Exploring the use of SEM-EDS analysis to measure the distribution of major, minor, and trace elements in bottlenose dolphin (*Tursiops truncatus*) teeth

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Abstract

Dolphin teeth contain enamel, dentin, and cementum. In dentin, growth layer groups (GLG's), deposited at incremental rates (e.g., annually), are used for aging. Major, minor, and trace elements are incorporated within teeth; their distribution within teeth varies, reflecting tooth function and temporal changes in an individual's exposure. This study used a scanning electron microscope (SEM) equipped with energy dispersive X-ray spectroscopy (EDS) to determine the distribution of major (e.g., Ca, P), minor (e.g., Cl, Mg, Na), and trace elements (e.g., Cd, Hg, Pb, Zn) in teeth from 12 bottlenose dolphins (*Tursiops truncatus*). The objective was to compare elemental distributions between enamel and dentin and across GLG's. Across all dolphins and point analyses, the following elements were detected in descending weight percentage (wt %; mean ± SE): O (40.8 ± 0.236), Ca (24.3 ± 0.182), C (14.3 ± 0.409) P (14.0 ± 0.095), Al (4.28 ± 0.295), Mg (1.89 ± 0.047), Na (0.666± 0.008), Cl (0.083± 0.003). Chlorine and Mg differed between enamel and dentin; Mg increased from the enamel towards the dentin while Cl decreased. The wt % of elements did not vary significantly across the approximate location of the GLG's. Except for Al, which may be due to backscatter from the SEM stub, we did not detect trace elements. Other trace elements, if present, are below the detection limit. Technologies with lower detection limits [e.g., laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)] would be required to confirm the presence and distribution of trace elements in bottlenose dolphin teeth.

Keywords

Elemental microanalysis; Scanning electron microscopy; Energy dispersive x-ray spectroscopy; Bottlenose dolphin; Enamel; Dentin

Declarations

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Conflict of interest/Competing interests

The authors have no conflicting or competing interests to declare.

Availability of data and material

Data for each dolphin is available in the supplementary information. Any data and images not published in this paper can be requested from Meaghan McCormack at mmccormack@txstate.edu.

Code availability

Not applicable

Author's contributions

Meaghan A. McCormack: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Writing - original draft, Writing – review & editing. Wayne E. McFee: Data curation, Methodology, Formal analysis, Writing - review & editing. Heidi R. Whitehead: Sample acquisition, Writing – original draft, Writing - review & editing. Sarah

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1. Introduction

Dolphins have evolved homodont dentition; their simplified cone shaped teeth are also greater in number compared to terrestrial mammals [1-4]. The evolution of dolphin dentition is likely a consequence of their foraging behavior and the absence of mastication [2]. Further, in contrast to most terrestrial mammals, which are diphyodonts and produce two sets of teeth (deciduous and permanent), dolphins are monophyodonts and develop only one set of teeth [1, 5-6]. In marine mammals, teeth grow incrementally, and once incorporated within the tooth structure, major [e.g., calcium (C), phosphorous (P)], minor [e.g., chlorine (Cl) magnesium (Mg), sodium (Na)], and trace elements [e.g., cadmium (Cd), mercury (Hg), lead (Pb), zinc (Zn)] remain unaltered, thereby reflecting an organism's physiology, ambient environmental conditions, dietary intake, and exposure to trace elements including contaminants [e.g., Cd, chromium (Cr), Pb, Hg] [7-17]. The chemical composition of teeth and the spatial distribution of major and minor elements within teeth influences tooth function [18-19]. For example, in human teeth, a decrease in tooth hardness has been associated with increases in the weight percentage (wt %) of Na_2O and MgO and decreases in the wt % of P_2O_5 and CaO [20]. Additionally, the pattern of trace element deposition within dolphin teeth may reflect the maternal transfer of contaminants, the timing of life-history events [e.g., Zn to estimate age at maturity], and habitat use [e.g., barium (Ba) as a proxy for salinity] [8, 10, 15].

Like other mammalian teeth, dolphin teeth consist of three primary components: enamel, dentin, and cementum [1, 3-5, 19]. Structurally, the tooth consists of nested layers. On the exterior, the enamel and cementum line the tooth crown and root, respectively. Following the enamel and cementum is the dentin, which surrounds the central pulp cavity [21]. Development of the enamel and dentin begins while the dolphin is *in utero*, while cementum begins developing after birth [1]. In dolphins, dentin layers accumulate along the edges of the pulp cavity at predictable rates (e.g., annually), slowly decreasing the volume of the pulp cavity; collectively, the layers of dentin are referred to as growth layer groups (GLG's) [21-24].

Enamel, dentin, and cementum are comprised of water, inorganic components, primarily hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], and organic components (e.g., proteins) [18-19, 25-26]. Although these three dental tissues have a similar mineral composition, the proportion of inorganic and organic materials varies among the tissues; notably, the enamel is the harder of the tissues, comprised of 95-96% inorganic material, while the dentin and cementum are softer tissues comprised of a lower percentage of inorganic material (e.g., 70% inorganic material in dentin) [27-29]. Calcium and P are the main components of hydroxyapatite and, as a result, are the major elements present in teeth. The structure of hydroxyapatite includes several cationic and anionic sites; therefore, a variety of minor and trace elements can be incorporated within its chemical structure [30-31]. For example, cations such as Cu^{2+}, K^{+}, Mg^{2+}, Na^{+}, Ni^{2+}, Pb^{2+}, or Zn^{2+} may substitute for Ca^{2+}, while anions such as CO_3^{2-} and SiO_4^{4-}, or Cl^{-} and F^{-} may replace PO_4^{3-} and OH^{-}, respectively [30-36]. In addition to being incorporated within the mineral structure itself, elements (e.g., Zn) associated with macromolecules on the surface of the crystalline lattice may become trapped as new mineral layers are deposited [37]. In marine mammals, more than 20 elements have been reported in dental tissues including Ba, carbon (C), Ca, Cd, Cl, copper

(Cu), Cr, cobalt (Co), fluorine (F), iron (Fe), Pb, Mg, Hg, P, selenium (Se), Na, strontium (Sr), vanadium (V), and Z [8-13, 15,17, 19, 38-41]

To determine the elemental composition within the tooth structure, several *in situ* analytical methods are currently available. Some techniques involve the use of electron or proton microprobes with X-ray emission detectors, such as scanning electron microscopes (SEM) equipped with energy dispersive X-ray detection (EDS) and particle-induced X-ray fluorescence (PIXE), respectively [19, 38, 40-41]. These techniques are advantageous because they require little sample preparation and have the spatial resolution necessary to measure the concentration or wt % of elements within GLG's. Further, for studies with methodologies that do not require tooth sectioning or studies that utilize teeth that have been previously sectioned, the methods are non-destructive. However, they often lack the sensitivity to detect elements present at low concentrations, although technologies have improved and detection limits can be optimized with proper sample preparation and analytical settings [26, 42-44]. An alternative approach combines the use of laser ablation and inductively coupled plasma mass spectrometry (LA-ICP-MS), which allows for fine-scale spatial resolution (e.g., tens of microns) and high levels of sensitivity (< 1 ppm) but is destructive as it requires ablating the surface of the sample [42-43].

In this study, we used SEM-EDS analysis to explore the distribution of major, minor, and trace elements within teeth from twelve bottlenose dolphins (*Tursiops truncatus*) that stranded along the northern Texas coast in Galveston County between 1987 and 2014. The primary objectives were to explore whether the distribution of major, minor, and trace elements in dolphin teeth 1) differed between the enamel and dentin and 2) varied across the dentin GLG's within individuals, which may reflect physiological changes and exposure to major, minor, and trace elements over time. Finally, although our sample size was limited, we sought to

148 qualitatively assess multi-decadal temporal trends in the wt % of trace elements, particularly
149 those of anthropogenic origin (e.g., Cd, Hg, Pb).
150

151 **2. Methods**

152 *2.1. Teeth collection and preservation*

153 We analyzed teeth from six male and six female bottlenose dolphins that stranded
154 between 1987 and 2014 in Galveston County, TX (Table 1). We preferentially chose individuals
155 with straight-line body lengths between 221 cm and 245 cm. In doing so, we aimed to study
156 dolphins that were at least five years old so we could analyze dolphins that had several GLG's
157 but had not yet reached their asymptotic body length [45]. In older dolphins, GLG's become
158 increasingly irregular and can be challenging to decipher [21]. Furthermore, in some cases, the
159 pulp cavity may become occluded. If this occurs, dentin layers no longer accumulate; therefore,
160 if a dolphin lived beyond the time of pulp occlusion, a complete dentin record would not be
161 available [1, 21].

162 Teeth were extracted from the left mandible of dead stranded bottlenose dolphins using
163 an elevator to loosen the gum and connective tissue, and for most dolphins, an extractor was used
164 to lift the tooth free. For most samples, tooth number eight from the proximal end of the
165 mandible and several surrounding teeth were collected. In some cases, a section of the mandible
166 with teeth still intact was cut from the carcass and frozen for subsequent processing and
167 extraction. If teeth were not available from the left mandible, they were extracted from the right
168 mandible. Teeth were either fixed in 10% neutral buffered formalin or stored at -20°C. A large-
169 scale cleaning/preparation project was undertaken in 2017 wherein teeth were removed from

formalin prior to preparation. Therefore, some teeth may have been stored in formalin for several decades before 2017; however, no records were kept for which teeth were frozen and which teeth were stored in formalin. In 2017, formalin-fixed teeth were removed from solution and thoroughly rinsed in running tap water. Water maceration was performed on all teeth with attached soft tissue, using separate containers for each dolphin. Any soft tissue that did not detach after soaking was gently brushed away. Teeth were then rinsed and air-dried in a temperature-controlled room and stored in individually labeled Whirl-Pak bags (Nasco; Fort Atkinson, WI) at room temperature. Since a detailed storage history for the teeth was not available, it was not possible to explore the influence of preservation methods on major, minor, and trace elements. Despite disparate storage conditions, we utilized all teeth for both age estimation and SEM-EDS analysis. Formalin preservation may influence tooth elemental composition; however, to the best of our knowledge there have been no studies that investigated the effect of formalin fixation on elemental concentrations in teeth.

2.2. Teeth sectioning and age estimation

Teeth were initially sectioned down the center mid-line of the longitudinal buccal-lingual axis. One half of the tooth was used for SEM-EDS analysis, and the other half was prepared for sectioning for age determination using standard procedures [21-22]. Teeth for age determination were fixed in 10% neutral buffered formalin for 48 hours, rinsed in water, and dried before sectioning. Slabs were cut off the longitudinal buccal-lingual axis of each tooth using a diamond wafer blade mounted on a Buehler Isomet low-speed saw (Emerson Industrial Automation, Lake Bluff, IL). The slabs were continuously rinsed in tap water for approximately 6 hours and then

192 decalcified in RDO (rapid decalcifying agent of acids; Apex Engineering Products Corporation,
193 Aurora, IL) for 6-12 hours based on the thickness of the resulting center slab remaining (1-2
194 mm). The slabs were continuously rinsed overnight and thin-sectioned on a Leica SM2000R
195 sledge microtome (Leica, Inc., Nussloch, Germany) attached to a Physitemp freezing stage
196 (Physitemp, Inc., Clifton, New Jersey). Thin sections (25 µm thick) were stained in Mayer's
197 hematoxylin, blued for 30 seconds in a weak ammonia solution, dried on a slide, and mounted in
198 100% glycerin. All sections were read three times by the same reader (Wayne McFee) using a
199 Nikon SMZ1500 stereomicroscope (Nikon Instruments, Inc., Lewisville, Texas); at least one
200 week elapsed between readings to eliminate bias. Teeth were aged based on Hohn et al. [22]; if
201 two of the three readings were the same, this was used as the age estimate, whereas if differences
202 between readings were >2 GLG's, a fourth reading was made. Age estimates >1 GLG were
203 rounded to 0.50 GLG. Most teeth >5 GLG's were estimated to the last GLG.

204

205 *2.3. SEM-EDS analysis*

206 Before SEM-EDS analysis, teeth were rinsed with Milli-Q water (Millipore, Burlington,
207 MA), placed in trace metal clean 50 ml plastic tubes, and ultrasonically cleaned in 95% ethanol
208 for 5 minutes. Teeth were then triple rinsed with Milli-Q water, placed in trace metal clean 15 ml
209 plastic tubes, and air-dried in a clean fume hood for 48 hours. Two analyses on each tooth were
210 performed using an SEM (JSM-6010 PLUS/LA; JEOL USA Inc., Peabody, MA) equipped with
211 EDS at Texas State University. The SEM produces images by scanning the sample with a
212 focused electron beam; the incident electrons interact with the sample, resulting in the production
213 of secondary electrons, backscattered electrons, and characteristic x-rays. Backscattered

electrons reflect the composition of the sample, and when examined using an SEM in backscattered electron (BSE) imaging mode, color variation in the sample is indicative of variation in chemical composition [46]. For example, in BSE imaging mode, the enamel, which is more heavily mineralized compared to dentin, appears as a bright band [19, 41]. Characteristic x-rays are generated when the high-energy electron beam ejects an electron from its shell and an electron from a higher energy state transitions to a lower energy state to fill the space. This transition releases characteristic x-rays that are specific to individual elements. Energy dispersive x-ray detectors are often used in conjunction with an SEM to convert characteristic x-rays to electrical voltages to qualitatively and semi-quantitatively describe the distribution of elements in calcified tissues [19, 26, 41-42]. Using SEM-EDS, qualitative and semi-quantitative elemental information at an individual point (point analyses) and across an area (elemental maps) can be obtained, reporting detected elements in wt % or atomic % (at %); when coupled with BSE imagery, one can begin to understand the elemental distribution across the sample.

In the first analysis, selective point analysis on three points on the enamel (point 1 = outer enamel, point 2 = mid-enamel, and point 3 = inner enamel) and two points on the pre-natal dentin [point 4 = dentin near the enamel-pre-natal dentin junction (EDJ) and point 5 = inner pre-natal dentin] were performed, following the general methodology outlined by Loch et al. [19] for *in situ* analysis using wavelength dispersive x-ray spectroscopy (WDXS or WDS) (Figure 1). The procedure was repeated for two additional transects, approximately 50 μm apart. Combining the data from the three transects, the mean and standard error (SE) wt % of each element for each point was calculated. A 20 kV accelerating voltage and a working distance of between 10-12 mm was used. In each tooth, point analysis was performed approximately halfway between the tooth

neck and the top of the tooth crown. In a subset of teeth (n = 7), elemental maps were generated to visualize the distribution of elements across the enamel and pre-natal dentin.

In the second analysis, the potential differences in the wt % of elements across the GLG's were explored. Point analysis began halfway between the tooth neck and the bottom of the tooth root. The goal was to obtain measurements from the GLG's; however, GLG's were not visible. Therefore, the approximate location of the GLG's was identified by referencing the images from the thin-crossed sectioned teeth used for aging. Starting from the exterior of the tooth and moving toward the interior, point analyses were performed approximately every 300-350 µm until reaching the pulp cavity (Figure 2). When the pulp cavity was not visible, points were analyzed across half of the tooth width. On average, across all teeth, 7 points per transect were measured; the process was repeated for two more transects approximately 100 µm apart. The mean and SE wt % of the elements detected at each point were calculated. The analytical setting used a 20 kV accelerating voltage, 100 µm aperture size, and a working distance between 9-12 mm. Again, for a subset of the samples (n=2), elemental maps of the area of interest were generated to qualitatively assess the distribution of elements across the GLG's.

2.4. Statistical analysis

For both analyses (enamel vs. dentin and GLG's analysis), a repeated-measures linear mixed effects analysis of variance (ANOVA) and Tukey's post-hoc test was used to explore the potential spatial differences in elemental distribution within the teeth. The repeated-measures design was used because we measured several points on each tooth. In all models, the response variable was the element measured, the fixed effect was the point location [enamel vs. pre-natal

dentin (points 1-5) and GLG's analysis (points 1-7)], and the random effect was the individual dolphin (sample). Models with varying intercepts and varying intercepts and slopes were considered, and the model that best fit the data was selected. Residual plots were explored for violations of normality, and homoscedasticity and data were natural log-transformed when necessary. The level of significance was set at $\alpha = 0.05$, and the analysis was performed in R version 4.0.2 using the following packages: lme4 and eemeans [48-50]. For descriptive and inferential statistics, a value of one-half the detection (0.05 wt %) was applied to elements below the detection limit [46, 51].

3. Results

For ten dolphins, age estimates ranged between 4.5 to 18 years (Table 1). Hypermineralization precluded precise age estimates for two individuals; these individuals were estimated to be >11 and >16 years old, respectively (Table 1). Tables 2 and 3 provide a summary of the major, minor, and trace elements at each point measurement for all dolphins combined. Data pertaining to each individual dolphin, including the mean and SE calculations, for each point measurement are provided in Supplementary Tables 1-6.

Using SEM-EDS, we first compared the distribution of elements across the enamel and pre-natal dentin. The mean ± SE wt % for all point measurements and dolphins combined, were as follows: O (39.6 ± 0.373), Ca (25.0 ± 0.229), P (14.6 ± 0.091), C (10.4 ± 0.287), Al (8.29 ± 0.507), Mg (1.39 ± 0.070), Na (0.639 ± 0.013), and Cl (0.130 ± 0.007). For all elements, there were significant differences in wt % values among the five points in the enamel and pre-natal dentin (Table 2; Figure 3). For all models except for Mg, the random intercept model fit the data

280 better than the random intercept and slope model. Oxygen, Ca, and P were measured in lower wt
281 % values in the outer enamel (point 1) compared to the other points (points 2-5). Aluminum was
282 measured in the highest wt % in the outer enamel (point 1) and progressively decreased toward
283 the inner pre-natal dentin (Figure 3 and 4). For C, wt % values were higher in the outer enamel
284 and inner pre-natal dentin compared to points in the inner and mid enamel. The wt % of Mg was
285 lowest in the outer enamel and increased towards the inner pre-natal dentin. On average, the wt
286 % values of Mg wre 2.21 and 0.84 in the enamel (points 1, 2, and 3) and pre-natal dentin (points
287 4 and 5), respectively. Sodium increased from the outer enamel to the EDJ and then decreased in
288 the inner pre-natal dentin; on average, the wt % values of Na in the enamel and pre-natal dentin
289 were 0.593 and 0.703, respectively. Finally, Cl was present in the greatest wt % in the outer
290 enamel (point 1); while Cl was also detected at lower wt % values in the mid-enamel and
291 innerenamel, it was not observed in the pre-natal dentin (points four and five). Elemental maps
292 showed differences in the distribution of elements between the enamel and pre-natal dentin
293 (Figure 4).

294 In the second analysis using SEM-EDS, we performed point analyses at seven points
295 approximating where GLG's would occur; a summary of the major, minor, and trace elements at
296 each point measurement for all dolphins combined is shown in Table 3. The mean ± SE wt % for
297 all point measurements and dolphins combined, were as follows: O (41.6 ± 0.295), Ca (23.8 ±
298 0.260), C (16.9 ± 0.608), P (13.5 ± 0.141), Mg (2.24 ± 0.053) Al (1.43 ± 0.215), and Na (0.698
299 ±0.011) (Figure 5). For all elements, the intercepts model was a better fit than the intercepts and
300 slope model. Except for O, there were significant differences in the wt % values of the elements
301 across the seven points. The most common difference was between the tooth edge (point 1) and
302 the interior points (points 2-7). At the tooth edge (point 1), Ca, P, Mg, and Na were measured in

lower wt % values than the interior points (points 2-7). In contrast, C and Al were present at higher wt % values closest to the in tooth edge (point 1) than in the interior points (points 2-7). Visually the only differences that could be determined in the elemental maps were between the dentin and pulp cavity (Figure 6).

4. Discussion

Using SEM-EDS, we were able to visualize the microstructure of dolphin teeth, distinguishing between the enamel and dentin in the tooth crown, and explore the variation in major (C, Ca, O, and P) and minor elements (Cl, Mg, Na) between the enamel and dentin. Except for Al, no trace elements were detected. Although we could not visually distinguish GLG's based on the SEM-EDS, we made use of images from tooth sections used the aging to approximate the location where GLG's occurred and performed EDS analysis to investigate the potential variation in major, minor, and trace elements across the GLG's. Except for the point closest to the edge of the tooth, the wt % values of C, Ca, P, O, Mg, and Na did not vary substantially across the dentin transect. Except for Al, we did not observe any other trace elements; therefore, we could not examine how contaminants changed over time within the lifespan of an individual or temporally across the decades among individuals. While technologies with lower detection limits (e.g., LA-ICP-MS) may be required to explore the presence and distribution of trace elements in bottlenose dolphin teeth, the information provided in the current study will be valuable to other analyses such as LA-ICP-MS that rely on an Ca as an internal standard [10-12,52]. Further, for some elements reported in this study (e.g., O, P, C, and Cl), it is either not

possible or very challenging to analyze using LA-ICP-MS; therefore, SEM-EDS can serve as a complementary analysis [53].

The major elements detected in the dolphin teeth were C, Ca, P, and O. Calcium and P are the primary components of hydroxyapatite; across all samples, the mean ± SE wt % of C and P was 24.2 ± 0.228 and 14.6 ± 0.091, respectively. Murphy et al. [37] reported similar wt % values for Ca (24.9) and P (11.2) in bottlenose dolphin dentin, also measured by SEM-EDS. However, our values were lower than those reported by Loch et al. [19]; in analyzing the elemental distribution in the enamel and dentin from ten dolphin species using WDX, Loch et al. [19] reported wt % values of 46.9 and 36.2 for Ca and P, respectively for the single bottlenose dolphin tooth analyzed. Unlike Loch et al. [19] and Brügmann et al. [31], which reported the element concentrations in the enamel and dentin of hippopotamid teeth, we did not determine that Ca or P were consistently present in greater wt % values in the enamel compared to the dentin. Brügmann et al. [31] explain that the higher concentration of Ca and P in the enamel is a result of the reduced porosity and increased mineralization of the enamel compared to the dentin. Since the dentin has a higher percentage of organic components than enamel, when comparing the enamel and dentin, we expected to find a higher weight percentage of the O and C in the dentin, which are common elements found in proteins [26]. Oxygen followed this general pattern, but C did not. Other major elements of proteins (e.g., collagens), such as nitrogen (N) and hydrogen (H), were not detected. Hydrogen is too light to be detected using SEM-EDS, and N generally produces too weak of a signal to be detected [26]. In the GLG's analysis, an additional concern arose regarding the point closest to the tooth edge. In BSE mode, the cementum was indistinguishable from the dentin; consequently, the points closest to the tooth edge may have been cementum and not dentin. It is uncertain how wide the cementum layer was

in our samples; we also could not find an average cementum width in the literature for bottlenose dolphins.

Overall, except for points analyzed closest to the tooth edge, the major elements (C, Ca, O, and P) did not vary significantly across the tooth, making them good candidates for internal standards in future LA-ICP-MS analyses. In contrast to SEM-EDS methodologies, which are standardless, quantification in LA-ICP-MS involves external calibration using a standard reference material (SRM) (e.g., NIST 612 glass or NIST 1486 bone meal for teeth samples). In addition to external SRMs, signals are frequently normalized to an internal standard (e.g., Ca for teeth), and studies often assume homogeneous distributions of the internal standard [53]. The information provided here can help provide baseline information with respect to the wt % and distribution of major elements in bottlenose dolphin teeth. The consistent distribution of major elements across teeth supports their use as internal standards along with external CRMs.

The EDJ is a transition phase for major and minor elements. Cations and anions (e.g., Cl^-, Mg^{2+}, and Na^+) may also be incorporated into the hydroxyapatite structure of the enamel or dentin during the pre-eruptive period [32]. In the case of the enamel, they may also be incorporated post-eruption on the surface of the enamel (up to 150 μm depth) from the surrounding saliva [55-56]. In the present study, we observed the same trends in the variation of Cl and Mg across the enamel and pre-natal dentin as was previously reported in dolphin [19], hippopotamus [31], and human teeth [26]. Chlorine decreased from the enamel towards the dentin, while Mg increased from the enamel towards the dentin. Although the trends were not consistent across individual dolphins, on average, we found that Na followed a similar "umbrella" trend as was observed by Loch et al. [19], in which Na initially increased from the outer enamel towards the inner enamel and then decreased moving further towards the inner

dentin. Throughout the secretory and maturation stages of enamel formation, elements enter the enamel fluid; as the bioapatite crystallizes, the enamel becomes depleted in Mg and Na and enriched in Cl. Therefore, Mg and Na are present in the greatest wt % near the EDJ, while Cl is present in the greatest wt % in the outer enamel [31]. The incorporation of minor elements within the tooth structure can alter the tooth function. More research is required to fully understand how changes in minor elements alter the chemical structure and functionality of dental tissues. However, previous studies focused on human, bovine, porcine, and ovine teeth have shown that the incorporation of Mg^{2+} helps to regulate hydroxyapatite crystallization. For example, Mg is present in higher concentrations in the dentin and the inhibition of crystallization may explain why crystals are smaller and less frequently observed in the dentin compared to the enamel [26, 35].

During the mineralization phase of tooth development, trace elements can be incorporated within the crystalline apatite [40, 56]. Previous studies have used trace element concentration in marine mammal teeth to identify the timing of life-history events, identify the maternal transfer of contaminants, explore habitat utilization, and assess the spatial and temporal changes in environmental trace element concentrations, particularly those of anthropogenic origin [10-12, 15, 58]. Except for Al, we did not observe trace elements [e.g., Cd, Cu, Hg, Pb, Zn], which have been previously reported in marine mammal teeth [8-13, 15-17, 38-41]. Given that Al decreased in wt % moving from tooth exterior towards the tooth interior, and Al is the main component of the SEM stub, we suspect that the Al detected was related to the SEM stub and not the tooth itself. Caceres-Saez et al. [42], measuring the major, minor, and trace elements in Commerson's dolphin (*Cephalorhynchus c. commersonii*) and Franciscana dolphin (*Pontoporia blainvillei*) bone samples using SEM-EDS also detected Al and came to a similar

conclusion. Our results indicate either 1) the abovementioned trace elements were not present in our samples, or 2) they were present at concentrations below the detection limit. Based on the findings of other studies, many trace elements that we expected to find (e.g., Cu, Cd, Hg, Zn) but did not detect are likely present in the teeth but at wt %s below the detection limit of EDS (approximately 0.1 wt%) [46].

Low sensitivity due to high elemental detection limits is a significant disadvantage of using SEM-EDS technology to measure major, minor, and trace elements in dolphin teeth. Detections can be optimized if the sample is properly prepared, and the scan parameters, such as the vacuum conditions, accelerating voltage, spot size, and working distance are adjusted [26]. Ideally, the surface of the sample should be smooth and flat [54]. To minimize contamination, we did not polish our samples; however, variation in the sample topography may have affected the path of the x-rays exiting the surface and negatively influenced our ability to detect elements [26,54]. Non-conductive samples are generally coated with carbon or gold-palladium (Au-Pd) to reduce surface charging. After performing preliminary scans, we determined that there were no issues with surface charging. Therefore, to avoid contamination, we did not coat the sample in carbon or Au-Pd; however, the surface coating could have potentially increased the signal strength and improved the signal-to-noise ratio [26]. Because teeth are non-homogenous samples, it can be misleading to measure only one point, as some areas may have a greater percentage of elements than others. We attempted to overcome this limitation by taking measurements along several transects and averaging results. To maximize the detection of characteristic x-rays, the accelerating voltage must be 2- to 3-times higher than the energy required to eject an electron from its shell; in some cases, 20kv may not have been great enough to optimize detections but using a higher voltage was not possible while working in low vacuum

mode, which is required for any samples that have not been dehydrated. Further, the working distance, or the distance between the sample and the final piece of the lens, must be adjusted so that the angle of the outgoing characteristics x-rays intersects the detection system. Finally, as the mass percentage of the element decreases, the ability to correctly assign elemental peaks decreases due to reduced counts of associated characteristic x-rays [54]. Although SEM-EDS has several disadvantages, the technique provides a relatively quick method for elemental analysis; in addition, when study methodologies do not require tooth sectioning, or utilize teeth that have previously been sectioned, the method is non-destructive, making it appropriate for museum specimens [40-42,46-467. To understand how trace element deposition in bottlenose dolphin teeth may be used to create a timeline of life history events and exposure to trace elements, particularly pollutants (e.g., Cd, Hg, Pb), additional research is required using technologies with lower detection limits (e.g., LA-ICP-MS).

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620 **Table 1** Stranding year, straight-line body length, sex, and estimated age of bottlenose dolphins

621 used in the study

Sample ID	Stranding year	Length (cm)	Sex	Estimated age (years)
GA 159	1987	235	Female	>11[a]
GA 260	1989	233	Female	8
GA 277	1989	245	Male	>16[a]
GA 279	1989	244	Male	8
GA 345	1990	225	Female	4.5
GA 710	1995	238	Male	18
GA 737	1996	222	Male	8
GA 830	1996	237	Female	16
GA 1599	2009	221	Female	11
GA 1603	2009	241	Male	11
GA 1755	2012	224	Male	9
GA 1856	2014	226	Female	10

622 [a]hypermineralization near the pulp cavity precluded a more precise age estimate

Table 2 Weight percentage (wt %) of major, minor, and trace elements across the enamel and pre-natal dentin (PND) for all dolphins combined (mean ± standard deviation; range of wt % in parenthesis) EDJ = enamel dentin junction

Element	Outer enamel Point 1	Mid-enamel Point 2	Inner enamel Point 3	PND near (EDJ) Point 4	Crown PND Point 5
Major elements					
C	11.6 ± 6.40	8.69 ± 2.28	9.30 ± 2.84	10.2 ± 2.21	12.0 ± 2.48
	(5.92 - 33.6)	(6.08 - 16.1)	(6.16 - 20.4)	(6.86 - 14.1)	(8.70 - 19.3)
Ca	23.7 ± 4.91	25.7 ± 3.19	25.5 ± 2.03	25.0 ± 1.73	26.0 ± 1.74
	(16.1 - 37.7)	(19.2 - 36.3)	(21.5 - 29.4)	(22.1 - 27.8)	(21.8 - 28.0)
O	34.1 ± 5.80	38.9 ± 5.02	41.4 ± 3.05	41.7 ± 2.53	41.5 ± 2.56
	(20.5 - 44.2)	(25.4 - 47.3)	(32.5 - 46.4)	(36.9 - 46.1)	(33.4 - 45.4)
P	13.44 ± 1.83	15.0 ± 0.939	15.1 ± 0.803	14.8 ± 0.737	14.8 ± 0.504
	(9.50 - 16.6)	(12.7 - 17.1)	(13.1 - 16.4)	(13.1 - 15.8)	(14.0 - 15.8)
Minor elements					
Cl	0.238 ± 0.057	0.208 ± 0.042	0.106 ± 0.050	0.053 ± 0.011	0.050 ± 0.00
	(0.110 - 0.390)	(0.130 - 0.340)	(0.050 - 0.170)	(0.050 - 0.100)	(0.050 - 0.050)
Na	0.477 ± 0.153	0.598 ± 0.110	0.705 ± 0.108	0.742 ± 0.162	0.668 ± 0.174
	(0.230 - 1.06)	(0.400 - 0.840)	(0.540 - 0.980)	(0.570 - 1.21)	(0.550 - 1.39)
Mg	0.298 ± 0.394	0.734 ± 0.623	1.42 ± 0.414	1.86 ± 0.362	2.57 ± 0.384
	(0.050 - 1.06)	(0.050 - 1.80)	(0.050 - 2.07)	(1.27 - 2.70)	(1.77 - 3.40)
Trace elements					
Al	16.2 ± 8.45	9.80 ± 5.65	6.50 ± 3.80	5.79 ± 3.35	3.52 ± 1.67
	(0.83 - 30.67)	(0.480 - 20.1)	(0.460 - 13.3)	(0.420 - 11.1)	(0.360 - 6.54)

Table 3 Weight percentage (wt %) of major, minor, and trace elements at seven points approximating where growth layers groups (GLG's) would occur in the dentin moving from point 1 (edge of tooth) towards the pulp cavity for all dolphins combined (mean ± standard deviation; range of wt % in parenthesis)

Element	Point 1	Point 2	Point 3	Point 4	Point 5	Point 6	Point 7
Major elements							
C	34.7 ± 17.1	17.24 ± 1.85	14.5 ± 1.64	13.5 ± 1.52	14.2 ± 3.52	13.0 ± 1.72	12.9 ± 2.22
	(15.3 - 74.6)	(13.67 - 23.0)	(11.8 - 20.5)	(10.9 - 19.2)	(11.0 - 30.2)	(10.3 - 20.3)	(9.82 - 23.4)
Ca	16.58 ± 6.44	23.5 ± 1.59	24.8 ± 2.16	25.3 ± 1.75	24.80 ± 2.05	26.2 ± 2.25	25.2 ± 1.10
	(2.23 - 23.3)	(19.2 - 26.4)	(21.0 - 30.8)	(23.1 - 31.0)	(19.7 - 28.6)	(23.3 - 31.6)	(21.4 - 27.7)
O	33.7 ± 7.00	41.5 ± 3.01	42.6 ± 2.66	43.2 ± 2.19	43.34 ± 2.42	42.3 ± 3.07	43.8 ± 1.73
	(20.1 - 44.53)	(34.4 - 47.3)	(35.8 - 46.5)	(37.4 - 46.5)	(33.7 - 48.9)	(34.9 - 46.1)	(38.1 - 45.8)
P	9.33 ± 3.60	13.2 ± 0.681	14.0 ± 0.703	14.4 ± 0.543	14.2 ± 1.02	14.8 ± 0.532	14.51 ± 0.492
	(1.27 - 12.8)	(11.8 - 15.0)	(12.0 - 15.4)	(13.5 - 15.8)	(11.4 - 15.4)	(13.7 - 16.1)	(12.5 - 15.4)
Minor elements							
Mg	0.818 ± 0.698	2.06 ± 0.484	2.37 ± 0.664	2.55 ± 0.608	2.57 ± 0.639	2.62 ± 0.661	2.60 ± 0.421
	(0.050 - 2.29)	(1.32 - 2.88)	(0.005 - 3.67)	(1.85 - 4.05)	(1.73 - 4.19)	(1.00 - 3.90)	(1.96 - 3.56)
Na	0.501 ± 0.276	0.667 ± 0.109	0.711 ± 0.148	0.724 ± 0.122	0.719 ± 0.134	0.709 ± 0.113	0.748 ± 0.099
	(0.050 - 1.06)	(0.540 - 1.01)	(0.520 - 1.22)	(0.540 - 1.03)	(0.480 - 1.08)	(0.550 - 1.03)	(0.600 - 0.980)
Trace elements							
Al	4.97 ± 7.54	1.84 ± 2.68	1.14 ± 1.63	0.777 ± 1.20	0.654 ± 1.14	0.506 ± 1.02	0.404 ± 0.913
	(0.050 - 23.80)	(0.050 - 8.47)	(0.050 - 6.25)	(0.050 - 4.84)	(0.050 - 4.53)	(0.050 - 4.32)	(0.050 - 3.81)

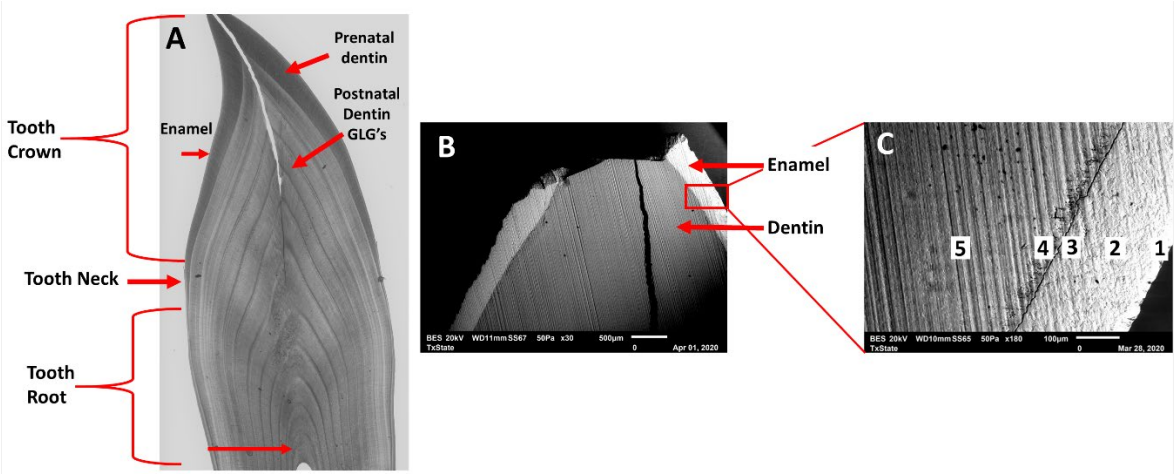

Figure 1 Cross-sectioned image of the top half of tooth of GA1603 (the split in the tooth was likely a result of being frozen in long-term storage; arrows represent the general location of the elemental analyses) (A), SEM backscattered imaging showing (B) the enamel and pre-natal dentin (PND), along with a rectangle that indicates the approximate area of SEM-EDS analysis, and (C) a zoomed in image of the area of the EDS analysis showing the locations for point analysis (point 1 = outer enamel, point 2 = mid enamel, point 3 = inner enamel, point 4 = pre-natal dentin near the enamel pre-natal dentin junction, and point 5 = inner pre-natal dentin)

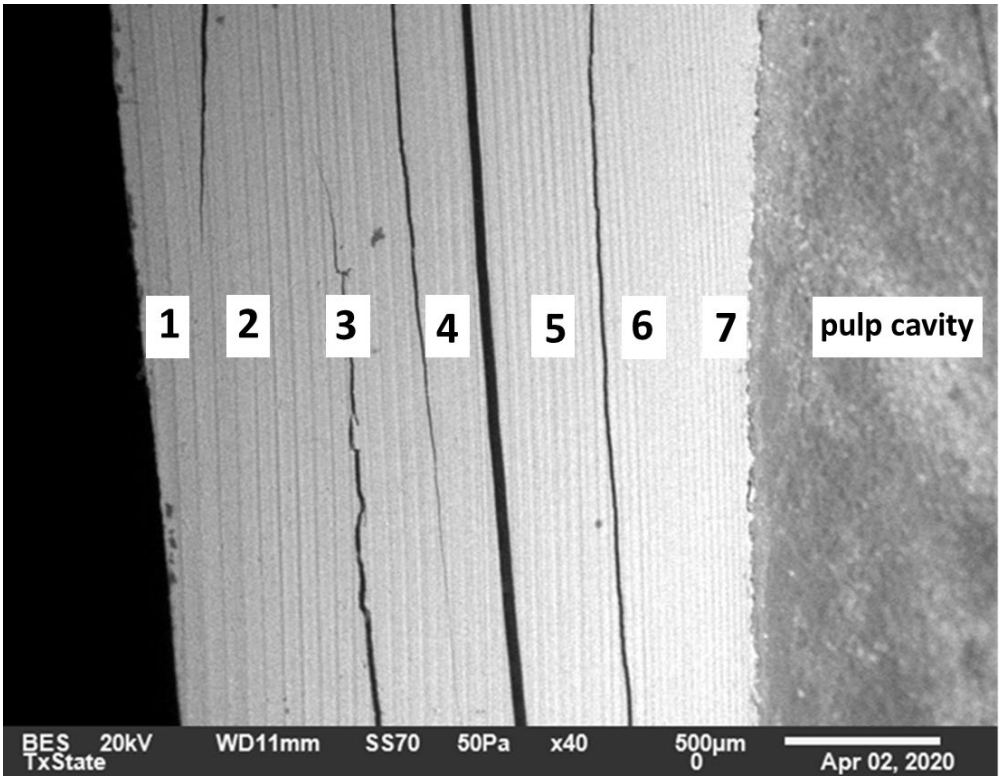

Figure 2 SEM backscattered image showing the locations of point analyses (points 1 - 7) used to explore the distribution of elements across the approximate location of the growth layer groups (GLG's).

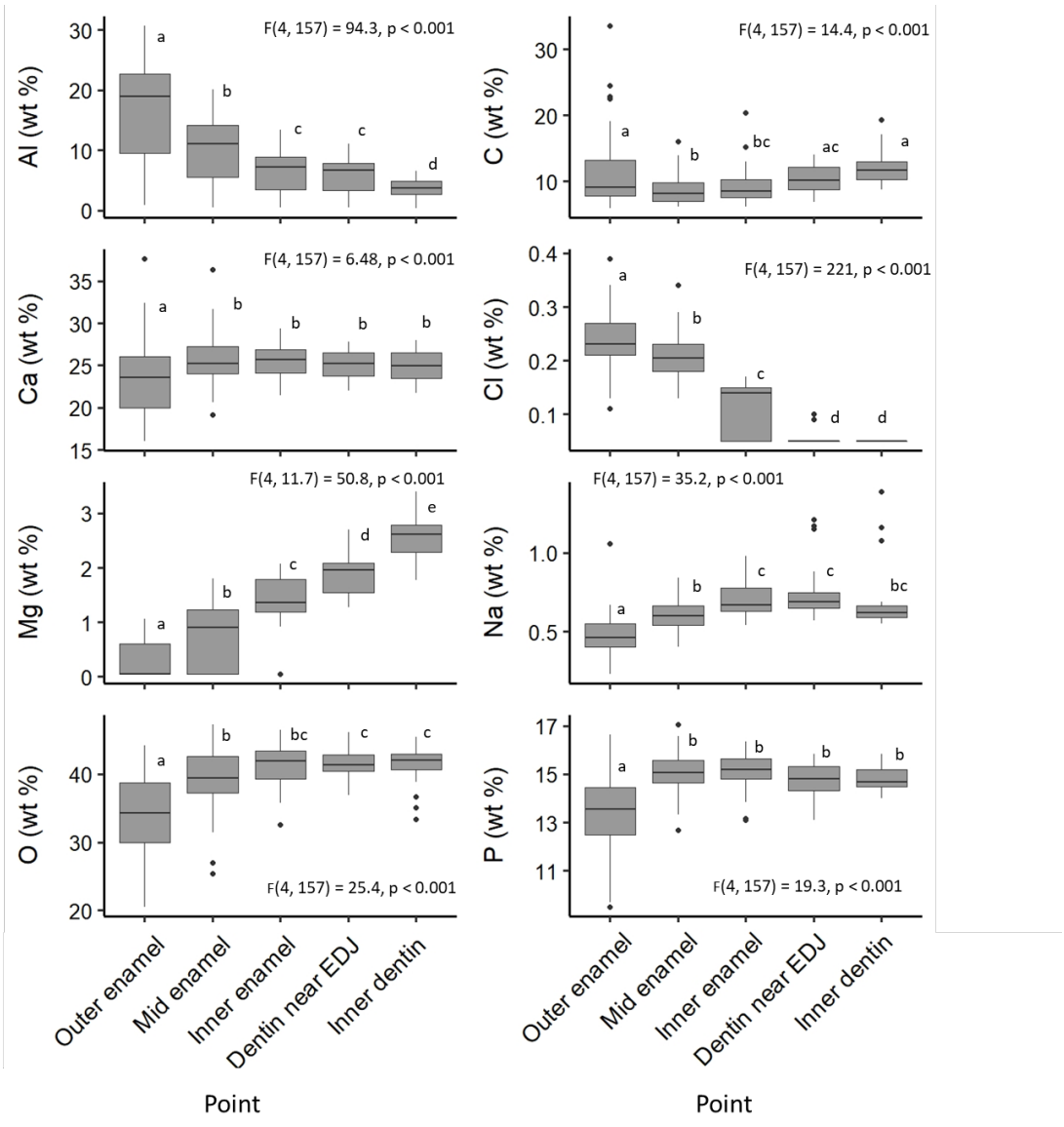

Figure 3 Selective point analyses for elements in the enamel and pre-natal dentin (PND) expressed as weight percentage (wt %): outer enamel (point 1), mid enamel (point 2), inner enamel (point 3), pre-natal dentin near enamel dentin junction (EDJ) (point 4), inner pre-natal dentin (point 5). Results of the repeated- measures linear mixed effects ANOVA and Tukey's post-hoc test are shown in each panel. Lowercase letters indicate points grouped by statistically similar wt % values. Data pertaining to each individual dolphin including the mean and SE wt % at each point are provided in the supplementary tables S1-S3.

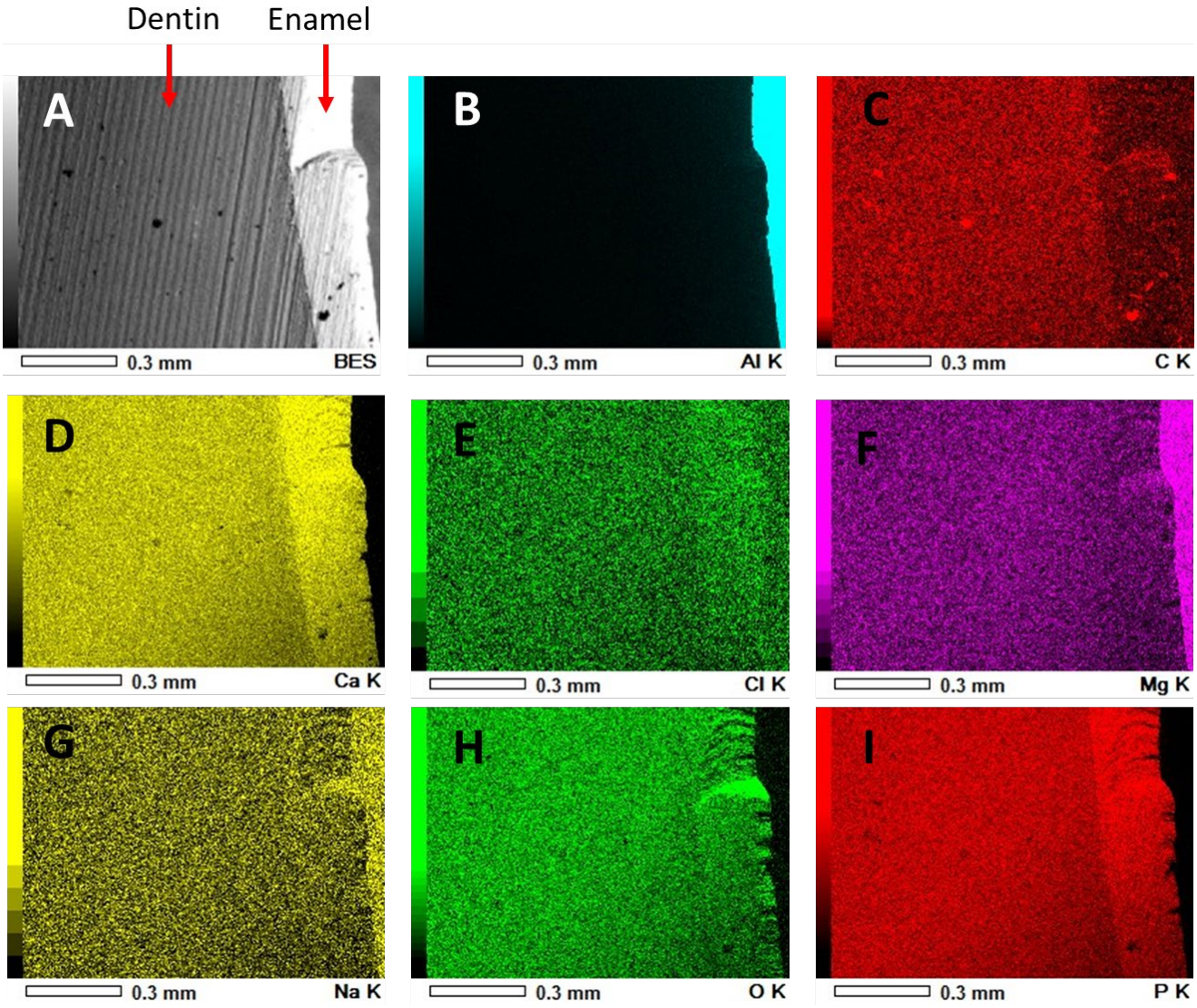

Figure 4 Backscatter SEM image of analysis area of sample GA 260 showing the enamel and pre-natal dentin (PND) (panel A) and elemental maps for Al (panel B), C (panel C), Ca (panel D), Cl (panel E), Mg (panel F), Na (panel G), O (panel H), and P (panel I). The intensity of the color is proportional to the number of x-ray counts in which higher intensity colors correspond to higher x-ray counts or greater wt %. For references to color please refer to the online version of this article

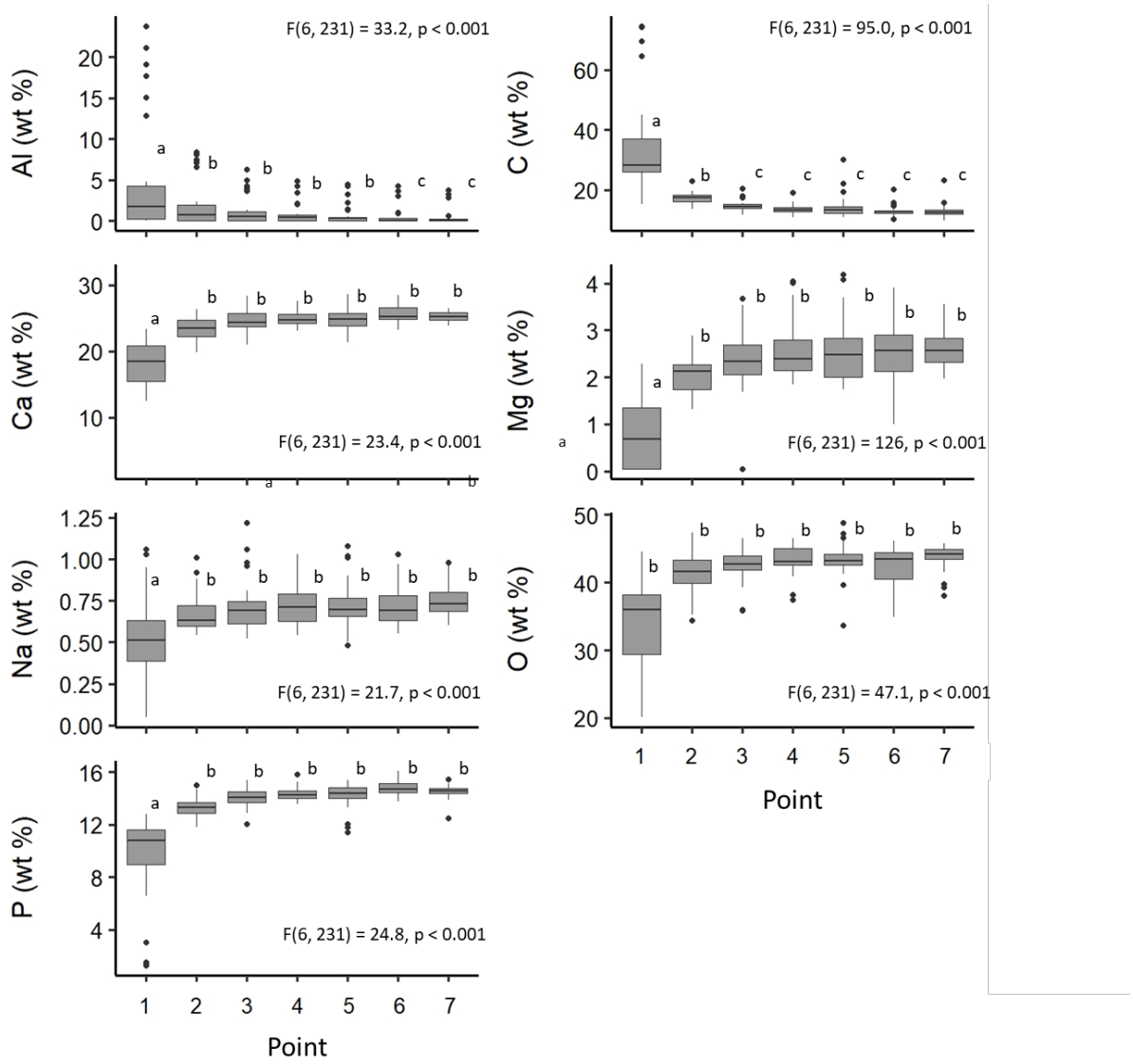

Figure 5 Selective point analyses for elements approximating where growth layers groups (GLG's) are present. Elements presented as a weight percentage (wt %), moving from the outer tooth edge (point 1) towards the tooth center (point 7), with points closest to the tooth edge being the oldest deposited dentin layers and points closest to the tooth center being the newest deposited dentin layers. Results of the repeated-measures linear mixed effects ANOVA and Tukey post-hoc test are shown in each panel. Pairwise comparisons for Al are not included

because the confidence intervals of the marginal means included zero. Lowercase letters indicate points grouped by statistically similar wt % values. Data pertaining to each individual dolphin including the mean and SE wt % at each point are provided in the supplementary tables S4-S5.

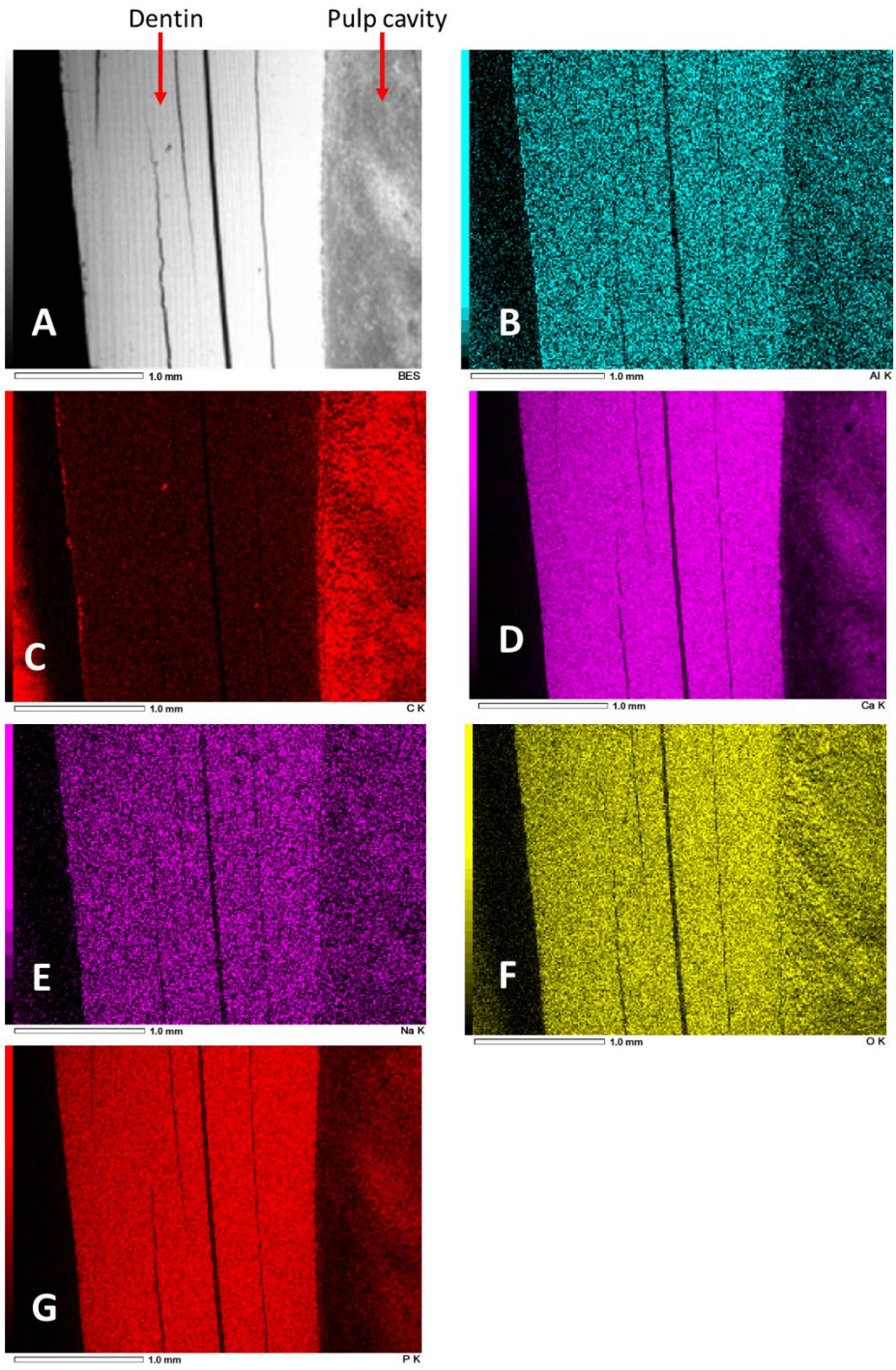

Figure 6 Backscatter SEM image of analysis area of sample GA 1755 showing the dentin and pulp cavity (panel A) and elemental maps for Al (panel B), C (panel C), Ca (panel D), Na (panel

E), O (panel F), and P (panel G). Magnesium and Cl were not detected in this particular sample. The intensity of the color is proportional to the number of x-ray counts in which higher intensity colors correspond to higher x-ray counts or greater wt %. Striations in the tooth may be a consequence of the cross-sectioning process. For references to color please refer to the online version of this article.