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Morphology and elemental components of sea turtle eggshells using scanning electron microscopy

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ABSTRACT

This research analyzed the morphology and elemental components of sea turtle eggshells by using quantitative and qualitative research methods. Results showed that sea turtle eggshells were composed of three layers: outer cuticle, middle, and inner fibrous layers. The morphology of the outer cuticle layer was thick, porous, and composed of aragonite to form $CaCO_3$. The middle layer was compact and thick, and the inner layer was compact and thin. The total layer thicknesses of the green turtle (*Chelonia mydas*), hawksbill sea turtle (*Eretmochelys imbricate*), and leatherback turtle (*Dermochelys coriacea*) were 251.92 ± 20.86, 236.94 ± 19.26, and 237.57 ± 18.23 μm, respectively. Elemental analysis of the eggshells of all species revealed the presence of C, O, Ca, S, F, Al, Na, Cl, Si, K, P, Mg, and I. Among the elements detected, C, O, and Ca were found at high percentages to form $CaCO_3$, which accumulated in the outer cuticle layer. Contamination of metals and non-metals, such as Mo, Br, Cu, Pb, and Pd, was found in the three types of sea turtle eggshells. This study is fundamental in providing data to manage sea turtle conservation in the future.

Keywords**:** eggshell; *Chelonia mydas*; *Eretmochelys imbricate*; *Dermochelys coriacea*; scanning electron microscope

1. INTRODUCTION

The sea turtle population is dramatically decreasing due to some human activities, such as seining, illegal egg harvesting, pollution, and spawning area destruction. Furthermore, humans are consuming sea turtle meat, extracting oil from their kidneys, livers, and fat for medicinal ingredients, and even using tortoiseshells for decorative purposes (Eckert et al., 1999). The Convention of International Trade in Endangered Species of Wild Fauna and Flora declared all sea turtle species as endangered wildlife species type 1 and forbade sales of sea turtles. In 1994, the Royal Thai Navy headquarters at Sattahip Naval Base in Chonburi, Thailand initiated the project to secure Cram, E-ra, and Jan islands, and also to conserve Thai turtle species *Chelonia mydas* and *Eretmochelys imbricata.* In 1995, the Royal Thai Navy assigned Navy Section 3 to secure Son-ngam beach and Por cape, which are actually obligations of Phang Nga Naval Base in Phang Nga, Thailand. They initiated the conservation of three sea turtle species, namely, *C. mydas*, *Lepidochelys olivacea*, and *Dermochelys coriacea.* The navy conducted research on the biology, spawning, migration, and nourishment of the species. Then, they set up a sea turtle exhibition to promote awareness and knowledge of the sea turtles. Previous reports on the eggshell morphology of side-necked turtles were used for phylogenetic tree analysis (Winkler, 2006). Several groups also studied environmental contamination by monitoring sea

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turtle eggshells (Bishop et al., 1998; Lam et al., 2006; Tryfonas et al., 2006). These studies reviewed several high-level contaminants, such as polychlorinated biphenyl, polychlorinated dibenzodioxins, Al, Zn, Mn, Pb, Sn, Cd, Cr, Cu, and Zn, which accumulate in the livers and eggshells of sea turtles. These contaminations cause the abnormal hatching of neonate turtles.

This research quantitatively and qualitatively analyzed the morphology and elemental components of sea turtle eggshells. Scanning electron microscopy (SEM) was performed using a secondary electron detector and an energy dispersive X-ray detector. The results of this study can be applied to categorize and model the sea turtle eggshells that were collected from the Andaman Seashore and the Gulf of Thailand.

2. MATERIALS AND METHODS

2.1 Materials

One hundred and eleven sea turtle eggshell samples were collected from the green turtle (*C. mydas*), the hawksbill sea turtle (*Eretmochelys imbricate*), and the leatherback turtle (*D. coriacea*) at the Sea Turtle Conservation Centre, the Air and Coastal Defense Command, the Sattahip Naval Base (Chonburi Thailand), and Phuket Marine Biological Centers (Phuket, Thailand) during the nesting season in 2010.

2.2 Sea turtle eggshell collection and preparation for SEM

The sea turtle eggshell samples were placed in beakers in an ultrasonic bath and washed with distilled water for 10 min. Then, they were dried at room temperature and stored at 29°C-30°C in a desiccator before analysis. The ultrastructure and elemental composition of sea turtle eggshells were analyzed using SEM with a secondary electron detector and an energy dispersive X-ray detector. For the ultrastructure study, dry eggshell samples were broken into pieces and stored in liquid nitrogen. First, three pieces, sized at 0.5×0.5 cm^2, were attached to a stub using carbon tape A sputter coater was used to coat the sample surface to conduct electricity. The sample was charged with 10-15 mA for 2 min. Finally, the eggshell structure of all samples was analyzed using SEM with the secondary electron detector under vacuum conditions, and the thickness of the sea turtle eggshell layer was measured with the Smile View program.

For elemental analysis of the sea turtle eggshells, the samples were placed in a way that the newly broken side is on the top. SEM was performed with the energy dispersive X-ray detector to analyze the elemental components under vacuum conditions.

2.3 Statistical analysis

Statistical analysis was conducted using SPSS software for Windows, and the thicknesses of each eggshell layer was analyzed and compared using SPSS for Windows (significant value = 0.05).

3. RESULTS AND DISCUSSION

3.1 Morphology of sea turtle eggshells

The ultrastructure of the sea turtle eggshells were observed using the secondary electron detector. The SEM images showed the outer cuticle layer or calcareous layer, the middle layer or middle multistrata layer, and the inner fibrous layer (Figure 1). The outer cuticle layer was thick and porous, which appeared like clusters of branching needle-like crystals, the middle layer was compact and thick, and the inner layer was compact and thin.

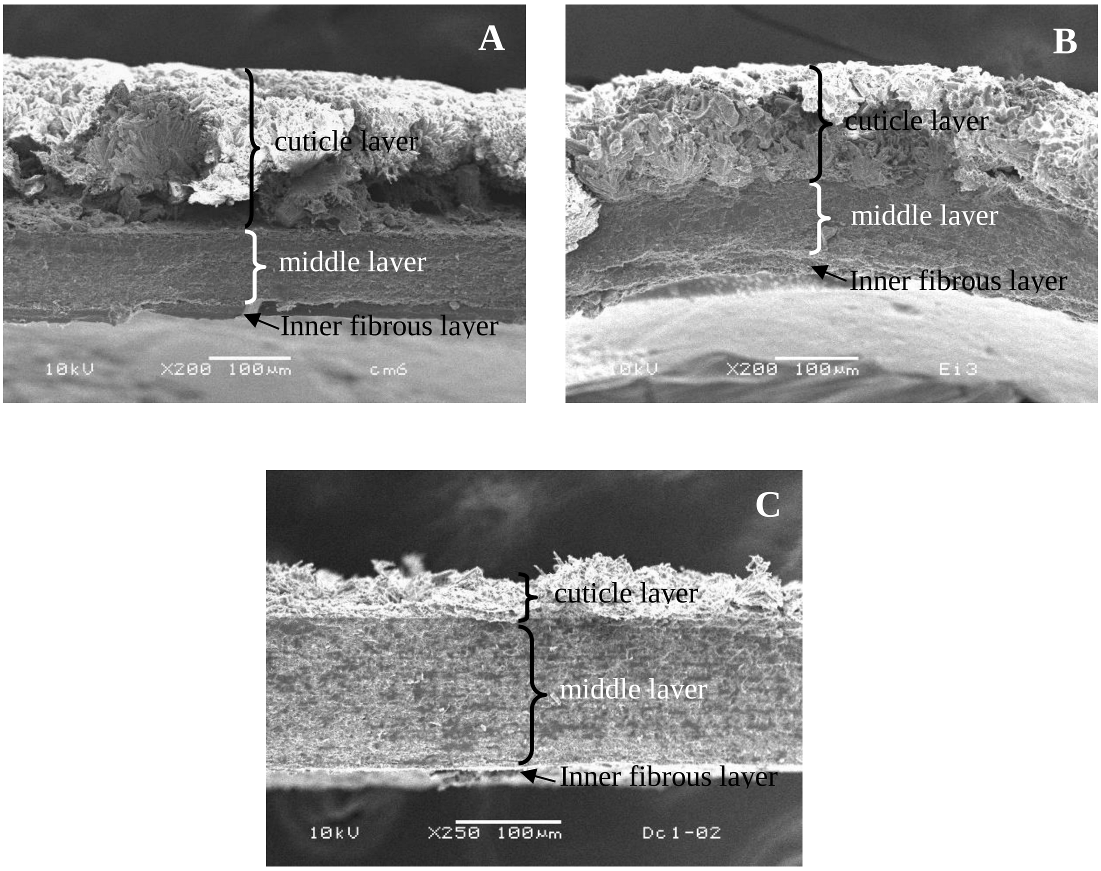

Figure 1 Cross-sectional view of SEM micrographs of the ultrastructure of the outer cuticle layer, middle layer, and inner fibrous layer of eggshells from the green turtle (A), the hawksbill sea turtle (B), and the leatherback turtle (C).

3.2 Thickness of three eggshell layers

The thicknesses of the three eggshell layers differed depending on the species of sea turtle. The outer cuticle layer thicknesses of the eggshells of the green turtle, the hawksbill turtle, and the leatherback turtle are shown in Table 1.

The ultrastructure of the green turtle eggshell showed that the outer cuticle layer had groups of spiked crystals originating at the top of the middle layer and formed a circular shape (Figure 2A). The hawksbill sea turtle eggshell had a spiked crystal structure with the center of each group of the crystals attached to the top of the middle layer. More than one pore could exist in the linkage of these two layers (Figure 2B). The ultrastructure of the outer cuticle layer of the leatherback turtle eggshell showed that the branching needle-like crystals were clearly tapering, which slightly differed from those of the green turtle and hawksbill sea turtle eggshells (Figure 2C).

Table 1 Thickness of eggshells of three turtle species

Type	Number of samples	Outer cuticle layer thickness (Mean ± S.D.; micrometer)	Middle layer thickness (Mean ± S.D.; micrometer)	Inner layer thickness (Mean ± S.D.; micrometer)	Total thickness (Mean ± S.D.; micrometer)
Green turtle (*Chelonia mydas*)	54	129.88 ± 14.22^{c}	118.32 ± 20.06^{b}	3.72 ± 0.86^{a}	251.92 ± 20.86^{b}
Hawksbill sea turtle (*Eretmochelys imbricata*)	27	117.51 ± 16.19^{b}	108.06 ± 13.00^{a}	11.38 ± 3.07^{c}	236.94 ± 19.28^{a}
Leatherback turtle (*Dermochelys coriacea*)	30	83.67 ± 17.15^{a}	144.20 ± 2.20^{c}	9.69 ± 2.53^{b}	237.57 ± 18.23^{a}

[*]a, b, c applied Duncan's test to categorize with $p \leq 0.05$

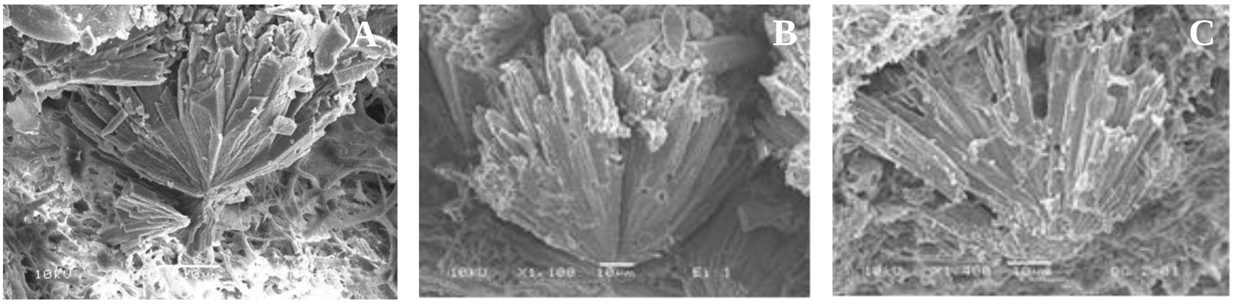

Figure 2 SEM micrographs (cross-sectional view) of the ultrastructure on the outer cuticle layer of the green turtle eggshell (A) showing the groups of spiked crystal originating at to the top of the middle layer (hawksbill sea turtle (B), and leatherback turtle (C)).

Micrographs of the ultrastructure show reduced redundancy in the outer layer of the green turtle eggshell, which consists of crystals of different sizes. These crystals were mixed and tightly coupled (Figure 3A). The outer layer of the hawksbill sea turtle eggshell consisted of 0.2-µm spherical crystals surrounded by spiked crystals. The hawksbill eggshell's spiked crystals were relatively smaller than the green turtle eggshell's crystals (Figure 3B). The tapering and small cylindrical crystals in different sizes in the outer layer of the leatherback turtle eggshell are also shown in Figure 3C.

The reticulate fibers in the middle layer (or middle multistrata layer) of the eggshells of the green turtle, the hawksbill sea turtle, and the leatherback turtle featured the same characteristics. The diameter of reticular fibers ranged from 0.3 to 2.0 µm (Figure 4A-C). The numerous pores in the reticular fibrous participated in the exchange of gas from the embryo through the eggshell to the outer environment. Primary spherite (PS) was also found between the middle and cuticle layers of the leatherback turtle eggshell layer (Figure 5). The result showed the same characteristic of a thin and compact tiny inner fibrous layer within all three species of the sea turtle eggshells (Figure 6A-C).

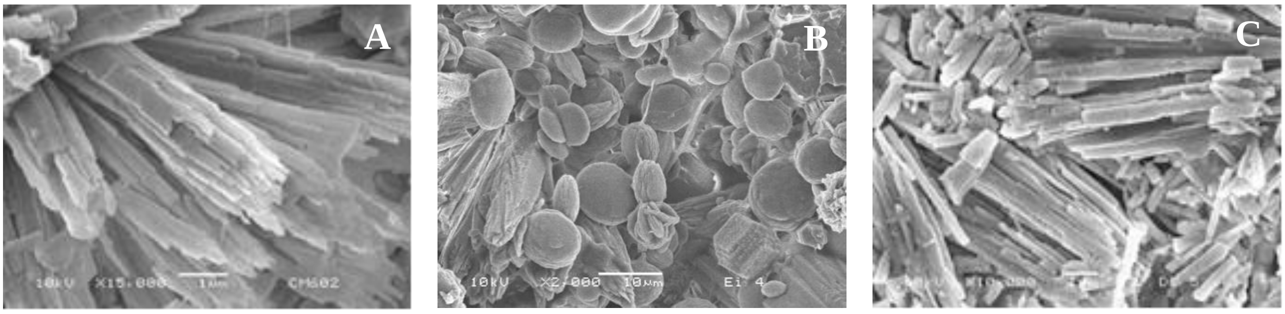

Figure 3 SEM micrographs of the ultrastructure of the outer cuticle layer of the green turtle eggshell with crystal formations (A), the hawksbill sea turtle (B) with spherical crystals between the groups of spiked crystals, and the leatherback turtle (C) with tapering and small cylindrical crystals.

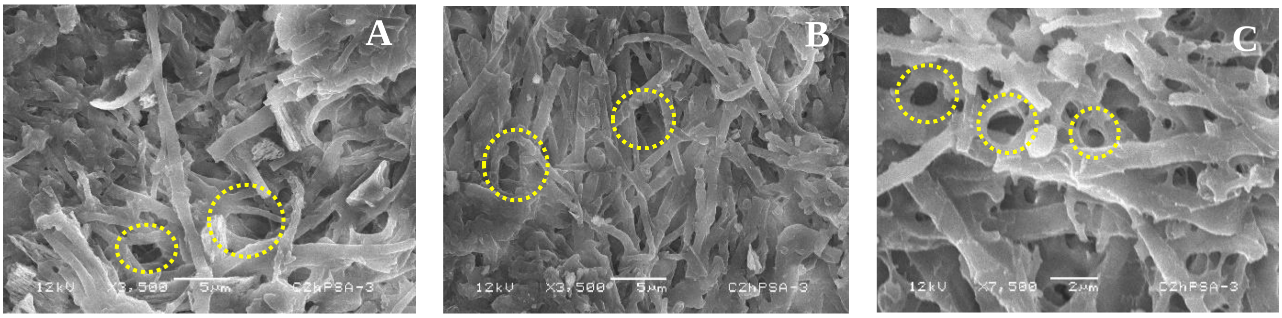

Figure 4 SEM micrographs (cross-sectional view) of the reticulate fiber in the middle layer of the green turtle (A), the hawksbill sea turtle (B), and the leatherback turtle (C) with numerous pores (in the circles).

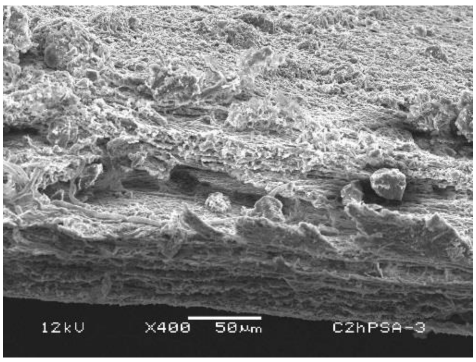

Figure 5 SEM micrographs showing the middle layer of the leatherback turtle eggshell with numerous fibrous layers and the primary spherite between the middle and cuticle layers.

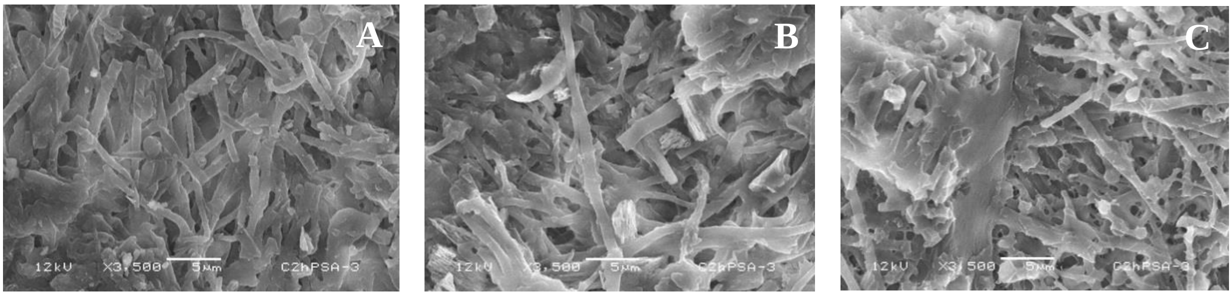

Figure 6 SEM micrographs (cross-sectional view) showing the fibrous inner layer of the green turtle (A), the hawksbill sea turtle (B), and the leatherback turtle (C).

3.3 Elemental composition of three turtle eggshells

The energy dispersive X-ray detector revealed that the energy spectrum of the X-rays emitted from the elements of C, O, Ca, S, F, Al, Na, Cl, Si, K, P, Mg, and I. The percentage of all elements detected was in the following order: C> O >Ca > S > F > Al > Na > Cl > Si > K > P > Mg > I.

The experiment revealed that the majority of elements included C, O, and Ca, which formed as $CaCO_3$ and were essential for the turtle's embryonic development in the outer cuticle layer (p<0.05).

The contamination of hazardous metals and non-metals, such as Mo, Br, Cu, Pb, and Pd, was found in the three turtle eggshell samples; however, Cd was only found in the green turtle sample (Table 2).

Table 2 Quantitative analysis of elements in three turtle eggshells

Element	Percentage of elemental compositions (Mean ± S.D.)		
	Green turtle	**Hawksbill sea turtle**	**Leatherback turtle**
C	54.85 ± 13.77	57.76 ± 14.00	48.46 ± 18.05
O	39.33 ± 10.47	35.95 ± 11.84	40.70 ± 11.34
Ca	2.54 ± 6.70	3.72 ± 13.07	8.42 ± 11.29
S	2.16 ± 2.73	1.98 ± 1.60	1.93 ± 1.42
F	0.49 ± 0.62	0.32 ± 0.34	0.25 ± 0.19
Mo	0.34 ± 0.09	0.39 ± 0.34	0.41 ± 0.28
Br	0.30 ± 0.45	1.42 ± 2.56	0.39 ± 0.30
Al	0.21 ± 0.38	0.88 ± 2.43	0.16 ± 0.57
Na	0.96 ± 1.23	0.96 ± 1.23	0.12 ± 0.08
Cu	0.21 ± 0.14	0.55 ± 0.62	0.22 ± 0.22
Pb	0.21 ± 0.07	0.55 ± 0.62	0.29 ± 0.10
Cl	0.21 ± 0.39	0.72 ± 1.11	0.09 ± 0.20
Si	0.21 ± 0.46	0.12 ± 0.27	0.18 ± 0.15
K	0.12 ± 0.12	0.07 ± 0.06	0.12 ± 0.16
Pd	0.14	0.08	0.31
P	0.17 ± 0.22	0.09 ± 0.13	0.07 ± 0.07
Mg	0.10 ± 0.10	0.30 ± 0.35	0.06 ± 0.04
I	0.08 ± 0.04	0.11 ± 0.03	0.19 ± 0.10
Cd	0.10 ± 0.03	-	-

4. DISCUSSION

The sea turtle eggshell is separated into layers: outer cuticle layer, middle layer, and inner fibrous layer (Ewert, 1985; Kitimasak et al., 2003; Al-Bahry et al., 2009; Nuamsukon et al., 2009; Sikiwat et al., 2015). The thickness of the eggshell can be used to classify animal species (Osborne & Thompson, 2005; Winkler, 2006). In the present study, the species of turtle can be identified on the basis of the thickness of their eggshells. The total thickness of the green turtle eggshell was higher than those of the hawksbill sea turtle and the leatherback. Morphological study of the eggshells of the green turtle, the hawksbill sea turtle, and the leatherback turtle by using SEM revealed that the eggshells consist of three layers: outer cuticle, middle, and inner layers. This result was supported by the work of Al-Bahry et al. (2009), who reported that the green turtle eggshell has three layers under SEM observation. The outer layer has groups of spiked crystals and formed a circular group that originated at the top of the middle layer.

Different species of turtles can be identified from the different shapes and patterns of eggshells. Green turtle and leatherback turtle eggshells had spiked circular crystalized shapes. However, the hawksbill sea turtle eggshell had dense circular shapes in between the spikes. The crystal on the outer shell of the leatherback turtle had a smaller size and was thinner than that on the outer shell of the green turtle. It also had a different diameter and height of each crystal. The eggshell of the green turtle formed a group of spikes with a similar height and the same alignment. The base of the crystal was connected to the middle layer and formed a circular shape. Holes connected to the middle layer of the eggshell. Single or multiple holes can also be found in all three eggshells. The middle layer was densely fibrous and gaps were present between each fiber, which formed a reticular gap similar to the pore of sponge. These holes could exchange gases with the external environment.

The inner layer eggshell has a reticular fibrous structure, which is denser than the middle layer (Al-Bahry et al., 2009; Carpenter, 1999; Osborne & Thompson, 2005). In the proper condition, $CaCO_3$ can crystallize into aragonite form in the middle layer as the origin. The outer cuticle layer is induced by $MgCO_3$ and water to form spiked crystallized structures. (Baird & Solomon, 1979; Packard & Packard, 1988; Solomon & Baird, 1976).

Considering that we obtained these eggs from the nature, we found that the crystallization can only be aragonite, which also matches with the research of Baird and Solomon (1979). They studied the eggshells of green turtles that were collected from nature and from farms by using SEM. Eggshells found in nature can only crystallize into aragonite, but eggshells from farms have a crystallized calcite structure. However, the difference in crystallization is because of the different amounts of nutrition that the turtles received. Under normal conditions, turtles should receive an appropriate amount of nutrition and Ca. Deficiency in Ca and nutrition can affect eggshell construction and can change the eggshell structure of turtles (Packard & Packard, 1988; Romanoff & Romanoff, 1949). In addition, the calcite or aragonite crystallization may be changed because of the level of acid/base and the changes in ions, such as PO_4^{3-} and Mg^{2+}. These ions can cause calcite crystallization in eggshell construction (Solomon & Baird, 1976).

The results revealed the presence of several trace elements in the turtle eggshells: O, C, Ca, S, Na, Cl, Mg, and Si (Sahoo et al., 199) (Al-Bahry et al., 2009; Kitimasak et al., 2003; Osborne & Thompson, 2005; Tryfonas et al., 2006). Metal and non-metal elements such as Pb, Mo, Al, Br, and I, are also found (Alava et al., 2006; Lam et al., 2006; Osborne & Thompson, 2005; Sakai, Ichihashi, Suganuma, & Tatsukawa, 1995; Tryfonas et al., 2006). However, the contamination is relatively small compared with other components.

C, O, and Ca are the main components, which is supported by Al-Bahry et al. (2009). In their research, they analyzed the components of the green turtles' eggshell with energy dispersive X-ray technique. They found that the eggshell consists of C, O, and Ca. However, some other components are found in small amounts, such as Si, Cl, Al, K, P, Mg, and I.

Ca is an important component for the development of the embryo, which is mostly found in the eggshell. It is also supported by the work of Bustard and Greenham (1968). They claimed that digestion occurs because CO_2 from the embryo is released. CO_2 reacts with water and becomes a carbonic acid. The structure of $CaCO_3$ in the outer layer of the shell changes to Ca $(HCO_3)_2$, a form that can be easily absorbed.

The amount of Pb, Pd, and Mo was about 0.10% - 0.56% in the three types of eggshells. Cu was found in the eggshells of green turtles and leatherback turtles. Cd, Br, and I were found in the eggshells of hawksbill sea turtles. The heavy metals might occur during shell construction because of the environment of its habitat. For example, the sea might be contaminated by these heavy metals, which then infiltrate the turtle's body. The metals may attach with the ions residing in the body. During egg formation, these ions and metals might be used in the process.

From the empirical study, Pb, Pd, Cu, and Cd bond well with Ca. Thus, the contamination can be detected in the study by Guirlet et al. (2008). They also studied the concentration levels of non-toxic metals (Cu, Zn, and Se) and toxic metals (Cd, Pb, and Hg) in the blood and eggs of leatherback turtles during nesting season. They found a relationship of the level of this substance within the bloodstream and eggs. Therefore, the blood from the maternal can be transferred to the eggs. Selenium plays an important role in utilizing protein in embryo development. During the nesting season, the level of Cu in the turtles bloodstream decreases while the level of Pb increases because Ca is used in egg formation, which simultaneously occurs with Pb decomposition. Thus, it increases the levels of Pb in the bloodstream during the nesting season. However, the concentration of the toxic metal is less than that of the non-toxic metal. The contamination may affect the development of the embryo and change the levels of thyroid hormones thyroxine (T4) and triiodothyronine (T3), leading to the death of the embryo. The heavy metals can enter into embryo by active transport (Brasfield et. Al., 2004; Van Meter 2006). The results indicate that the eggs of these turtles can be used to discover contaminants in the sea. This work showed the merit of the secondary electron detector and energy dispersive X-ray detector in SEM. It is a powerful and suitable method to analyze the percentage of trace element accumulation and transition metal contamination.

5. CONCLUSION

Three sea turtle species; green turtle (*Chelonia mydas*), hawksbill sea turtle (*Eretmochelys imbricata*) and leatherback turtle (*Dermochelys coriacea*) were studied on morphology and elemental components. Sea turtle eggshell was formed with 3 layers, which were outer cuticle layer, middle layer, and inner fibrous layer. The morphology of the outer cuticle layer is thick, porous, and composed of aragonite to form $CaCO_3$. The middle layer is compact and thick, and the inner layer is compact and thin. Results of elemental analysis in three sea turtle types showed that C, O, and Ca were found at high percentages to form $CaCO_3$, which accumulated in the outer cuticle layer. Contamination of metals and non-metals, such as Mo, Br, Cu, Pb, and Pd, was found in the three types of sea turtle eggshells. The results from this study would be the fundamental data for further works to manage sea turtle conservation.

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