strong interest in Mg, Ba, and Sr of fish otoliths as well, usually regarding to questions besides hypoxia. For instance, Walther and Limburg (2012) suggested the high potential of using Ba and Sr to characterize migrations. In freshwater habitat, the $[Mg/Ca]_{otolith}$, $[Ba/Ca]_{otolith}$, and $[Sr/Ca]_{otolith}$ showed 100% accuracy of track down to the original capture locations of trout (Wells et al. 2003). Thorrold et al. (1997) found these three elements varied across otoliths in Atlantic croaker (*Micropogonias undulatus*). Above suggests that Mg, Ba, and Sr might be sensitive to some factors, corresponding to environment or not. In particular, $[Mg/Ca]_{otolith}$ was positively affected by salinity, especially under relatively low salinity conditions (Lowe et al. 2009). Otolith Ba and Sr could remain physically stable even under acid-wash and ultrasonic cleaning (Campana et al. 2000), thus, these two elements are not easily affected by experimental preparation and correlated closer to fish itself. A number of researches used these two elements to determine movements (Gillanders 2005), identify habitats (Radigan et al. 2018), distinguish species (Radigan et al. 2018, Hamer and Jenkins 2007), resolve life history (Patterson et al. 2005), and reconstruct migratory patterns (Elsdon and Gillanders 2003). Otolith Ba might be able to indicate temperature changes (Miller 2009). $[Sr/Ca]_{otolith}$ also seemed to be positively correlated to salinity in largemouth bass (*Micropterus salmoides*, Lowe et al. 2009), and change of $[Sr/Ca]_{otolith}$ of gulf killifish (*Fundulus grandis*) otoliths fitted the best when the model was built with both salinity and temperature positively involved (Nelson et al. 2017), suggesting that temperature could affect $[Sr/Ca]_{otolith}$ too.

Multiple species in Tokyo Bay have been found altering behaviors to avoid hypoxia (Kodama and Horiguchi, 2011; Tachibana et al., 2019). However, marbled flounder (*Pseudopleuronectes yokohamae*), as a common commercial species in Japan and having large numbers in Tokyo Bay (Chiba Prefecture, 2019), is a demersal species and moves relatively slow. Marbled flounder has a wide distribution, covering Hokkaido to Hiroshima (Hata et al., 2016; Lee et al., 2016; Shafieipour et al., 2004). Although studies have found that the marbled flounder in Tokyo Bay reacts to the high temperatures and hypoxia in summer by immigration (Kodama and Horiguchi, 2011; Mitamura et al., 2020), the species could still experience hypoxia due to the large hypoxic area in summer.

In this chapter, our main aim is to use $[Mn/Ca]_{otolith}$ of marbled flounder captured from different locations to understand the hypoxic conditions around the Tokyo Bay area. In addition, to examine the changes of other environmental factors, such as temperature and salinity, in Tokyo Bay though otolith microchemistry.

2.2 Materials and methods

2.2.1 Sample collection

Thirty-two marbled flounder were randomly selected from fishery captures in the spring of 2012 and 2014 from four locations inside and outside Tokyo Bay. Choshi (Cho), located outside of the Bay, and two mouth sites of the Bay: Kaneda (Ka) and Takeoka (Ta), and the inner Bay site Funabashi (Fu). Standard length was determined by the fishery staff, and the otoliths were transported to Tohoku University. Three water samples from the inner Tokyo Bay (139°59.6’E, 35°34.2’N, close to Fu) were collected on August 19, 2019 (Figure 2-1).

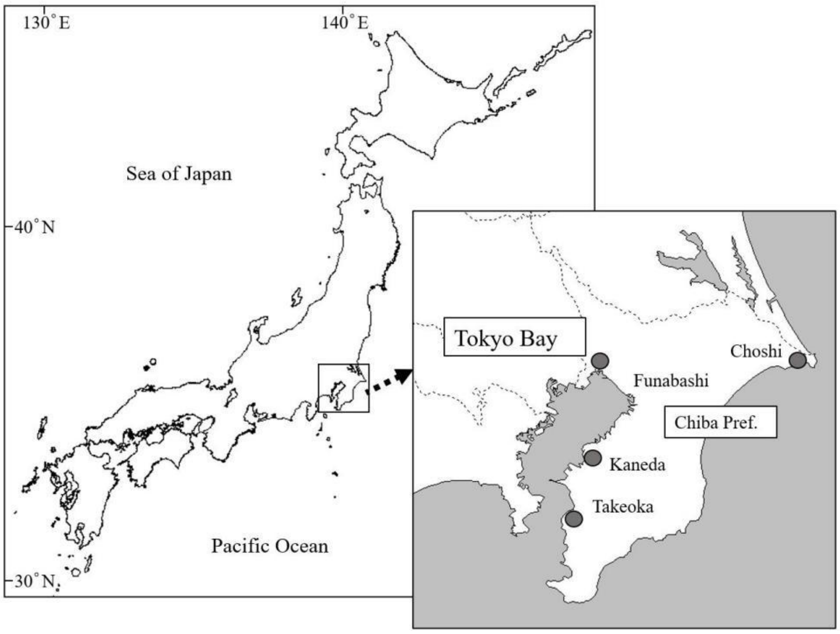

Figure 2-1 Map of capture locations. Fish samples were collected from Tokyo Bay (Funabashi, Kaneda and Takeoka) and outside (Choshi), belonging to Chiba Prefecture of Japan. White area in detailed map shows land and gray area is water. Water sample was collected near Funabashi.

2.2.2 Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA ICPMS) analysis

To avoid asymmetric impacts, only the blind-side otolith was used in the ICPMS. Otoliths were embedded in one epoxy mold with the potential core lined up. Sections were made along the width of each otolith, and a 600-μm section was kept using a low-speed saw. All sections were glued onto slides and polished with sandpaper to reach the core area. Fish age of the field samples was determined by three readers reading photos of the sections. Results were recorded only when two or more readings were identical.

Sections were later analyzed using laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS). In total, the content of eight elements (Ca, Sr, Na, K, Mg, Ba, Cu, Zn, Cd, and Mn) was measured with LA-ICPMS by the JFE Techno-Research Corporation (Kawasaki, Tokyo, Japan). An Agilent 7900 (Agilent Technologies, Japan) was used to perform the ICPMS analysis with an NWR213 laser (ESI designed and built). A line connecting the longest distance on the section was lasered with a 30-μm diameter spot at 4 Hz with an energy output of 2.0-2.3 J/cm^2. The distance between the two centers of the spot was 50-μm. NIST 614 glass was used as the standard.

2.2.3 Statistical analyses

For the Tokyo Bay samples, to eliminate outliers, the z-score of $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ was calculated, and $-3<z<3$ was defined as acceptable. After excluding outliers, $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ was grouped by age, and a boxplot was applied along different capture locations separated by age.

In addition, to reveal the potential temporal trend of the Tokyo Bay samples (except Cho), all data points of $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ were plotted along the distance of otolith radius. Studies have shown that the different structural characteristics of otolith represent different environments fish experienced: the opaque zones are usually formed under low temperature, while the translucent zones are formed under high temperature (Hong et al. 2021). Due to the different distance of rings formed by each individual, the trend might be overwrote by different ages when plotted together. Thus, the data was separated by age for point plot. The age was read by three readers, and at least two of the same reads on one sample were required for age determination.

To avoid the potential impacts of seasonal changes caused by individuals, the averages of each annual ring were also calculated by sample. Because areas after year 2+ were not completed by all fish, which means the period of time covered (late winter or middle spring) was not constant among all samples, only the 1st (age 0) and 2nd (age 1) annual rings were mainly focused.

One-way ANOVA was applied on all data of ages 0 and 1 respectively to compare the differences among capture places. The results would be followed by a Tukey's test when significant differences were observed. $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ in age 0 was normally distributed, thus be involved into ANOVA directly; age 1 was not following normality, so $\ln([\mathrm{Mn/Ca}]_{\mathrm{otolith}})$ was transformed and used in age 1 analysis.

Boxplot was applied to reveal the potential patterns of $[\mathrm{Mg/Ca}]_{\mathrm{otolith}}$, $[\mathrm{Ba/Ca}]_{\mathrm{otolith}}$, and $[\mathrm{Sr/Ca}]_{\mathrm{otolith}}$ in and outside Tokyo Bay. All points were grouped by locations. One-way ANOVA would be used to show the difference caused by locations if any pattern could be recognized through the boxplot.

All analyses were performed in R, mainly using the packages “ggpubr” and “multcomp”.

2.3 Results

2.3.1 Tokyo Bay and fish conditions

Sample fish were mainly collected during spring, aged from 2 to 4 years old, with a standard length of 232-289 mm (Table 2-1). Regardless of the year of capture, fish from the mouth sites of the Bay seemed to grow the largest within same period of time, compared to the inner or outside of the Bay (Table 2-1). The SL between the inner Bay station (Fu) and the station located outside of the Bay (Cho) were similar (Table 2-1). Because all samples were collected during fishery instead of scientific cruise, environmental factors were not recorded.

Table 2-1 Fish and capture data of the field experiment. Marbled founders were captured from outside (Choshi) or inside (Funabashi, Kaneda and Takeoka) of Tokyo Bay. Capture date and SL were reported by the fishery staff, and age was determined by annual ring examination. SL was represented as mean ± standard deviation (SD).

Location	N	Capture Date	SL (mm)	Age (year)
Choshi	5	Jan 21 2014	254.4±12.7	3-4
Kaneda	8	Mar 12 2014	287±25.5	3
Takeoka	6	May 31 2012	288.8±40.4	2-4
Funabashi	5	Jan 19 2012	255.4±29.8	2-3
	8	Jan 22 2014	232±13.5	3

2.3.2 Otolith Mn in Tokyo Bay

$[Mn/Ca]_{otolith}$ showed considerable difference among locations, with in-Bay regions higher than the outside one (Table 2-2, Figure 2-2). $[Mn/Ca]_{otolith}$ of age 0 (Shapiro test Pr = 0.182) and $\ln([Mn/Ca]_{otolith})$ of age 1 (Shapiro test Pr = 0.617) were analyzed. Results of ANOVA at age 0 revealed that the difference in $[Mn/Ca]_{otolith}$ was significant (age 0: Pr = 0.0191; age 1: Pr = 0.000771) among the four locations, while the associated Tukey's test showed that the inner Bay (Fu) was not significantly different but higher in $[Mn/Ca]_{otolith}$ than the mouth areas (Ka and Ta), and the outside-Bay site (Cho) had the lowest $[Mn/Ca]_{otolith}$ (Tables 2-3 and 2-4, Figure 2-2). On the other hand, $\ln([Mn/Ca]_{otolith})$ of age 1 showed a change in pattern, with the inner Bay similar to the outside location, and the mouth areas having the highest $[Mn/Ca]_{otolith}$ (Tables 2-3 and 2-4, Figure 2-2).

A larger variance could be observed at locations inside of Tokyo Bay (Fu, Ka and Ta, Figure 2-2). Age seemed to affect $[Mn/Ca]_{otolith}$, showing a decreasing trend as fish grew up annually (Figure 2-2).

Table 2-2 Average of otolith chemistry of the field experiment. Data was collected by LA-ICPMS, and average calculation was separated by location (outside-Bay: Choshi; mouth-Bay: Kaneda and Takeoka; inner Bay: Funabashi). Values were represented as mean ± standard deviation (SD).

Location	Mn (μmol)	Ca (mol)	Mn/Ca (μmol/mol)	Mg (μmol)	Ba (μmol)	Sr (mmol)
Choshi	4.85±3.72	0.99±0.00	4.92±3.78	67.83±25.19	1.49±1.32	2.14±0.42
Kaneda	12.71±9.76	0.98±0.00	12.94±9.97	75.35±27.74	1.28±0.88	2.05±0.43
Takeoka	8.00±7.64	0.99±0.00	8.12±7.76	74.76±28.15	1.46±2.16	2.10±0.41
Funabashi	11.70±12.51	0.98±0.00	11.90±12.73	67.77±26.51	1.09±0.82	1.94±0.39

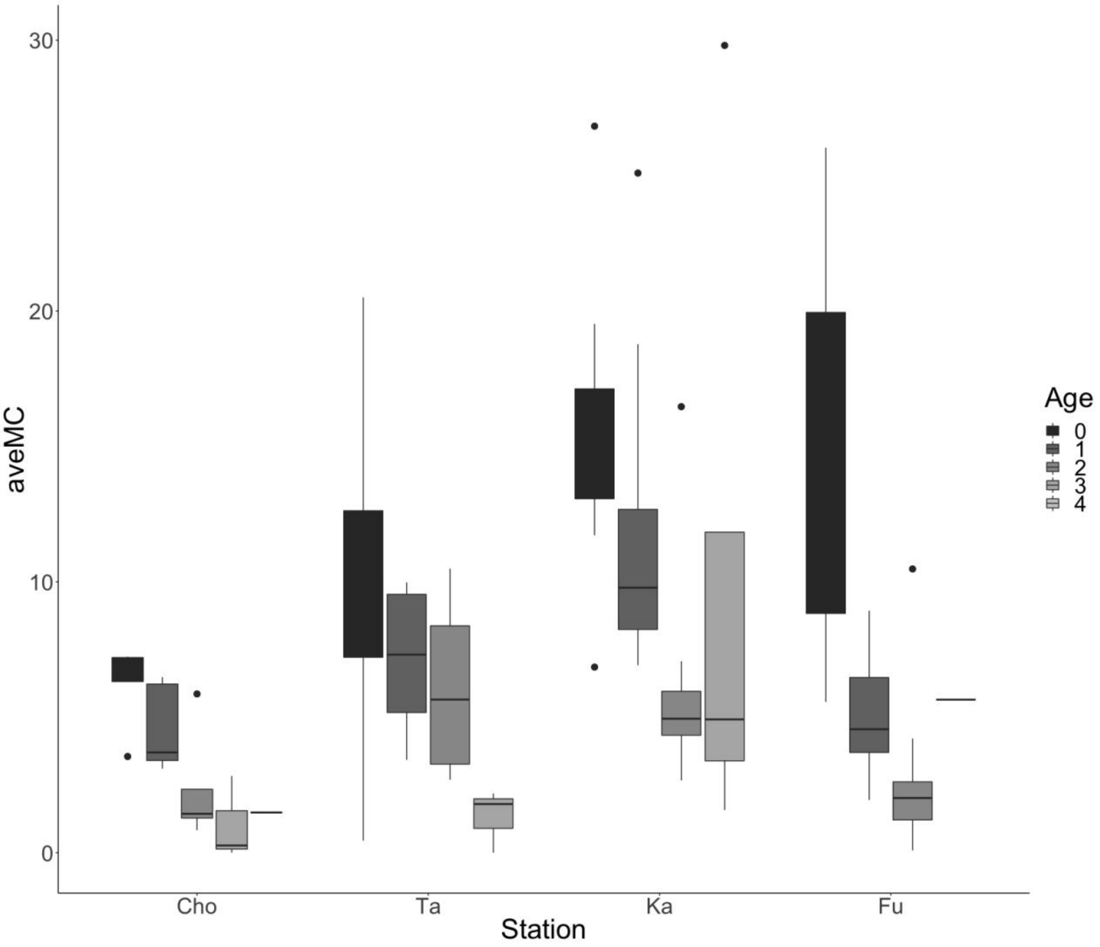

Figure 2-2 Boxplots of average [Mn/Ca]$_{otolith}$ (μmol/mol) of otoliths from outside (Cho) to mouth (Ta and Ka) and inner Bay (Fu). Each box showed the [Mn/Ca]$_{otolith}$ averaged for different ages within each station. Gray scale represents ages, with age 0 as the darkest black to age 4 as the lightest gray.

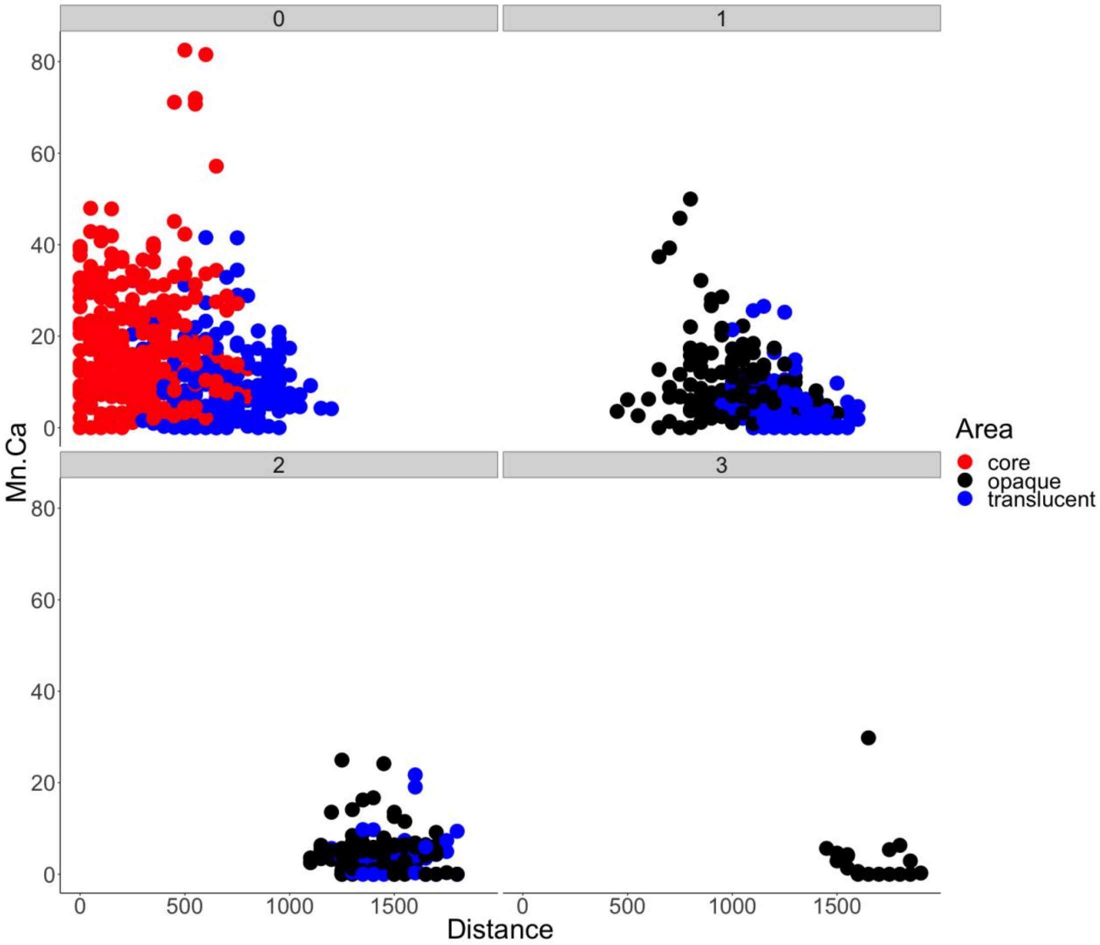

Figure 2-3 [Mn/Ca]$_{otolith}$ of Tokyo Bay samples (Fu, Ka, & Ta) in each age stage. The x-axis represents the distance from core to edge on otolith. Colors indicate different structures as core (the center), opaque (white and unclear areas), and translucent (clear areas).

Values of [Mn/Ca]$_{otolith}$ remained relatively high at the core area. A decreasing trend could be clearly seen at the edge of translucent areas connecting to an increase of [Mn/Ca]$_{otolith}$ as otolith transforming from translucent to opaque areas, especially during age 0 and 1 periods. Age 2 and 3 regions were not finished by all samples, meaning the data included might be covering different length of time of a year, thus might not be accurate enough for revealing patterns.

Table 2-3 Results of one-way ANOVA of $[Mn/Ca]_{otolith}$ in age 0 (core to the 1st annual ring) and $\ln([Mn/Ca]_{otolith})$ in age 1 (the 1st to the 2nd annual ring) in relation to locations.

		Df	Sum Sq	Mean Sq	F value	Pr
Age 0	Location	3	455.2	151.72	3.899	0.0191**
	Residuals	28	1089.6	38.91		
Age 1	Location	3	4.323	1.4410	7.516	0.000771***
	Residuals	28	5.368	0.1917		

Asterisk represents significance: Pr < 0.1*, Pr < 0.05**, Pr < 0.01***

Table 2-4 Results of Tukey's test of $[Mn/Ca]_{otolith}$ in age 0 and $\ln([Mn/Ca]_{otolith})$ in age 1 compared among locations (outside-Bay: Cho; mouth-Bay: Ka, and Ta; inner Bay: Fu). Letters represent difference.

	Cho	Ka	Ta	Fu
Age 0	b	ab	ab	a
Age 1	b	a	ab	b

2.3.3 Other elements

The distributions of $[Mg/Ca]_{otolith}$, $[Ba/Ca]_{otolith}$, and $[Sr/Ca]_{otolith}$ seemed similar among locations (Figure 2-4). Thus, no further analysis was applied.

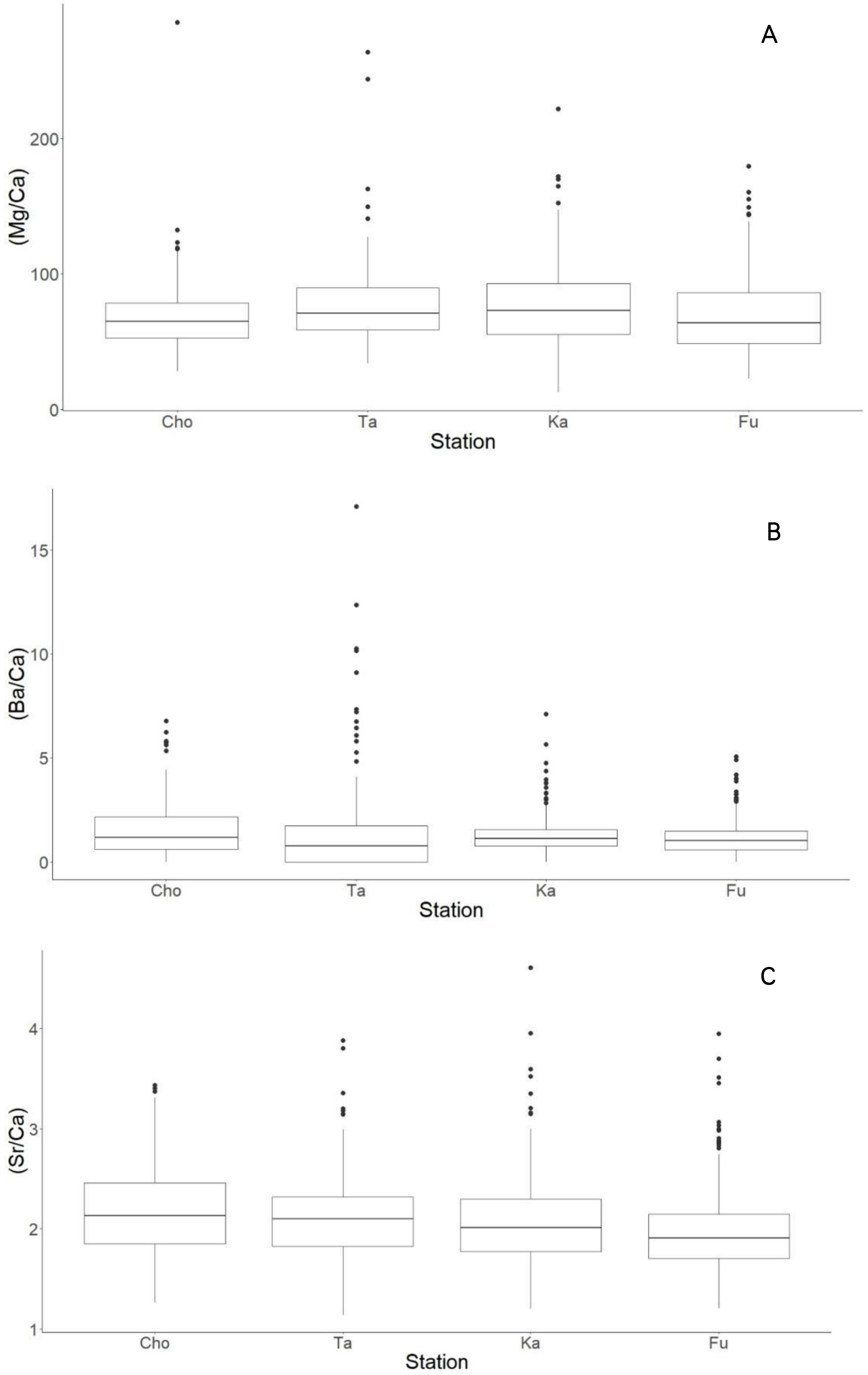

Figure 2-4 Boxplots of otolith element/Ca compared by locations, indicating normoxic (Cho) or hypoxic (Ta, Ka, and Fu) conditions. A: $[Mg/Ca]_{otolith}$ (μmol/mol);

B: $[\mathrm{Ba/Ca}]_{\mathrm{otolith}}$ (µmol/mol); and C: $[\mathrm{Sr/Ca}]_{\mathrm{otolith}}$ (mmol/mol).

2.4 Discussion

Our results found significant difference in $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ among locations around the Tokyo Bay area. The most possible cause based on results from other researchers is the difference in hypoxic conditions among these areas. Studies have revealed that fish from hypoxic conditions showed a higher $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ than fish captured from normoxic conditions (Limburg et al. 2011, Limburg et al. 2015). The seasonal trend of $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ in Figure 2-3 supported this correlation between hypoxia and otolith chemistry. The trend showed that $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ of the Tokyo Bay samples tended to increase when the structure of otoliths turned from opaque to translucent, indicating the temperature change from low to high (Hong et al. 2021). This matches the fact that hypoxia often occurs around July to September, the highest temperature in Tokyo Bay around a year. Thus, the difference in $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ among locations found by our results could suggest that Tokyo Bay is suffering more severe hypoxia than the outside, and the inner Bay area is not the same as the mouth area.

The correlation between $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ and hypoxia has been applied in many studies (Altenritter et al. 2018, Altenritter and Walther 2019, Limburg and Casini 2019), but the mechanism has not been clearly examined. The most detailed explanation available now is probably the one introduced by Limburg et al. (2015).

Due to the different solubility among Mn ions, the oxidation from Mn^{2+} to Mn^{3+} under normoxic conditions decreased the accessible Mn^{2+} in water for fish to uptake. Thus, the higher concentration of Mn^{2+} in water under hypoxia compared to normoxia made fish to uptake more Mn into their otoliths. Another factor which might connect to $[Mn/Ca]_{otolith}$ was suggested to be diet by Mohan and Walther (2016). Instead of affecting fish, the elevated Mn in water might increase the Mn in pray species to fish, then to be consumed into otoliths. Lastly, $[Mn/Ca]_{otolith}$ seemed to have a positive correlation to growth rate (Turner and Limburg 2015). This means the elevated $[Mn/Ca]_{otolith}$ under hypoxia might also correlate to faster growth in fish. However, Atlantic croaker (*Micropogonias undulatus*) reared under constant or periodic hypoxia did not show significant difference in total length of fish or mass of otoliths among treatments (Mohan et al. 2014), suggesting that fish growth was not influenced by hypoxia. Thus, the elevated $[Mn/Ca]_{otolith}$ under hypoxia seemed not to be caused by growth.

The significant difference among locations showed different patterns under different ages of fish in our results. By age 0, amount of $[Mn/Ca]_{otolith}$ was largest in the inner Bay (Fu), followed by the mouth areas (Ta and Ka), and the least in the outside (Cho). This seems to represent the expected hypoxic conditions around the Tokyo Bay areas perfectly: the inner Bay was the most hypoxic due to its difficulty in water supply; the mouth areas were experiencing a mixture of hypoxic and normoxic supply from both sides; and the outside was not affected by hypoxia at all. However, data of age 1 showed a different and more confusing pattern as the inner Bay having a similar low value of $[Mn/Ca]_{otolith}$ as the outside, and the mouth areas were high in $[Mn/Ca]_{otolith}$. There are three possible explanations we could come up to this difference. Firstly, the hypoxic conditions changed in different years. Thus, the different years in fish's life might be under different hypoxic situations. Ishii (2021) illustrated that the ratio of hypoxia area in Tokyo Bay was different for age 0 and age 1. With the Tokyo Bay samples mainly from 2008, 2009, and 2011 year classes, hypoxia of the age-0 years (2008: 0.57, 2009: 0.51, 2011: 0.57) were under larger hypoxia ratios than the age-1 years (2009: 0.51, 2010: 0.48, 2012: 0.46). Thus, the pattern of age 0 might be caused by the more severe hypoxia in Tokyo Bay. When the hypoxic situation became better, Tokyo Bay tended to have a similar value in $[Mn/Ca]_{otolith}$ to the outside. In addition, studies have found that South-west wind swept Tokyo Bay could increase the water exchange and refill the DO in the inner Bay (Nakayama et al. 2010, Sato et al. 2012). Thus, the different states of climate between age-0 years and age-1 years might cause a different DO level in Tokyo Bay, thus switch $[Mn/Ca]_{otolith}$ of different locations. Secondly, age might affect the way fish uptake or respond to hypoxia. Figure 2-2 showed a annual decline in $[Mn/Ca]_{otolith}$ while marbled flounder grew. The annual decline in $[Mn/Ca]_{otolith}$ has also been found in Baltic cod (Limburg et al. 2015). This annual decline with age of fish might neutralize the significant difference and turn all locations similar in $[Mn/Ca]_{otolith}$. In addition, adult fish might be less influenced when experiencing same stress as juvenile. They should have stronger physiological tolerance than young fish, and they could move further to escape from the hypoxic zones as well. However, this is hard to explain the high $[Mn/Ca]_{otolith}$ of age 1 flounders found in the mouth areas of Tokyo Bay. Thirdly, although studies and results have shown that the correlation between $[Mn/Ca]_{otolith}$ and hypoxia is promising, Mohan et al. (2014) did not find significant correlations between $[Mn/Ca]_{otolith}$ and hypoxia at laboratory, suggesting the possibility that there might be other factors which could affect $[Mn/Ca]_{otolith}$ in field. Temperature was revealed to have a negative impact on $[Mn/Ca]_{otolith}$ in black rockfish (Miller 2009). Salinity, on the other hand, resulted in an increasing $[Mn/Ca]_{otolith}$ in King George whiting (*Sillaginodes punctatus*) while increasing (Mazloumi et al. 2017). Temperature and salinity also showed a significant impact on Mn in otoliths of Atlantic cod (*G. morhua*, Stanley et al. 2015). The difference of other factors which affect $[Mn/Ca]_{otolith}$ might lead to the different patterns shown between age 0 and 1 in our results.

The core area of otoliths seemed to have a relatively large variance in $[Mn/Ca]_{otolith}$ in our study (Figure 2-3). Other researchers found some correlated results too. Spiked Mn has been found in multiple species, but under different scales (Ruttenberg et al. 2005), indicating that Mn concentration might be a species-specific characteristic of fish otoliths. Brophy et al. (2003) revealed a peak of Mn in the core area, regardless of species or original incubation conditions. In addition, it seemed that the spiked area in core might be very small (Brophy et al. 2003), suggesting the large variation observed in our results could be an overlap of multiple core-peaks from different individuals. The reason caused this elevated Mn in core area remains unclear. Although Brophy et al. (2003) found that reared under laboratory or wild conditions did not influence the occurrence of Mn peak, but the two peaks seemed to be under different levels of value. In addition, otolith-core Mn was found to be significant different among capture sites, suggesting the connection to nursery areas of fish (Avigliano et al. 2017). Thus, it is still highly possible that the value of $[Mn/Ca]_{otolith}$ in core area could represent the environment fish experienced.

Besides Mn, none of Mg, Ba or Sr seemed to be different among locations around Tokyo Bay. The results could be caused by the similar conditions, which these elements correlated to, among Tokyo Bay areas. Otolith Mg was shown correlated to salinity (Lowe et al. 2009), Ba to temperature (Miller 2009), and Sr might be influenced by both (Lowe et al. 2009, Nelson et al. 2017). Other than directly connected to salinity, $[Sr/Ca]_{otolith}$ was commonly used as an indicator to freshwater, estuarine and marine habitats (Coffey et al 1997, Lowe et al. 2011, Nims and Walther 2014), also correlating the element to salinity. These two environmental factors might not be hugely affected by the geography of Tokyo Bay like DO, thus, inside or outside did not influence the value of these factors much enough. In addition, besides environment, there are other possible causes to change the values of these elements uptake into otoliths of fish. $[Mg/Ca]_{otolith}$ was also revealed a negative correlation to otolith mass and somatic growth rate in spot (Martin and Thorrold 2005). Most studies found a strong impact of $[Ba/Ca]_{water}$ and $[Sr/Ca]_{water}$ on $[Ba/Ca]_{otolith}$ and

$[Sr/Ca]_{otolith}$, with over 60% of otolith elements were contributed by water (Farmer et al. 2013, Izzo et al. 2015, Reis-Santos et al. 2013, Walther and Thorrold 2006). Macdonald and Crook (2010) found a linear correlation between water and otolith Sr:Ca, while Ba:Ca was exponential. These possible causes might overwrite the changes in otolith microchemistry due to the differences of temperature or salinity, and led to similar results among different locations around Tokyo Bay.

Chapter 3

Relationship of Mn concentrations between water and otolith

3.1 Introduction

As mentioned in Chapter 2, many studies have found that fish caught from hypoxic regions showed a higher $[Mn/Ca]_{otolith}$ than normoxic regions. About the reason which caused this phenomenon, Limburg et al. (2015) explained it through the chemical characteristics of Mn. Because the dissolved Mn is the reduced form (Mn^{2+}), when DO returns to normal levels, Mn^{2+} is oxidized and precipitated, thus decreasing the Mn levels in ambient water and limiting its conversion into otoliths (Limburg et al., 2015). This explanation suggests that water Mn will increase under hypoxic condition when sediment Mn decreases, and both turn opposite under normoxia, and these changes make $[Mn/Ca]_{otolith}$ to vary following DO changes.

Based on the correlations found in multiple species (Limburg et al. 2011, Limburg et al. 2015) and the explanation above, otolith Mn has already been used as a proxy for hypoxia in some studies (Limburg and Casini, 2019, Altenritter and Walther 2019, Altenritter et al. 2018). However, the mechanism has remained a deduction and not been well understood. Fang et al. (2017), Abesser and Robinson (2010), and Kristiansen et al. (2002) found the release of Mn from sediment was greatly affected by DO, while Pakhomova et al. (2007) found no impact. The relationship between water and otolith Mn seems complicated as well. A positive correlation between $[Mn/Ca]_{otolith}$ and $[Mn/Ca]_{water}$ was found in spotted seatrout (*Cynoscion nebulosus*) collected from July to September in Chesapeake Bay (Dorval et al. 2006), where has been found experiencing hypoxia for long term (Hagy et al. 2004, Murphy et al. 2011, Breitburg 1992). Fish under marine environment had higher otolith Mn than riverine (Franco et al. 2013), also suggesting a positive correlation between water and otolith Mn. However, when salinity differed between approximately 14 and 20 ‰, Mn concentrations did not show correlation between sediment and otolith of Atlantic croaker (*Micropogonias undulatus*, Hanson and Zdanowicz 1999). No significant correlation was found in either snapper (*Pagrus auratus*) or sand flathead (*Platycephalus bassensis*) between otolith Mn and ambient water Mn (Hamer and Jenkins 2007). $[Mn/Ca]_{otolith}$ was not related to $[Mn/Ca]_{water}$ in black bream (*Acanthopagrus butcheri*), when water Mn concentration varied from approximately 4 to 80 μmol/mol (Elsdon and Gillanders 2003); nor even higher concentration at 50 to 110 μmol/mol in black rockfish (*Sebastes melanops*, Miller 2009). In addition, other factors, such as temperature (Miller 2009), diet (Sanchez-Jerez et al. 2002), and species difference (Clarke et al. 2011, Hamer and Jenkins 2007), showed impacts on $[Mn/Ca]_{otolith}$ sometimes as well.

Limburg and Casini (2019) found that fish with low body condition, defined as Fulton's condition factor K [(total weight (g) / (total length^3 (mm))) ×10^5] < 0.9, might be easier and longer to hypoxic exposure than fish with high body condition, indicating that physiology of fish might have impacts on $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ too. In addition to escaping from hypoxia, fish could have physical adjustment responding to constant hypoxia, thus, based on the mechanism, constant water Mn level. But how does otolith chemistry react to rapidly changing water chemistry has not been much studied.

To examine the potential species-specific patterns of $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ corresponding to $[\mathrm{Mn/Ca}]_{\mathrm{water}}$, marbled flounder, as an important species which has been mentioned in the former chapters, was used as the main model species, and olive flounder (*Paralichthyidae olivaceus*) and red sea bream (*Pagrus major*) were reared under different $[\mathrm{Mn/Ca}]_{\mathrm{water}}$ treatments too. Olive flounder, which could be found from south-eastern Russia to the South China Sea (Hu et al. 2014), passing Korea (Jung et al. 2008) and Japan (Tateda et al. 2015), is also a very important commercial fish in Japan. The annual catch once reached 250 tons in Tottori Prefecture in 1995 (Shirakashi et al. 2006). Because of the wide distribution, olive flounder could survive under a large range of temperature, causing the spawning season to shift between January and August depending on the water temperature (Danield and Watanabe 2010). The species was chosen to show if there could be observed difference between the two flatfishes. In addition, as the second most commonly cultured marine fish in

Japan (Foscarini 1988), red sea bream could be found in the coastal regions from north to south of Japan (Yamamoto et al. 2020). The catch of this species showed an increase from 297 to 2,039 tons during the recent forty years to 2010 in eastern Seto Inland Sea (Yamamoto et al. 2020). Red sea bream usually spawns during May with temperature between 14.8-17.4 °C and salinity between 32.0-33.0 (Zenitani et al. 2009). Compared to flatfishes, red sea bream is more pelagic with agile movement, thus is possible avoiding hypoxia via different strategy (Xiao 2015), leading to a different pattern corresponding to otolith Mn.

Thus, in this chapter, we are mainly aiming to examine the correlation between water and otolith Mn concentrations, subsequently discussing the impacts other factors might have on otolith Mn content, looking for the mechanism of elevated $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ under hypoxia.

3.2 Materials and methods

3.2.1 Uptake under constant Mn concentrations

Juvenile marbled flounder from the Kudamatsu City Center for Stock Enhancement hatched around mid-January 2020 were transported to the laboratory on April 22, 2020. The fish settled for 2 weeks in artificial seawater (Aquarium Systems, France, at a concentration of 33 g/L). All fish were marked with alizarin complexone (ALC, Dojindo Laboratories, Japan, at a concentration of 30 mg/L) on May 6, 2020, for 24 h. Room temperature was maintained at 15 °C to guarantee survival rate, and light was provided from 8 AM to 4 PM to simulate daytime. Fish were fed commercial fish food (Scientific Feed Laboratory Co. Ltd, Japan) twice a day during the three-month experiment (from May 11 to August 12, 2020).

After a three-day recovery from the ALC marking, fish were divided into different treatment groups on May 11, 2020. Three groups were designed: control with a normal Mn level of approximately 0.011 mg/L, Mn 30x with extra $MnCl_2 \cdot 4H_2O$ (Wako Pure Chemical Industries, Ltd., Japan) 1.149 mg/L added, and Mn 50x with 1.942 mg/L $MnCl_2 \cdot 4H_2O$. Each treatment consisted of six fish reared, in 3 L assigned water, with 2/3rd of the water volume being changed weekly. The environmental conditions (temperature, DO, salinity, and pH) were measured and water samples from each tank (50 mL) were collected weekly before the water was changed (Table 3-1). Water samples were frozen to store after filtered with a 0.20-μm filter and acidified with 2% HNO_3.

Table 3-1 Water conditions of the rearing experiment. Juvenile marbled flounders were kept under control (water Mn 0.011mg/L), Mn 30 (water Mn 30x of control), and Mn 50 (water Mn 50x of control) for two months. Dissolved oxygen (DO), pH, salinity and temperature were recorded weekly. Values were represented as mean ± standard deviation (SD).

	DO (mg/L)	pH	Salinity (‰)	Temperature (°C)
Control	10.3±0.15	7.9±0.2	30.4±1.47	12.8±0.64
Mn 30	10.1±0.26	7.9±0.3	29.3±0.51	12.8±0.64
Mn 50	10.1±0.33	7.8±0.3	30.0±0.44	12.9±0.63

3.2.2 Qualitative experiment with weekly increasing concentrations of water Mn

The same assignment of juvenile marbled flounder arrived laboratory on June 6, 2020, settled for 12 days and marked with ALC on June 18 for 24 h. Ten fish were set in a 12 L tank with 7 L artificial seawater on June 22, 2020. The starting concentration of Mn was 30x, 1.2 mg/L $MnCl_2 \cdot 4H_2O$ added, then increased weekly (30x – 50x 1.9 mg/L– 70x 2.8 mg/L– 90x 4.2 mg/L– 110x 4.3mg/L– 120x 5.3mg/L– 140x 5.8 mg/L– 150x 5.9 mg/L– 170x 7.0 mg/L– 190x 7.7 mg/L- 190x). Fish were kept under the same experimental conditions as in 3.2.1 for 11 weeks.

3.2.3 Species difference

Juvenile red sea bream (hatched on April 4-5 at the Kanagawa Prefecture Fish Farming Center, arrived laboratory on June 11) and olive flounder (hatched in late April at the Iwate Prefecture Fish Farming Center, arrived laboratory on July 14) were allowed to settle for 2 weeks in two 100 L tanks. All fish were marked with $SrCl_2 \cdot 6H_2O$ (Wako Pure Chemical Industries, Ltd., Japan) at a concentration of 302.5 mg/L (Sr is approximately 11x to control) for one week ahead the experiments. Room temperature was maintained at 18-20 °C, and light was provided from 8 AM to 4 PM to simulate daytime. Fish were fed 0.2 mL commercial fish food twice a day during the four-week experiment (red bream from July 2 to July 30, 2021, olive flounder from July 30 to August 27, 2021).

After Sr marking, fish were divided into three groups: control with a normal Mn level of approximately 0.011 mg/L, Mn 10x with extra $MnCl_2 \cdot 4H_2O$ 0.357 mg/L added, and Mn 30x with 1.149 mg/L $MnCl_2 \cdot 4H_2O$. Each treatment consisted of eight fish reared, in 9 L assigned water, with 2/3rd of the water volume being changed weekly. Temperature, DO, and salinity were measured and water samples from each tank were collected at the beginning, middle, and the end of the experiment.

3.2.4 Mn released from sediment to water under hypoxia

The results from this part was not reliable enough due to the condition control which was not well achieved during the experiment. However, it revealed some interesting possibility, thus is mentioned here as a prior study.

To examine the transformation of Mn between sediment and water under hypoxia, two 3-L tanks filled with sediment, collected from Ishinomaki Bay, Miyagi, and artificial seawater were settled for two days. Another tank contained only artificial seawater followed the same procedures as control. Three tanks were induced N_2 solely for 10 min to reach hypoxia, then sealed with plastic bags and left over a four-day period. After then, normal air was induced continuously to all tanks for the next three days. Approximately 50 ml of sediment and water samples were collected before the hypoxic treatment, after opening the sealed bag, and at the end of the experiment. Temperature, DO, and salinity were measured at the same time.

The experiment was done from July 18 to July 21 and repeated from July 28 to August 6, 2019. The differences between the two replicates were sediment contained and room temperature. The first experiment was with 400 g sediment and a constant room temperature as 24 °C. As the hypoxic condition was not well controlled during the treatment, the second experiment used 800 g sediment, and the room temperature was increased from 24 °C to 27 °C when treated with hypoxia.

3.2.5 ICPMS analyses

Fish were frozen to death at the end of the experiment, and the pair of sagittal otoliths was removed and cleaned. Three to five samples were embedded in one epoxy mold with core lined up along the length of each otolith, and a 1000-μm section was kept using a low-speed saw depending on the size of the otoliths. All sections were polished to reach the core.

All water samples, together with field samples collected during 2.2.2, were mainly measured for concentrations of Mn, Ca, Mg, Ba, and Sr. The analyses were performed at the School of Engineering, Tohoku University, using ICPMS (Agilent 8800, Agilent Technologies, Japan), following their inhouse procedures. Sediment samples were sent to JAPAN ENVIRONMENT SCIENCE CO., LTD (Yamagata, Japan) for ICP-ms analysis, following the procedures of this institution.

3.2.6 Statistical analyses

Because ALC did not stain well and was unreadable to determine the start point of our experiments, for 3.2.1, the experimental period was determined by an otolith Ba content > 0.001% in weight based on observations along the ICPMS laser line. Our data of water samples from laboratory (average Mn 271.4 ng/mL) and the institution (average <1 ng/mL) supported this way of distinguishing. In addition, the start of 3.2.2 was determined to be Ba > 5 μmol in otoliths.

The $[Mn/Ca]_{otolith}$ and partition coefficient of Mn (DMn) of the experimental period were then calculated and analyzed. Because the number of points analyzed by

LA-ICPMS was different among samples, the $[Mn/Ca]_{water}$ for each sample was matched to the exact one-week data instead of the average of the 13-week data. After quantile-quantile plot, neither $[Mn/Ca]_{otolith}$ nor DMn was normally distributed; thus, a natural logarithmic transformation was applied to both $[Mn/Ca]_{otolith}$ and DMn. Nested ANOVA was used to examine whether different categories of water treatment caused any differences in $[Mn/Ca]_{otolith}$. A value of 0 and outliers (defined as -2 > z-score or z-score > 2 due to the constancy of the experimental environment) were excluded during the analyses. When significance was found in ANOVA, Tukey' test was used to reveal the detailed results of differences.

The maximum $[Mn/Ca]_{otolith}$ within each sample from both Ch2 and Ch3 were recorded, and boxplots were drawn to visualize the data distribution. Maximum was chosen for revealing the highest value that $[Mn/Ca]_{otolith}$ could get under a specific water condition, and to avoid the potential impact of seasonal neutralization of the field data for better comparing to the laboratory data.

Water chemistry were compared, together with samples from Tokyo Bay. Because $[Mn/Ca]_{water}$ was not normally distributed, the non-parametric Kruskal-Wallis test was applied.

Due to the lack of data in 3.2.2, 3.2.3, and 3.2.4, only figures of raw data were made to have a visual view of tendency, potentially being a pioneer for future experiments.

All analyses were performed in R, mainly using the packages “ggpubr” and “multcomp”.

3.3 Results

3.3.1 No correlation between $[Mn/Ca]_{water}$ and $[Mn/Ca]_{otolith}$

Although the concentrations of water Mn did not reach the levels we set (Table 3-2), one-way ANOVA showed that the difference of $[Mn/Ca]_{water}$ among treatments was enough to show significance ($Pr < 2\ 10^{-16}$).

Table 3-2 Water chemistry of the laboratory and field experiments. Control, Mn 30 and Mn 50 were collected from the rearing experiment weekly, and water samples of the field experiment were collected near Funabashi. Values were measured using ICPMS, and represented as mean ± standard deviation (SD).

	Mn ($\times 10^{-3}$ mmol/L)	Ca ($\times 10^{-3}$ mol/L)	Mn/Ca (mmol/mol)	Mg ($\times 10^{-3}$ mol/L)	Ba ($\times 10^{-3}$ mmol/L)	Sr ($\times 10^{-3}$ mmol/L)
Control	0.50±0.16	4.45±0.68	0.12±0.06	87.8±15.7	3.27±0.57	81.8±10.9
Mn 30	6.94±1.10	4.48±0.84	1.57±0.20	87.0±16.5	2.92±0.55	82.0±14.5
Mn 50	10.4±1.92	4.23±0.74	2.47±0.10	81.4±15.4	2.76±0.71	76.4±13.1
Tokyo Bay	0.30±0.01	2.85±0.34	0.11±0.01	47.0±5.5	0.06±0.01	77.6±4.8

The SL of marbled flounder showed a decreasing trend, while the water Mn concentration increased from the control to Mn 30 and Mn 50 (Table 3-3). The difference in otolith Mn became much smaller compared to water Mn, with concentrations of 2.91 ± 1.71 μmol in the control, 4.77 ± 3.80 μmol in Mn 30 (1.6 fold of the control) and 6.01 ± 4.32 μmol in Mn 50 (2.1 fold of the control, Table 3-3). $[Mn/Ca]_{otolith}$ increased while $[Mn/Ca]_{water}$ increased, with the variance became larger (Figure 3-x). A disproportionate increase in the means of $[Mn/Ca]_{otolith}$ in each treatment could be observed following $[Mn/Ca]_{water}$, with D_{Mn} roughly calculated from the two means in Table 3-2 and 3-3 changed from 24.6 in control to 3.1 in Mn 30 then 2.5 in Mn 50.

Nested ANOVA revealed differences among treatments in $\ln([Mn/Ca]_{otolith})$ (Pr = 0.00566, Table 3-4). Tukey's test showed that the control had the lowest $\ln([Mn/Ca]_{otolith})$, but the highest was found in Mn 30 instead of Mn 50 (Table 3-5). The difference in $\ln(D_{Mn})$ was also significant among treatments in nested ANOVA (Pr < 2e-16), with Tukey's test revealing that the control was with the highest D_{Mn} compared to the two Mn treatments (Tables 3-4 and 3-5).

Table 3-3 Fish condition and otolith chemistry of the rearing experiment. Juvenile marbled founders were kept under three Mn treatments (control: water Mn 0.027 mg/L, Mn 30: water Mn 0.382 mg/L, and Mn 50: water Mn 0.575 mg/L) for two months. Values were measured using LA-ICPMS and represented as mean ± standard deviation (SD).

	SL (mm)	Mn (μmol)	Ca (mol)	Mn/Ca (μmol/mol)	Mg (μmol)	Ba (μmol)	Sr (mmol)
Control	38.64±0.76	2.91±1.71	0.99±0.0026	2.95±1.74	47.5±10.5	16.8±3.57	1.63±0.27
Mn 30	36.46±3.56	4.77±3.80	0.99±0.0024	4.84±3.86	51.6±17.7	14.3±3.17	1.64±0.27
Mn 50	33.91±1.71	6.01±4.32	0.99±0.0025	6.11±4.40	53.9±21.8	18.9±4.48	1.85±0.25

Table 3-4 Results of nested ANOVA of the rearing experiment. Individual difference (Fish) was nested into treatment difference (Water) during analysis. The two-month period for rearing experiment on otolith was determined by otolith Ba content > 0.001% in weight. Outliers were defined as otolith Mn content below detection limit or otolith Mn |z-score| > 2. $[Mn/Ca]_{otolith}$ and D_{Mn} were logarithmic transformed.

		Df	Sum Sq	Mean Sq	F value	Pr
ln[Mn/Ca]	Water	2	3.691	1.8453	5.675	0.00566***
	Water:Fish	6	5.979	0.9965	3.065	0.01141**
	Residuals	57	18.535	0.3252		
ln(DMn)	Water	2	81.25	40.63	80.591	<2e-16***
	Water:Fish	6	6.60	1.10	2.184	0.0577*
	Residuals	57	28.73	0.50		

Asterisk represents significance: Pr < 0.1*, Pr < 0.05**, Pr < 0.01***

Table 3-5 Results of Tukey's test of the rearing experiment. Logarithmic transformed $[Mn/Ca]_{otolith}$ and D_{Mn} were compared among Mn treatments. Letters represent difference.

	Control	Mn 30	Mn 50
ln[Otolith(Mn/Ca)]	b	a	ab
ln(DMn)	a	b	b

3.3.2 Difference between field and laboratory samples

The maximum of all samples from both Tokyo Bay and laboratory showed that, the highest concentration under rearing condition raised fish with an $[Mn/Ca]_{otolith}$ close to normoxic area in field. The locations in Tokyo Bay had much higher $[Mn/Ca]_{otolith}$ than the laboratory samples (Figure 3-1). Analyses of water samples showed significant difference (Pr = 6.399e-08) among water samples from the rearing experiment and Tokyo Bay. The non-parametric analysis suggested that the water sample from the inner Bay was similar to the control, but much less than Mn 30 and Mn 50 (Tables 3-2 and 3-6).

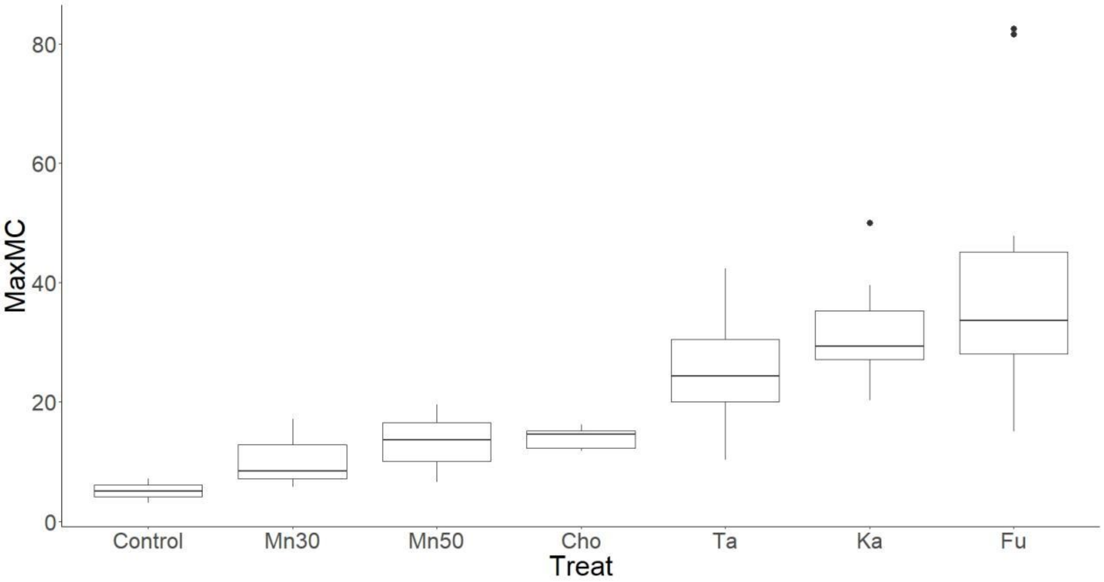

Figure 3-1 $[Mn/Ca]_{otolith}$ (μmol/mol) comparison between the rearing and field experiment. The x-axis contains all treatment groups from the laboratory experiment and locations from the field experiment. The y-axis is the maximum value of $[Mn/Ca]_{otolith}$ in each sample, and boxplot was applied on treatments or locations from the rearing experiment (Control, Mn 30 and Mn 50) and field experiment (normoxic: Cho; hypoxic: Ta, Ka, and Fu).

Table 3-6 Results of pairwise comparisons using Wilcoxon rank sum exact test of $[Mn/Ca]_{water.}$ Both rearing and field experiments were analyzed. Laboratory samples were collected weekly under three Mn treatments (control, Mn 30, and Mn 50) for two months, and three water samples of the field were collected near Funebashi.

	Tokyo Bay	Control	Mn 30
Control	1.0000	-	-
Mn 30	0.0043***	3.8 10^{-7}***	-
Mn 50	0.0043***	3.8 10^{-7}***	3.8 10^{-7}***

Asterisk represents significance: Pr < 0.1*, Pr < 0.05**, Pr < 0.01***

Sediment from five tanks was collected at the end of the experiment, and was verified to contain Mn concentrations of 130 mg/kg (control), 140 mg/kg (Mn 30), and 160 mg/kg (Mn 50), while the inner Tokyo Bay showed an average sediment Mn of 503 mg/kg.

3.3.3 Qualitative results of Exp. Sediment-water, Exp. Weekly increasing Mn, and Exp. Species difference

Exp. Sediment-water

Because Ca was not measured during this experiment, only Mn was exhibited in molecular level. It could be seen from Figure 3-2 that in control, water Mn kept a relatively low level and did not change much during the treatments; but two groups with sediment filled showed a high value only after the hypoxic treatment. These two tanks were from the second replicate experiment with increased sediment and temperature. Table 3-7 also revealed a lowest DO value in these two groups during the hypoxic treatment. However, Mn in sediment did not change much during the experiments.

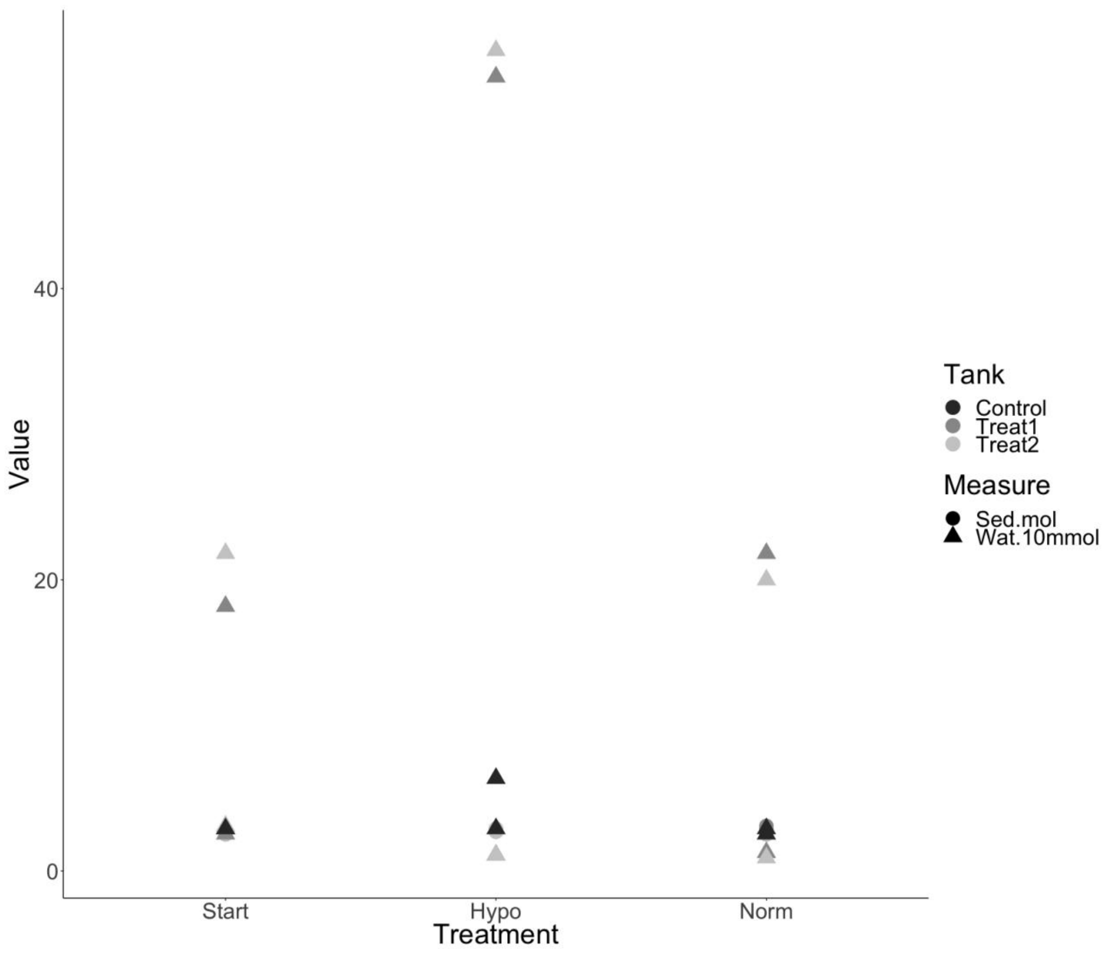

Figure 3-2 Mn concentrations of water (10x mmol) and sediment (mol). Black and gray (dark and light) represent control or tanks with sediment, and shape represents values from sediment or water samples. The x-axis is the different periods as the start of the experiment, after the hypoxic treatment, and after the normoxic condition.

Table 3-7 Environmental factors of Exp. Sediment-water. Control was only with water, and Treat 1 and 2 were replicates with water and sediment.

Exp.#	Tank	Treatment	Temperature (°C)	Salinity (‰)	DO (mg/L)
1	Control	Start	22.4	27.8	8.5
		Hypo	23.1	28.3	7.2
		Norm	22.2	28.2	8.6
	Treat1	Start	22.4	28.5	7.9
		Hypo	22.9	28.9	7.2
		Norm	22.8	28.6	8.5
	Treat2	Start	22.3	28.7	7.2
		Hypo	22.8	28.8	6
		Norm	22.9	29.1	8.4
2	Control	Start	22.6	27	8.6
		Hypo	26.1	27.3	6.7
		Norm	22.6	27.5	8.6
	Treat1	Start	22.2	28	5.6
		Hypo	24.9	28.7	1.3
		Norm	22.7	29.3	8.5
	Treat2	Start	22.1	28.1	6.7
		Hypo	25.0	28.9	1.6
		Norm	22.4	29.2	8.5

Start: measured right before the hypoxic treatment; Hypo: measured at the end of the hypoxic treatment when the sealed bag was just opened; Norm: measured at the end of the experiment when tanks were left under normoxic for three days.

Exp. Weekly increasing Mn

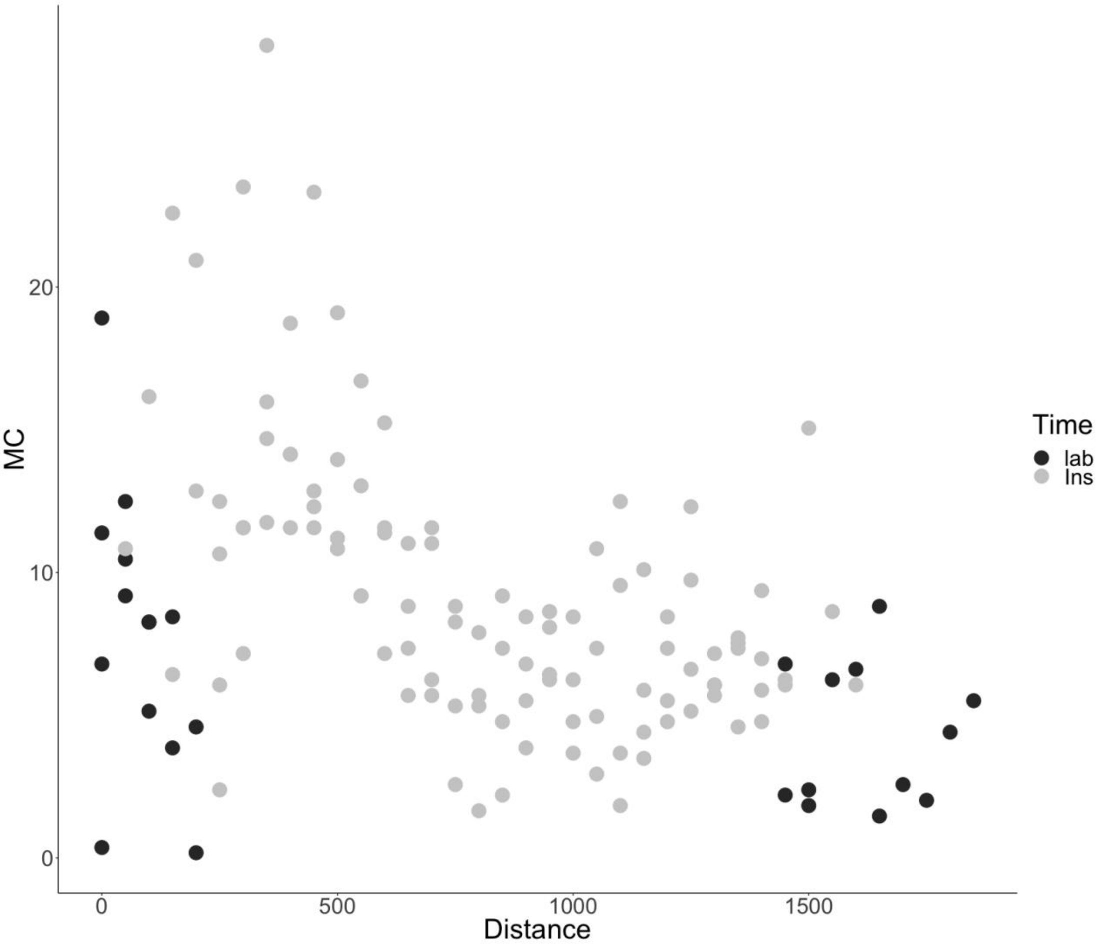

Figure 3-4 [Mn/Ca]$_{otolith}$ in the Exp. Weekly increasing Mn. The x-axis is the diameter of otoliths, and the y-axis is the [Mn/Ca]$_{otolith}$ (μmol/mol) along this diameter. The dark dots represent microchemistry formed under treatment, and the gray dots were formed before arriving laboratory as control.

From Figure 3-4, the edges of the Exp. Weekly increasing Mn did not rise up dramatically as expected. In addition, even the highest value was not collected from the treatment period.

Exp. Species difference

Only three samples of each species stayed alive along the four-week experiment, with control (1) and Mn 10 (2) for red sea bream, and control (1), Mn 10 (1), and Mn 30 (1) for olive flounder. Water concentrations of Mn seemed to be different enough following the set values (Table 3-8). Data was picked right after the dramatically increase of Sr in LA-ICPMS as the result of the Sr stain before experiments started.

Due to the lack of samples, no statistical analyses could be applied on this part of experiment, but the figures of plotting the data directly might have indicated some interesting possibilities for future explores. Figure 3-5 showed that the two species seemed to be different in $[Mn/Ca]_{otolith}$ under same treatments, with olive flounder having a higher value than red sea bream. In addition, it is again the middle treatment (Mn 10) which showed the highest value in $[Mn/Ca]_{otolith}$. Figure 3-6, with a near-linear decline of D_{Mn} especially in olive flounder, supported the former finding in marbled flounder which the contribution of water Mn into otolith Mn decreased while water concentration increased.

Table 3-8 Water chemistry of all treatments. RB represents red sea bream, and OF represents olive flounder. All samples from Mn 30 of RB dead within half of the experiment, thus, water sample was no longer collected or analyzed. Values were measured using ICPMS, and represented as mean ± standard deviation (SD).

	Mn (mmol)	Ca (mol)	Mn/Ca (mmol/mol)	Mg (mol)	Ba (mmol)	Sr (mmol)
RB.Control	0.27±0.02	7.00±0.46	0.04±0.00	53.4±4.0	1.63±1.42	72.6±3.0
RB.Mn 10	2.12±0.07	6.56±0.11	0.32±0.01	49.0±1.1	2.02±0.41	67.2±0.78
OF.Control	0.23±0.02	6.81±0.18	0.03±0.00	50.2±1.0	2.32±0.25	68.6±1.6
OF.Mn 10	2.07±0.13	6.39±0.09	0.32±0.02	45.0±0.5	2.09±0.34	64.5±1.4
OF.Mn 30	5.76±0.16	6.34±0.16	0.91±0.00	45.3±1.4	1.70±0.20	64.2±1.1

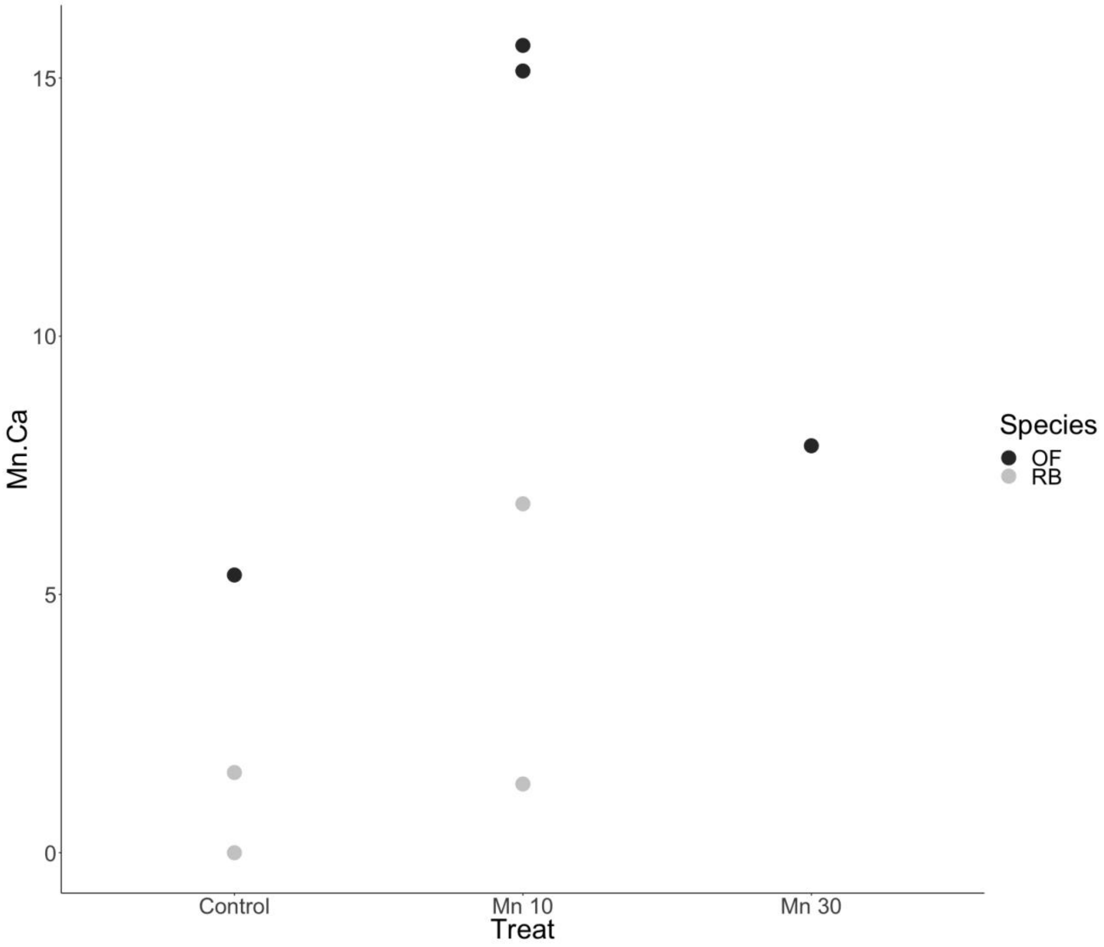

Figure 3-5 [Mn/Ca]$_{otolith}$ (μmol/mol) of different species under Mn treatments. The black dots represent olive flounder (OF), while the gray dots represent red sea bream (RB) in different concentrations of Mn from low (Control) to middle (Mn 10) then high (Mn 30). Data only included the experimental period under treatments.

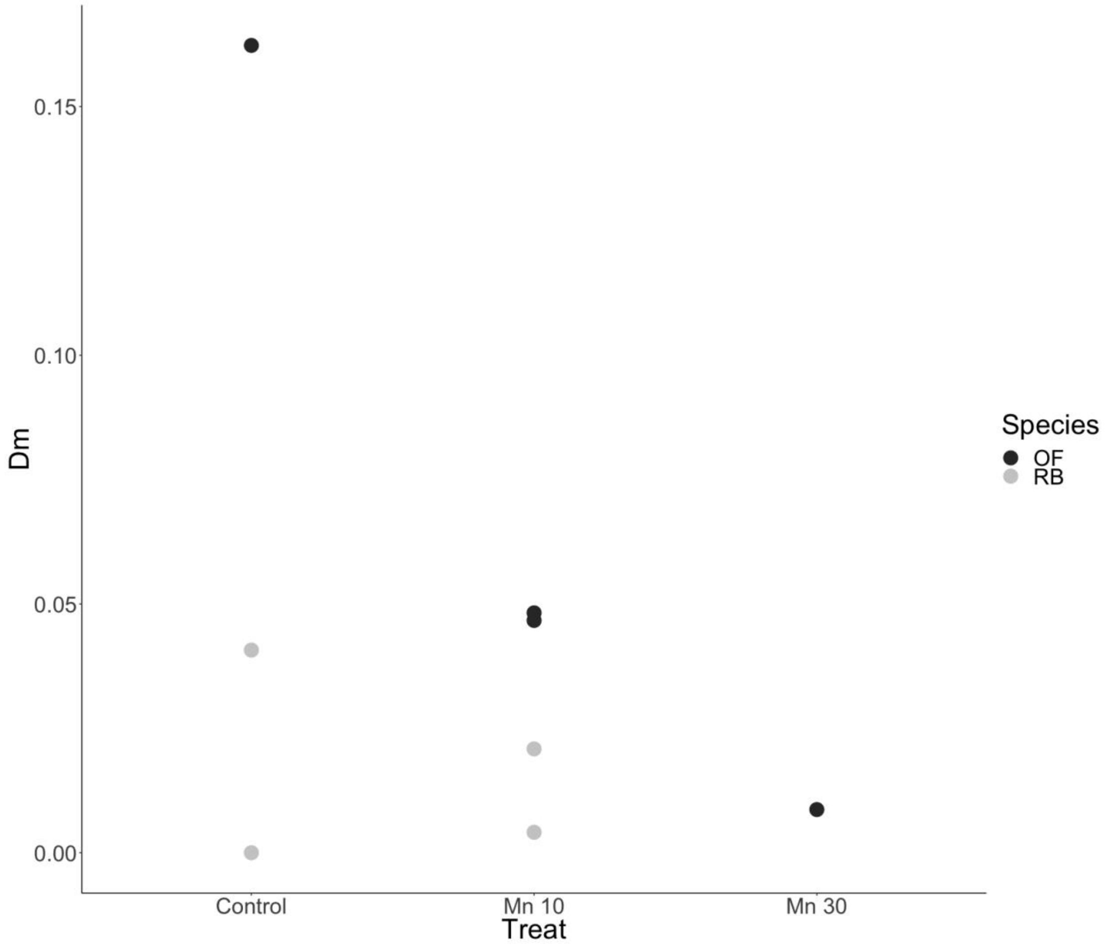

Figure 3-6 D_{Mn} difference based on species. Olive flounder (OF) and red sea bream (RB) were reared under three different Mn concentrations for four weeks. Black and gray represent different species, and the data only included the experimental period.

3.4 Discussion

Under controlled laboratory condition, our results did not reveal a positive correlation between $[Mn/Ca]_{water}$ and $[Mn/Ca]_{otolith}$. The constant Mn treatments showed the highest $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ occurred in the middle level, and the weekly-increasing Mn treatment did not find a continuous increase in $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ either. In addition, by comparing laboratory and field samples, similar results could be found. Water from the Tokyo Bay showed a same level of $[\mathrm{Mn/Ca}]_{\mathrm{water}}$ as control at laboratory, which was much lower than the other two Mn treatments. However, all locations in the field experiment, even including Choshi which is believed to be normoxic, had a much higher $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ than laboratory conditions (Figure 3-1, Table 3-2). These results suggested a more complicated correlation between $[\mathrm{Mn/Ca}]_{\mathrm{water}}$ and $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ that water chemistry is not the sole source to otolith chemistry.

One of the possible sources might be sediment. Our analyses revealed that sediment from the inner Tokyo Bay showed a much higher level than all treatment samples from laboratory. Results from Exp. Sediment-water also suggested that the Mn concentration in sediment might not decrease very much under hypoxia. If the different patterns in water Mn were caused by our experimental conditions in this experiment, results in Figure 3-2 and 3-2 might indicate that higher temperature and larger amount of sediment could enhance hypoxia happened in water, which matches the bottom ocean conditions during summers. However, if sediment was the main source of $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$, the difference among treatments from the laboratory experiment would be hard to explain due to their similar values of Mn in sediment. Thus, the best way to comprehend these might be to combine water and sediment impacts together. But the highest $[Mn/Ca]_{otolith}$ from Mn 30 remains unexplained. The correlation among chemistry of sediment, water, and otoliths has been long discussed but with no clear conclusion in other studies as well. Most experiments conducted under laboratory conditions on multiple fish species from larvae or juvenile did not show correlation between water concentration and $[Mn/Ca]_{otolith}$ (Elsdon and Gillanders 2003, Lloyd et al. 2008, Miller 2009), while Forrester (2005) found that $[Mn/Ca]_{otolith}$ of mudsuckers (*Gillichthys mirabilis*) ranked the same as water Mn concentration in filed. However, Forrester (2005) did not find correlation between Mn in otoliths and sediments, even mudsuckers were bottom dwellers that might ingest sediment directly. Salinity, possibly referring to changes in water Mn concentration, showed no correlation to $[Mn/Ca]_{otolith}$ either (Martin and Thorrold 2005, Martin and Wuenschel 2006). The assumption of positive impact from high water or sediment concentration to otolith chemistry, as normally accepted, might need further consideration than we expected.

Another factor which influences the impact from $[Mn/Ca]_{water}$ to $[Mn/Ca]_{otolith}$ might be the physiological adjustment of fish. In results of this study, D_{Mn} showed a different trend compared to $[Mn/Ca]_{otolith}$. Although Mn 30 group showed a highest $[Mn/Ca]_{otolith}$, $[Mn/Ca]_{water}$ of control contributed the most to $[Mn/Ca]_{otolith}$ with the lowest concentration, indicating that the uptake of Mn into fish otoliths was most efficient when Mn available in water was the lowest. The decreased uptake of Mn might support the occurrence of physiological adjustment in fish, responding to the environmental stress to keep homeostasis. D_{Mn} has been used in many studies, and sometimes more sensitive than $[Mn/Ca]_{otolith}$ to the change of environments. D_{Mn} seemed to have a relatively high value when $[Mn/Ca]_{water}$ was lower than 10 μmol/mol, then dramatically decreased until $[Mn/Ca]_{water}$ reached around 40 μmol/mol, and slowly decreased afterwards (Elsdon and Gillanders 2003, Miller 2009). Compared to our results, D_{Mn} showed a large decrease started from control with $[Mn/Ca]_{water}$ as 120 μmol/mol, and these might indicate a threshold but influenced by factors such as species or temperature. On the other hand, there was also research found no difference in D_{Mn} (Martin and Wuenschel 2006), possibly due to a more complex change in $[Mn/Ca]_{water}$ determined by salinity. In addition, the nested ANOVA on both $[Mn/Ca]_{otolith}$ and D_{Mn} revealed a significant difference on the interaction between water and fish, indicating that individuals responded to the same environment differently. Thus, physiological adjustment of each fish might play an important role in $[Mn/Ca]_{otolith}$ forming too, compared to the treatment experienced by grouped fish. Other studies also found that $[Sr/Ca]_{otolith}$ reacted differently when fish originally came from different environments, correlated to $[Sr/Ca]_{water}$ in fish caught from freshwater and estuary but not from marine systems (Brown and Severin 2009, Grønkjær 2016). In addition to $[Sr/Ca]_{otolith}$, when reared under the same environment for 12 months, Mn in blood plasma of fish was found to be positively affected by fish condition and growth rate but not by water Mn concentration (Sturrock et al. 2014), indicating that physiological impacts, not water chemistry, could influence plasma for otolith forming. However, Elsdon and Gillanders (2003) found no significant difference of $[Mn/Ca]_{otolith}$ on replicate tanks, possibly due to the difficulty of looking for difference between two tanks within each treatment. And mixed-effects modelling did not reveal relationship between $[Mn/Ca]_{otolith}$ and physiological factors such as age, otolith growth rate, fish size and sex (Martino et al. 2020). The last possible clue for physiological adjustment from our results is the observation of the weekly-increasing-$[Mn/Ca]_{water}$ impact on $[Mn/Ca]_{otolith}$. The much slower climbing of $[Mn/Ca]_{otolith}$ during the experimental period, together with the highest value occurring not under the laboratory condition, might suggest a physiological adjustment or threshold of chemical uptake in fish under extreme environments.

The mechanism of Mn uptake process in fish physiology has not been well studied. The available results from other research showed a possible support to this adjustment. Rouleau et al. (1995) used ^{54}Mn to examine the distribution of Mn in the brown trout (*Salmo trutta*). Along the six-week period of experiment, the trout showed a quick climbing of organ and tissue Mn in the first three weeks, then the concentration became relatively stable. This might be a prove to the self-adaption after three-week of exposure. However, Adam et al. (1997) found that the rainbow trout (*Oncorhynchus mikiss*) kept accumulating Mn during an eight-week experiment. The fluctuation of Mn in fish seemed more correlated to the fluctuation happened in the water concentration. The daily water concentration even dropped to 40 Bq/mL for once. These two researches did not well explain the mechanism of Mn uptake, but both studies revealed a relatively high Mn concentration in head (Adam et al. 1997) or the olfactory system and brain (Rouleau et al. 1995), suggesting that the in-fish environment of otoliths might be a high bioaccumulation region when water Mn concentration increased and possibly affected by the ambient water stronger than other tissues.

The different responds on similar water concentrations of Mn from different fish species revealed some interesting points as well. Our results showed that olive flounder had a higher $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ than red sea bream, which is opposite to our expectation. Xiao (2015) suggested that rapid-swimming fish should be more sensitive to hypoxia due to its dependence on aerobic metabolism, thus, we expected to see a more severe response on water Mn concentration (which was thought to be the result of hypoxia) from the red sea bream rather than olive flounder. However, although due to the small sample size, our results could not reveal any solid conclusion, Figure 3-5 and 3-6 showed a potential pattern that olive flounder had a relatively higher $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ than red sea bream under control, but also seemed to change more when under elevated water Mn. In the field, fish like red sea bream might be able to escape from the uncomfortable environments. At laboratory, the constant treatment should force the fish to experience and respond to the stress. This might be another support that the increase of $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ under hypoxia is not mainly caused by water Mn concentration. Species difference in $[\mathrm{Mn/Ca}]_{\mathrm{otolith}}$ is not firstly discussed in fish otolith studies. Di Beneditto and Franco (2018) found that captured from the same habitat, fish from the bottom tended to have higher

$[Mn/Ca]_{otolith}$ than surface. This might match the findings in this study, but the cause should not be difference between bottom and water column environment (eg. different levels of DO or water/sediment Mn). Not many studies on species difference were done at laboratory, and the explanation in field studies was almost habitat difference. The reason for olive flounder to have higher $[Mn/Ca]_{otolith}$ could not been well explained yet, but instead of species, Franco et al. (2013) found Mn in otoliths correlated to otolith shapes as elongated or rounded. Although the mechanism behind this correlation between otolith Mn and shape was not clear, due to the difference of shapes between olive flounder and red bream, this might be an explanation to our results. The highest value occurred in the middle treatment as in marbled flounder seemed interesting as well. These results could suggest the threshold of $[Mn/Ca]_{otolith}$ uptake depended on species (Rouleau et al. 1995), but it is hard to explain the decline under the high concentrations. One possibility might still be the involvement of physiological adjustment to respond stronger under extreme conditions.

Because $[Mn/Ca]_{otolith}$ seemed not to be the solely affected by water chemistry, the mechanism we are commonly accepting now for why hypoxia causes $[Mn/Ca]_{otolith}$ to increase is not accurate. However, studies together with our Chapter 2 have found an elevated $[Mn/Ca]_{otolith}$ under hypoxia (Limburg et al. 2011, Limburg et al. 2015). What might be the explanation to this phenomenon? We do not have a good answer to this question yet, but one hypothesis might be that instead of solely indicating hypoxia, $[Mn/Ca]_{otolith}$ could be a proxy to stressful experiences of fish. It seems that otolith Mn reacts to not only hypoxia but also other factors, such as temperature. Martin and Thorrold (2005) found a combined effect of temperature and salinity on D_{Mn} in early juvenile spot (*Leiostomus xanthurus*). A negative effect of temperature was also revealed on $[Mn/Ca]_{otolith}$ and D_{Mn} in black rockfish (Miller 2009). In addition, the increased $[Mn/Ca]_{otolith}$ under hypoxia often associated with larger variance in value compared to normal conditions. Our results revealed this observation in both laboratory and field experiments (Table 2-2 and 3-3), indicating that either chemical contamination or hypoxia could cause fish to respond individually. Limburg et al. (2011) also found a larger variance in $[Mn/Ca]_{otolith}$ of fish captured recently compared to Neolithic or early 1990s, when hypoxia was believed to be less severe than nowadays. The impacts from temperature and salinity on variance of $[Mn/Ca]_{otolith}$ seemed to be more complicated (Mazloumi et al. 2017, Martin and Wuenschel 2006), probably due to the relatively large range of the optimum value among species. The reason for $[Mn/Ca]_{otolith}$ to increase under hypoxia remained unclear, but our results suggested that water Mn concentration was not likely to be the main cause of it. The mechanism requires further study if we would like to keep involving $[Mn/Ca]_{otolith}$ into environmental studies in the future.

Chapter 4

Otolith morphology under ocean acidification (OA)

4.1 Introduction

As mentioned, OA is describing the decline of ocean pH caused by climate change, especially increasing release of CO_2. Oceans are the main carbon sink for atmospheric CO_2 and have absorbed approximately 1/3rd of the total CO_2 emissions over the last 250 years (Sabine et al. 2004). And this leads to a decrease by pH of 0.1 unit now compared to year 1900, while less than 0.05 unit of decline was observed during 11th to 19th century (Doney et al. 2011). The acidification process is still going on. Government data of the European Environment Agency has revealed that the west Mediterranean Sea and the Central North Pacific experienced a pH decline of approximately 0.03 unit during the past few decades (European Environment Agency 2020). Due to its exponential function form, a small pH unit could mean a huge increase in the H^+ dissolved. Under a future projection scenario, ocean pH will face a reduction by approximately 0.36 unit by the end of this century, together with an increasing of atmospheric CO_2 from 364 μatm to 877 μatm (Khangaonkar et al. 2021).

OA has impacts on many organisms, usually in a negative way. For example, species with calcium structures, such as corals, benthic mollusks, and algae, are experiencing difficulty in calcifying shells under decreasing pH (Guinotte and Fabry 2008). Shrimp and oysters responded to OA with astonishing regulatory, but this might cost their energy of reacting to other toxins or parasites (Burnett 1997). On the other hand, some species might benefit from OA. Macroalgae green tides (*Ulva rigida*) showed a better growth when pH decreased from 7.95 to 7.55 (Gao et al. 2016). To fish, the change occurred on size and morphology of their otoliths under acidification is hard to determine positive or negative now, but the difference is significant enough to draw researchers' attention. Increased otolith area (Checkley Jr. et al. 2009, Maneja et al. 2013), maximum length (Bignami et al. 2014), mass (Bignami et al. 2013), and volume (Bignami et al. 2013) has been observed in multiple species, and denser otoliths were reported by Bignami et al. (2013). In addition, the otoliths of Atlantic croakers, Micropogonias undulatus caught recently have less bumps than those of Atlantic croakers caught in the 1980s, thereby indicating a potential influence of OA leading to smoother otolith surfaces (Jiang 2017). These changes in otolith size and morphology might alter the way fish move and behave, thus affect the fish responds toward other organisms within the same population, or even at the ecosystem-level. However, opposite to expected, fish were found with larger and denser otoliths under OA conditions, showing that the inner structures are not dissolved by OA like what happened to shells and corals, and the mechanism of this unexpected response of fish has not yet been totally understood. The speculated cause is the increased H^+ in fish blood that triggers the reaction of the plasma HCO_3^- buffer, thus providing more available HCO_3^- to attract Ca^{2+} and secrete more $CaCO_3$ in otoliths (Alves 2020). On the other hand, the CO_3^{2-} concentration in the endolymph seems to have a positive correlation with the pH of the endolymph in rainbow trout, Oncorhynchus mykiss according to Takagi (2002). Thus, it is not clear whether the changes in otoliths are caused by H^+ or other irons. Acidification caused by elevated CO_2 levels has been studied extensively, this is not true for the effects of increased H^+ on fish. Therefore, the aim of our study was to reveal the direct impact of H^+ on fish otoliths.

Based on what is currently known, we formulated the following hypothesis: by directly adding H^+ to decrease the pH of ambient water, marbled flounder will grow larger and smoother otoliths.

4.2 Materials and methods

4.2.1 Rearing condition design

Juvenile marbled flounder from the Kudamatsu City Center for Stock Enhancement hatched around mid-January 2020 were transported to the laboratory on June 6, 2020 and kept in artificial seawater until middle September. Thirty-two fish were designed into five 6-L tanks. Two HNO_3 treatment groups were decided as:

Group pH 7.5 (HNO_3 2.00 10^{-3} mol/L) and Group pH 6.8 (HNO_3 3.99 10^{-3} mol/L). Each group had two replicates and started with six fish per tank. Group pH 7.8 was the control group and included eight fish at the beginning due to the lack of replicates. All fish were kept in 4 L of water under treatment conditions for two months (September 21, 2020 – November 21, 2020). Room temperature was maintained at 15 °C to guarantee survival rate, and light was provided from 8 AM to 4 PM to simulate daytime. Fish were fed commercial fish food twice a day.

Doses of HNO_3 were determined by a pre-experiment. Former experiment showed that the lethal dose of HNO_3 was 4.66 10^{-3} mol/L, and fish needed two to three days to adjust new acidic treatment after 2.00 10^{-3} mol/L, with 0.67 10^{-3} mol/L HNO_3 added each elevation. However, when the two replicates with 3.33 10^{-3} mol/L were supplemented to 3.99 10^{-3} mol/L HNO_3 on September 14, 2020, all fish died the following days. Thus, HNO_3 were added to the former two replicates 2.00 10^{-3} mol/L to reach 3.99 10^{-3} mol/L (to save time for extra adjustment), and two new replicates with 2.00 10^{-3} mol/L HNO_3 were created. All tanks, including the control, were supplemented with fish to initial numbers.

4.2.2 Fish measurements and otolith preparation

The fish were frozen to death and the standard length (SL) were recorded. The sagittal otolith pairs were dissected, cleaned and stored at room temperature. Photos were taken under an anatomical lens with an exposure time of 257.9 ms, microscope main objective magnification of 0.63, and microscope zoom magnification of 0.74. Background colors of the photos were inverted using Windows Paint. Area, perimeter, solidity (defined as Area/Convex hull area, Figure 4-1), and the maximum and minimum Feret's diameter were measured by ImageJ software. To obtain otolith length, width, and thickness measurements, we took photos of the flat surface and the lateral face of the otoliths. The maximum Feret's diameter of the lateral face photo was considered as length, and the minimum as thickness; the minimum Feret's diameter of the flat surface photo was the width (Figure 4-2). The lateral and flat areas, lateral and flat perimeters, and lateral and flat solidities were analyzed together with lateral length, thickness, and width.

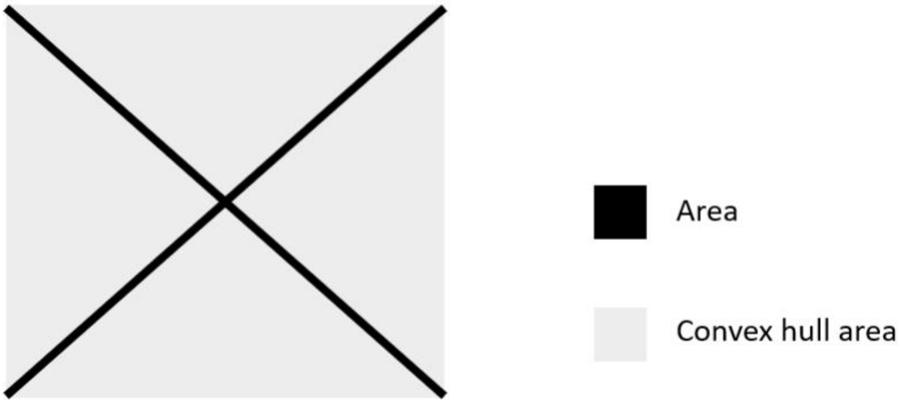

Figure 4-1 Definition of solidity. The black "X" represents the original objective, and the gray area is the convex hull area. In this case, solidity can be calculated as the ratio of areas black/gray.

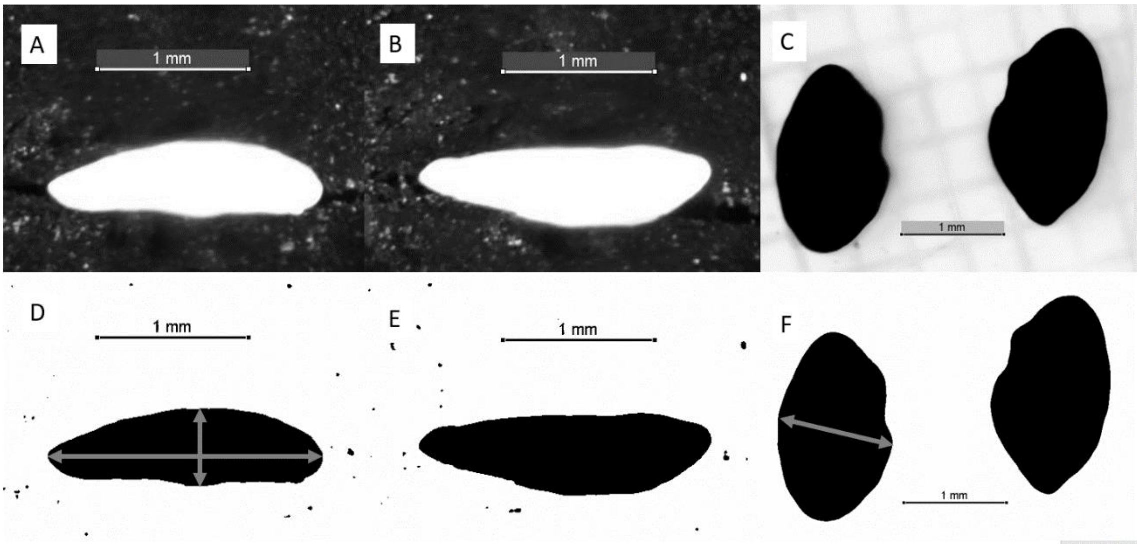

Figure 4-2 Otolith measurements taken as variables. All photos taken were of sample 24 (Group pH 7.5) on lateral face (A, B, D, E) and flat surface (C, F). A, D, and otoliths on the left in C and F are eye-side otoliths, and B, E, and the right ones in C and F are blind-side otoliths. A and B were taken using an anatomical lens. C is background reversed by Paint. D, E, and F are the final analyzed versions in Image J The solidity, area, and perimeter of each dark area in D, E, and F were directly measured by ImageJ, and the arrows represent the lateral length (long arrow on D), thickness (short arrow on D), and width (arrow on F).

4.2.3 Statistical analyses

To examine the asymmetry between the eye side and blind side of the otoliths, a *t*-test was applied on all otolith measurements. Measurements were first divided by SL to prevent the size impact of fish. Based on the otolith asymmetry results, only the blind-side otolith was used in the following analyses.

A principal component analysis (PCA) was employed to visually understand the data and decrease the dimensions for further analyses. The coordinates of each individual on PC1 were defined as size, and PC2 as shape. The analysis was run in R using package “factoextra.”

Analysis of variance (ANOVA) was applied to compare the differences in size and shape caused by treatments. After significance was found, a further ANOVA test was applied on the most contributed variables within the PC, and a Tukey’s test was used to determine which treatment was different. Both analyses were performed in R using the “stats” and “multcomp” packages.

4.3 Results

4.3.1 Fish otolith morphology

Environment remained relatively stable among treatments (Table 4-1). The fish from Group pH 6.8 exhibited the highest mortality rate, but also had the largest SL (Table 4-1). The mortality of the control fish was attributed to fish jumping out of the tank. In Group pH 6.8, two fish (16.67%) died because they jumped out of the tank and not of pH stress.

Table 4-1 Summary of water parameter and physical condition of fish. Water parameters were recorded weekly over the two-month experiment. N is the number of samples at the end of the experiment, and mortality rate is calculated by N dividing the sample size at the beginning. Standard length (SL), together with four parameters are represented in mean (±SD).

pH Treatment	N	Mortality Rate	SL (mm)	pH	Temperature (°C)	DO (mg/L)	Salinity (‰)
7.8	6	25%	60.99±4.29	7.8±0.08	12.1±0.86	10.2±0.23	30.2±0.64
7.5	12	0	59.65±4.79	7.5±0.11	11.9±0.95	10.2±0.37	30.4±0.66
6.8	7	41.67%	65.72±3.79	6.8±0.34	11.8±0.90	10.3±0.28	30.7±0.79

Table 4-2 Summary of otolith measurements by asymmetry. Solidity, area and perimeter were measured on both lateral face and flat surface of each otoliths. Length was represented by the maximum Feret’s diameter on lateral face, and the minimum Feret’s diameters of the lateral face and the flat surface were considered as thickness and width respectively. Values are mean (±SD)

pH Treatment	Eye/Blind Side	Solidity-lateral	Solidity-flat	Area-lateral (mm^2)	Area-flat （mm^2）	Perimeter-lateral (mm）	Perimeter-flat (mm)	Length-lateral (mm)	Width (mm)	Thickness (mm)
7.8	Blind	0.957±0.009	0.985±0.001	0.818±0.064	1.715±0.149	4.675±0.266	5.249±0.263	2.040±0.111	1.144±0.045	0.543±0.023
	Eye	0.957±0.012	0.982±0.004	0.736±0.068	1.581±0.076	4.434±0.192	5.056±0.143	1.935±0.102	1.118±0.029	0.521±0.051
7.5	Blind	0.969±0.006	0.983±0.003	0.877±0.086	1.707±0.244	4.716±0.303	5.276±0.371	2.061±0.139	1.151±0.098	0.570±0.020
	Eye	0.962±0.005	0.980±0.004	0.797±0.094	1.678±0.227	4.546±0.302	5.190±0.365	1.976±0.142	1.166±0.079	0.544±0.042
6.8	Blind	0.966±0.006	0.982±0.003	0.956±0.056	1.919±0.175	4.967±0.267	5.576±0.257	2.154±0.124	1.216±0.058	0.595±0.017
	Eye	0.964±0.015	0.98±0.003	0.838±0.069	1.809±0.200	4.686±0.255	5.410±0.323	2.044±0.123	1.197±0.065	0.550±0.026

Table 4-3 Result of t-test on asymmetry of otoliths. To limit the impact of fish somatic size, all measurements except solidity were divided by fish SL before analysis. Solidity, area and perimeter of both lateral face and flat surface, together with otolith length, width and thickness were compared between blind and eye side within each sample to examine the asymmetry. Asterisk represents significance: * P<0.1, ** P<0.05, *** P<0.01

pH Treatment	Solidity-lateral	Solidity-flat	Area-lateral /SL	Area-flat /SL	Perimeter -lateral /SL	Perimeter -flat /SL	Length-lateral /SL	Width /SL	Thickness /SL
7.8	0.941	0.187	0.093 *	0.007 ***	0.064 *	0.167	0.040 **	0.495	0.598
7.5	0.006 ***	0.057 *	0.002 ***	0.614	0.063 *	0.318	0.031 **	0.513	0.246
6.8	0.755	0.318	0.000 ***	0.166	0.034 **	0.174	0.052 *	0.533	0.026 **

Through Table 4-2 and Table 4-3, the averages of each measurement revealed difference, and the results of *t*-test showed at least four significantly different variables between the eye-side and blind-side otoliths within each treatment. Thus, to limit the impact of asymmetry, only the blind-side otolith was chosen for the further analyses.

4.3.2 PCA and ANOVA results on the blind-side otolith

In PCA, the first two components explained almost 80% of the variance (Figure 4-2). By viewing the contributions of each variable, PC1 was close to size, with perimeters, lengths, and areas explaining most of the variation. PC2, on the other hand, was more likely to be defined as shape, which explained more about solidity (Table 4-4 and Figure 4-3). Thus, the coordinates of PC1 were redefined as “size” and those of PC2 as “shape,” in order to apply a one-way ANOVA. As similar results were shown in the individual PCA plot (Figure 4-4), the ANOVA did not show any differences in terms of size; however, it indicated a significant difference in shape (Table 4-5).

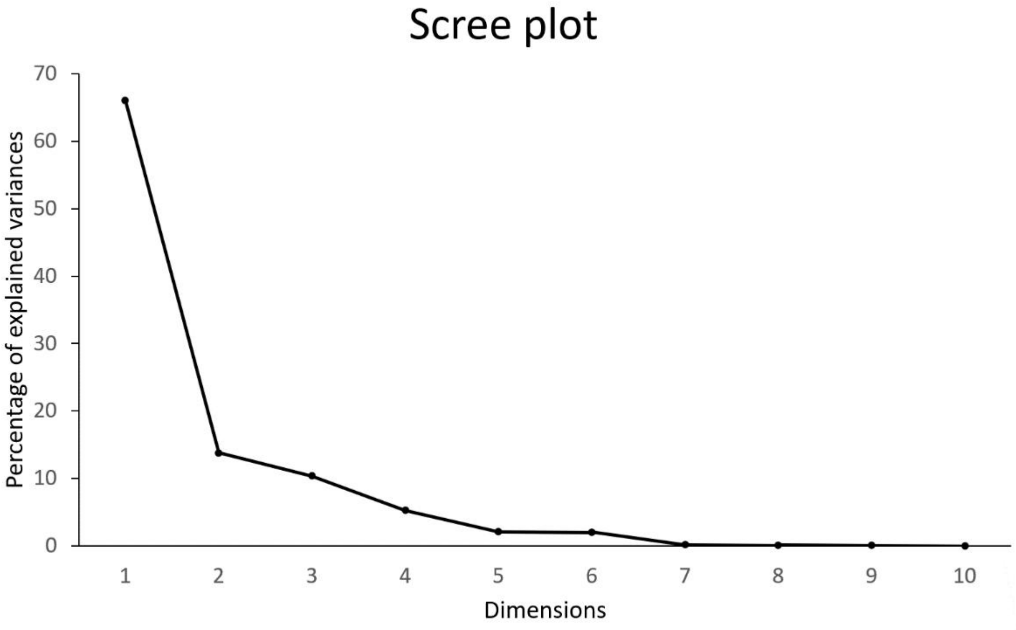

Figure 4-3 Scree plot of PCA on otolith measurements together with fish SL. To prevent the asymmetry impact, only the blind-side otolith was used. The solidity, area, and perimeter of both the lateral face and the flat surface, as well as the length, width, and thickness of each otolith were analyzed.

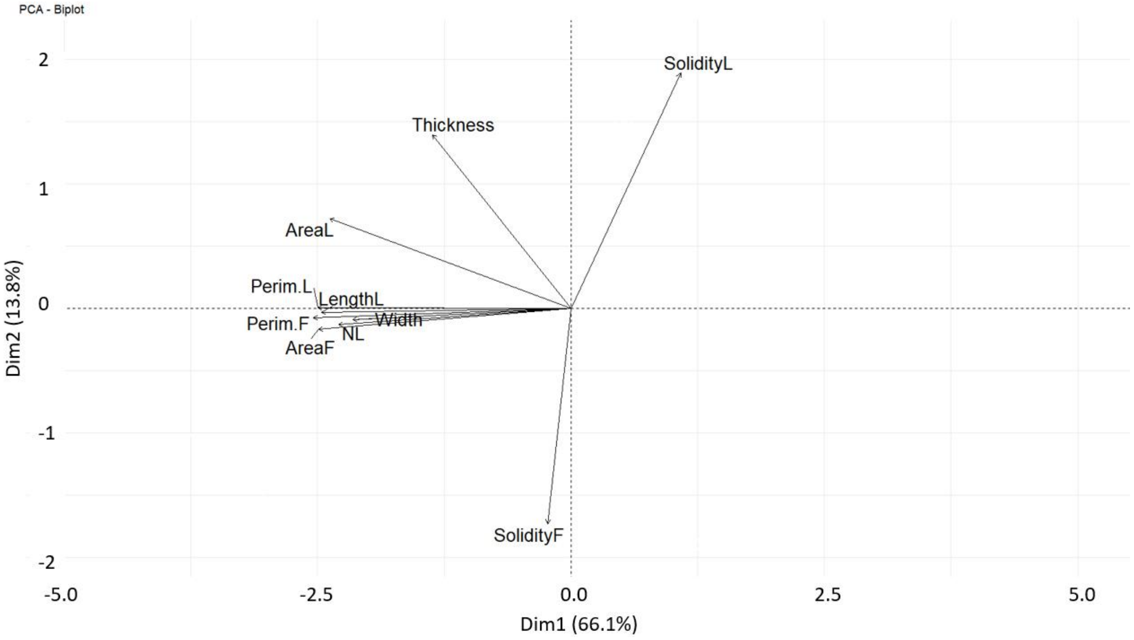

Figure 4-4 Biplot of PCA on otolith measurements together with fish SL (shown as NL). To prevent the asymmetry impact, only the blind-side otolith was used. The solidity, area, and perimeter of both the lateral face (shown as ~L) and the flat surface (shown as ~F), as well as the length (shown as LengthL), width, and thickness of each otolith were analyzed.

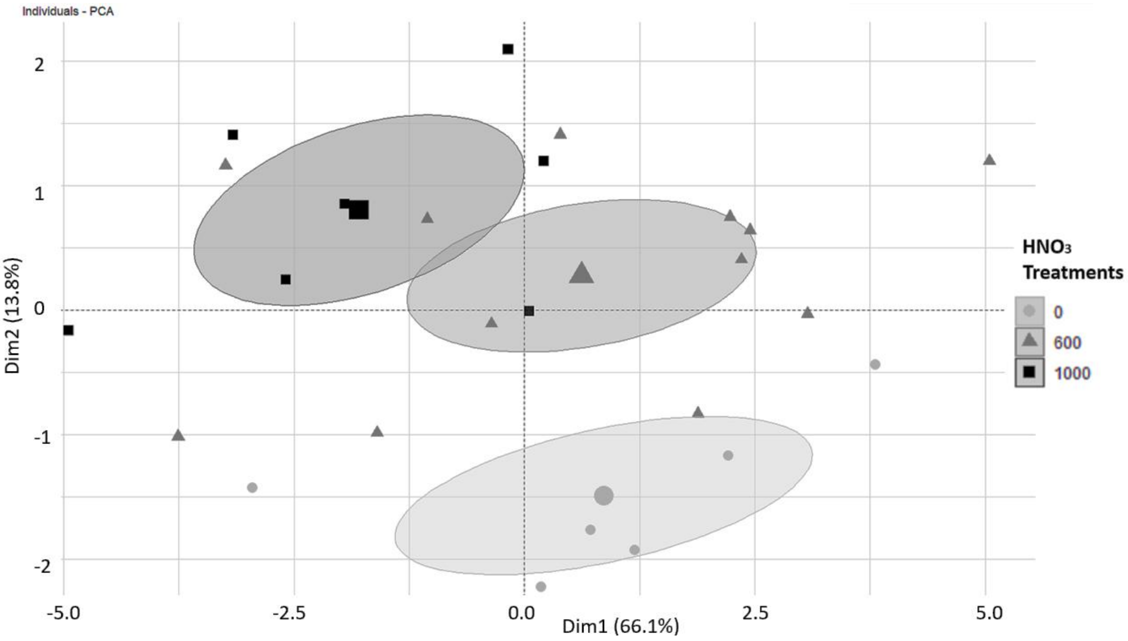

Figure 4-5 Individual plot of PCA on otolith measurements together with fish SL. Treatments refer to the amount of HNO_3 added during the experiment and 0 represents the control. Light gray circles represent Group pH 7.8 (shown as “0” on the right side), dark gray triangles represent Group pH 7.5 (shown as “600” on the right side), and black squares represent Group pH 6.5 (shown as “1000” on the right side) with the lowest pH. The small icons are individual samples, and the big circle, triangle and square in the central area of each ellipse represent the average of each treatment group. To prevent the asymmetry impact, only the blind-side otolith was used. The solidity, area, and perimeter of both the lateral face and the flat surface, as well as the length, width, and thickness of each otolith, together with fish SL were analyzed. A 95% confidence ellipse is used for each treatment to highlight data separation.

Table 4-4 Rotations of PCA calculated by R. Solidity, area and perimeter of both lateral face and flat surface, together with length, width and thickness of blind-side otolith of each sample were used as variables in PCA. Due to the impact of fish somatic growth, SL was analyzed at the same time. Areas, perimeters, length and width, together with SL contribute the most to PC1, so PC1 is defined as "size" in later analysis. PC2, with solidities the most important variables, is defined as "shape"

	PC1	PC2
Solidity-lateral	0.164	0.627
Solidity-flat	-0.035	-0.574
SL	-0.348	-0.043
Area-lateral	-0.360	0.239
Area-flat	-0.377	-0.056
Perimeter-lateral	-0.378	0.001
Perimeter-flat	-0.385	-0.025
Length-lateral	-0.373	-0.010
Width	-0.326	-0.030
Thickness	-0.207	0.462

Table 4-5 Result of ANOVA on size and shape. Difference was among HNO_3 treatment groups. Asterisk represents significance: * P<0.1, ** P<0.05, *** P<0.01

		Df	Sum Sq	Mean Sq	F value	Pr (>F)
Size	Treatment	2	31.5	15.77	2.73	0.0873
(PC1)	Residuals	22	127.1	5.78		
Shape	Treatment	2	18.7	9.36	14.36	0.0001***
(PC2)	Residuals	22	14.3	0.65		

ANOVA tests applied on solidity-flat, solidity-lateral, area-lateral, and thickness in PC2 showed that except solidity-flat (Pr = 0.271), all the other measurements on the lateral face of otolith were significantly different among treatments (Table 4-6). Following Tukey's tests confirmed that control was different compared to the pH treatments, with area-lateral and thickness changed more following gradients along pH (Table 4-7).

Table 4-6 Result of ANOVA on the most contributed variables in shape. Difference was among HNO_3 treatment groups, and asterisk represents significance: * $P<0.1$, ** $P<0.05$, *** $P<0.01$

		Df	Sum Sq	Mean Sq	F value	Pr (>F)
Solidity-flat	Treatment	2	2.257e-05	1.128e-05	1.385	0.271
	Residuals	22	1.793e-04	8.149e-06		
Solidity-lateral	Treatment	2	0.0005699	2.85e-04	5.632	0.0106**
	Residuals	22	0.0011131	5.06e-05		
Area-lateral	Treatment	2	0.06279	0.031396	5.13	0.0148**
	Residuals	22	0.13465	0.006121		
Thickness	Treatment	2	0.008734	0.004367	9.354	0.00115***
	Residuals	22	0.010270	0.000467		

Table 4-7 Result of Tukey's test on the most contributed variables in shape. Difference was among HNO_3 treatment groups

	pH 7.8	pH 7.5	pH 6.8
Solidity-lateral	b	a	ab
Area-lateral	b	ab	a
Thickness	b	ab	a

4.4 Discussion

The results of this study on the impacts of H^+ on fish otoliths showed the same outcomes as many researches which examined the impacts of elevated CO_2 on otoliths, suggesting that without CO_3^{2-}, otoliths reacted similar to solely H^+. In studies on elevated CO_2, most former researchers found significant difference in otolith size, usually larger under acidification. Definitions used to describe “size” showed a little variance, for example, area (Bignami et al. 2013, Bignami et al. 2014, Checkley Jr. et al. 2009, Di Franco et al. 2019, Hamilton et al. 2019, Munday and Hernaman et al. 2011), maximum length (Bignami et al. 2014, Munday and Hernaman et al. 2011), mass (Bignami et al. 2013), volume (Bignami et al. 2013), and daily increments (Coll-Llado et al. 2018). However, several studies have reported no effect of elevated CO_2 levels on fish otolith sizes. No significant difference was found in otolith area in larval Baltic cod (*Gadus morhua*) when comparing pH of 7.2 and 7.6 to 8.0 (Frommel et al. 2013). Similarly, otolith area, maximum length and maximum breadth did not be influenced that much under pH 7.8 and 7.6 compared to pH 8.15 in larvae of clownfish (*Amphiprion percula*, Munday and Gagliano et al. 2011). When pH decreased from 8.0 to 7.7, otoliths of juvenile spiny chromis damselfish (*Acanthochromis polyacanthus*) showed no difference in volume, aspect ratio (length/width), and area (Sundin et al. 2019). The reason for this different response of fish otoliths to OA could have to do with age impact and species difference. Juveniles may be more tolerant to environmental changes compared to larvae, and it seems like specific species (e.g., *A. polyacanthus*) may be more adaptable to pH differences than other species (Frommel and Schubert, 2013; Munday and Gagliano et al. 2011). In addition, juvenile fish seem to reflect stress on multiple dimensions, thus neutralizing the oversizing of otoliths. For example, otoliths of juvenile *Symphodus ocellatus* became 11% greater under a higher CO_2 environment, but due to the fish's greater somatic growth by 19.47% (Di Franco et al. 2019), thereby the difference of otolith growth caused by elevated CO_2 levels could be neutralized and became not significant. On the other hand, most studies on larvae with larger-sized otoliths showed limited somatic growth. These two opposite examinations might suggest that juvenile otolith growth could be neutralized by the positive impact on somatic growth of OA, thus occurred no significant results in otolith size. However, some studies on larvae found no significance either (Frommel et al. 2013, Munday et al. 2011). This might due to the second possible cause: the different pH levels caused by the introduction of different CO_2 amounts. Differences were observed when pH was below 7.6, while pH values of approximately 7.8 seemed to be tolerable by fish. Although Frommel et al. (2013) did not reveal significant difference in otolith area under pH 7.2, a clear positive trend between otolith area and pH could be observed when temperature was 7 °C, and pH 7.2 treatments were always with the largest otolith area and the largest standard deviation among all temperatures. Besides larvae and juvenile, fish otoliths may not be impacted dramatically by pH at the egg stage either (Frommel et al. 2013). These results seem to indicate that, based on the atmospheric CO_2 value predicted by different scenarios, OA may not have a huge impact on fish otoliths by the end of this century. However, it has already been monitored that the Bornholm Basin in the southwestern Baltic Sea is experiencing 1,100 μ atm CO_2 at pH 7.2 (Frommel et al. 2013), which has already been much higher than the expectation as 877 μ atm in year 2100. Thus, the changes happening in the field of OA might be more severe than we expected.

Otolith roughness has not been discussed in previous studies, and in the papers that discuss shape or morphology, variables vary. Rectangularity (Munday and Gagliano et al. 2011), circularity (Munday and Gagliano et al. 2011), roundness (Maneja et al. 2013), and aspect ratio (Sundin et al. 2019) have been used, but no significant difference has been found in terms of these characteristics. However, the solidity used in our study may indicate the ability to represent otolith roughness. Significant difference was found between control and pH treatments, showing that smoother otoliths were formed when H^+ increased. Less bumps on the otolith surface were observed in Atlantic croaker caught in 2016 compared to those caught in 1983 (Jiang, 2017), suggesting a smoother trend in change of roughness that occurred under OA conditions in the Gulf of Mexico. On the other hand, the correlation revealed by our results was not positive but showing the highest solidity occurred in the middle level of pH, suggesting the possibility that threshold might occur while otolith surface changing under elevated H^+. There has been a gap in otolith roughness examination, and our observation of significant difference caused by the H^+ treatment on solidity shed light on the potential that solidity may be a good indicator of OA impacts on fish otolith morphology.

The mechanism of smoother otoliths formed under acidic conditions remains unclear. Otoliths are formed in endolymphatic fluid which transfers its chemicals with blood plasma (Campana 1999). Studies have reported increased plasma HCO_3^- levels after exposure to elevated CO_2 levels (Alves et al. 2020, Perry et al., 2010), which is highly possible as a buffer reaction to the H^+ introduced into fish blood. Juvenile cobia *Rachycentron canadum* showed a decrease in blood pH of 0.34 when treated under pH 5.5 compared to 7.9 for only 24 h (Rodrigues et al. 2013). Brook trout *Salvelinus fontinales* experienced a drop in blood pH from 7.39 to 6.97 when exposed to more extreme environmental pH of 3.0-3.3 (Packer and Dunson 1970). Without acidified stress, the pH of plasma reaches a value of 7.21, lower than the pH value in the endolymph which is 7.97 in trout (Payan et al. 1997). Based on the results of Tohse and Mugiya (2004), the uptake of inorganic carbon in both endolymph and otoltith was limited at lower pH in vitro sacculi. Thus, the commonly accepted assumption that "HCO_3^- is absorbed from the plasma buffering to the endolymph when acidification happens" could happen but hardly the opposite way. However, the incorporation of calcium into the endolymph and otolith was found not influenced by pH in vitro sacculi (Tohse and Mugiya, 2004). Without a better understanding of the physiological reactions that occur in fish systems after growing in low pH conditions, the otolith changes under OA conditions remain difficult to explain.

Owing to the uncertainty of how the otolith formation mechanism works, the causes of the larger and smoother otoliths remain unclear as well. These effects could be caused by a change in density, as Bignami et al. (2013) reported that the otolith density of cobia larvae increased by 6% at pH 7.4, after only 20 days. Another possible cause could be the change in the crystals of calcium carbonate. How do calcium carbonate particles grow into a whole otolith? The process is unclear. An *in vitro* experiment of calcium carbonate formation suggested that when additives were involved, instead of developing a smooth surface, the crystal tended to grow ion-by-ion and was banded to the points where other crystals had already been growing, thus leading to an irregular-looking surface (Gal et al. 2015). Owing to pH stimulation, secretion patterns of additives might switch to secretion of other adjusting molecules, which could lead to a different formation on otolith surface compared to normal pH conditions. Another assumption behind the otolith growth mechanism is that multiple crystal particles are formed and finally attach to one another by fibrous or adhesive proteins (Lundberg et al. 2006). Thus, pH may affect the function of proteins, or the formation of properly secreted $CaCO_3$ particles. There are three types of crystals in fish otoliths: aragonite, calcite, and vaterite. Under pH 7.3, one fifth of the fish turned normal aragonite into calcite in otoliths (Coll-Llado et al. 2018). *In* *vitro* crystallization showed a size difference in calcium carbonate formation under different protein treatments (Tohse et al. 2009). If otoliths inside fish also have differences in crystal size, this could be one possible reason for the morphological changes that occurred under acidification due to the sensitivity of protein to pH. In addition, different protein matrices could enhance the formation of a specific type of calcium carbonate crystal (Tohse et al. 2009). This could lead to a change in otolith morphology because crystal types are in different shapes.

This chapter found that H^+ showed the same impact on otolith size as elevated CO_2 reported in other studies. Roughness has not been discussed often in former researches, and solidity used in this chapter could separate treatments of H^+ and control, indicating a change in roughness under low pH which could be measured by solidity in future studies of OA.

Conclusions

This study compared marbled flounder captured from different locations around the Tokyo Bay areas, and found that $[Mn/Ca]_{otolith}$ showed a higher value in the Bay than outside of the Bay, especially in young fish. This finding matched the occurrence of hypoxia happening in Tokyo Bay not the outside, together with seasonal trend observed in Tokyo Bay samples which further supported the hypoxic condition in summers. The results suggested the possibility that high $[Mn/Ca]_{otolith}$ could be used to indicate hypoxia.

Rearing experiment at laboratory did not find significant correlation between $[Mn/Ca]_{water}$ and $[Mn/Ca]_{otolith}$. No matter under constant or increasing treatments of water Mn concentration, the impact from water chemistry seemed not strong enough to cause the increase in $[Mn/Ca]_{otolith}$ happened under natural hypoxia in field. When comparing the laboratory data to the field, it became clearer that $[Mn/Ca]_{water}$ was not the main cause to the elevated $[Mn/Ca]_{otolith}$ happened under hypoxia, which had been discussed in former studies as the reason. Otoliths collected in Tokyo Bay showed a higher $[Mn/Ca]_{otolith}$ than all treatments at laboratory, but $[Mn/Ca]_{water}$ was only close to the Control. These results called for further studies on the mechanism behind the correlation between $[Mn/Ca]_{otolith}$ and hypoxia.

Increased H^+ showed an impact on otolith size of marbled flounder, with larger otoliths found under lower pH like former studies observed under elevated CO_2. In addition, this study revealed that otoliths became smoother under acidic conditions, and the measurement used to estimate this difference was solidity, with larger solidity indicating smoother surface. Changes in otolith size and shape might affect fish hearing and balance in movement. Thus, the results showed the potential impacts OA might have on fish, and suggested a new measurement on valuing otolith roughness as solidity.

Environmental changes in marine ecosystems have become a concern in recent decades, and this study found that hypoxia and OA seemed to affect microchemsitry and morphology of fish otoliths respectively. Thus, we might gain a better understanding on the environment through further study on fish otoliths.

Acknowledgments

I would like to thank Prof. Katayama for the kind and patient support on my study at Tohoku University, and all members from Laboratory of Fishery Biology and Ecology have helped me enjoy my life in Japan. It is grateful to receive many valuable advises from associate Prof. Kinuko Ito about experimental design. I also want to say thank you to my committee members Prof. Yoshihiro Ochiai and Waka Sato-Okoshi for their guidance in this dissertation, providing new ways to think about these experiments and pushing this study to a new level.

All juveniles of marbled flounder used in the rearing experiments were provided by Mr. Masaki Itohara from the Kudamatsu City Center for Stock Enhancement. In addition, I highly appreciate the technical support by Ms. Chikako Matuo, working at the Technical Division, School of Engineering, Tohoku University, on the ICPMS analyses of water chemistry.

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