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Bio-mineralization in Skeletons of Some Elasmobranches From Susah and Dernah Coasts, Libya

(Estimation Study Using Nano-Technique)

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التمعدن الحيوي في هياكل بعض الاسماك الغضروفية من سواحل سوسة ودرنة، ليبيا

(دراسة تقديرية باستخدام تقنية النانو)

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Abstract

Skeletons of five elasmobranches specimens: [*Galeorhinus galeus*, *Squalus acanthias*, *Rhinobatus rhinobatus*, *Raja clavata*, and *Taeniura grabata*] were collected from Susah and Dernah coasts between March and April 2019, and scanned by XRF with Bruker S2 Ranger 2008, to study the bio-mineralization in their skeletons. Calcium carbonates $CaCO_3$ ranged from 46.53% in Tope Shark to 50.69% in Dogfish. *G. galeus* peaked at its CaO (71.11%), compared to the nadir value of *T. grabata* (69.03%), the same species had a significant value of SO_3 (16.2%), which dropped to 9.21% in *R. clavata*, which had the same number as the zenith of Phosphates (P_2O_5), that was lower than 8% in *G. galeus*, as lower as Silicates (SiO_2) (7.80%). Also, *R. clavata* had maximized values of Magnesium, Aluminum, Iron, and Manganese oxide in this work till 0.32, 0.26, 0.42, and 0.89%, respectively. Tope sharks had the lowest sum of minerals 37.39%, insignificantly as much as the sum of the mineral in Dogfish's skeleton (39.95%). Also, *S. acanthias* recorded a significant Ca/P ratio (9.53:1.00), compare to *R. clavata* (7.70:1.00). These analyses are useful to understand and following the physiology of bio-mineralization and bio-accumulation of minerals in marine organisms in Libyan coast.

Keywords: *Elasmobranches, Galeorhinus galeus, Squalus acanthias, Rhinobatus rhinobatus, Raja clavata, Taeniura grabata, skeleton, Biomineralization, XRF technique, Susah & Dernah coasts, Libya*.

الملخص

في الفترة من مارس إلى أبريل 2019، من ميناءي سوسة ودرنة، تم إحضار عينات خمسة أنواع من الأسماك الغضروفية: المتسولا *Galeorhinus galeus*، كلب بوشوكة *Squalus acanthias*، محراث *Rhinobatus rhinobatus*، حرشاية داكنة *Raja clavata* والبقرة المدورة *Taeniura grabata* لدراسة التمعدن الحيوي لهياكلها الغضروفية بواسطة تقنية ال XRF على جهاز الـ Bruker S2 Ranger 2008، وقد تراوحت كربونات الكالسيوم $CaCO_3$ من 46.53٪ في المتسولا إلى 50.69٪ في البوشوكة. وقد بلغ CaO لدى ال *G. galeus* أعلى قيمة (71.11٪) مقارنة مع قيمة دنيا لدى ال *T. grabata* (69.03٪) والذي زادت فيه SO_3 (16.2٪)، في حين انخفضت إلى 9.21٪ في *R. clavata*، وبنفس الرقم كانت أعلى نسبة فوسفات (P_2O_5)، والتي كانت أقل من 8٪ في *G. galeus* التي حملت أقل سيليكات (SiO_2) (7.80٪). أيضا، كانت الحرشاية

عالية في نسب أكاسيد المغنيسيوم والألومنيوم والحديد والمنجنيز في هذا العمل حتى 0.32 و0.26 و0.42 و0.89٪ على التوالي. كانت المتسولا صاحبة أقل كمية معادن في هياكلها حتى 37.39٪ بقدر ضئيل عن مجموع معادن هيكل البوشوكة (39.95٪). أيضًا، سجلت الـ *S. acanthias* نسبة Ca/P مرتفعة (9.53: 1.00)، مقارنة بـ *R. clavata* (7.70: 1.00). هذه التحليلات مفيدة لفهم ومتابعة فسيولوجية التمعدن حيوي والتراكم الحيوي للمعادن في الكائنات البحرية في الساحل الليبي.

الكلمات الدالة: الغضروفيات، المتسولا، كلب بوشوكة، محراث، حرشاية داكنة، البقرة المدورة، الهيكل العظمي، التمعدن الحيوي، تقنية الاشعة المتفلورة، سواحل سوسة و درنة، ليبيا.

1. Introduction

Bio-mineralization is a process to produce minerals by living organisms, often to harden or stiffen existing tissues, as a "mineralized tissue" (Skinner & Jahren, 2004; Harris & Edward, 2012; Vert *et al.*, 2012; and Vengadesan & Kumar, 2016). This phenomenon widespread extremely; more than sixty different minerals are able to be formed in members of most organisms (Weiner & Lowenstam,1989; Sigel *et al.*, 2008; and Cuif *et al.*, 2011).

The majority of bio-minerals are divided into three major sections: carbonates, silicates and phosphates (Knoll, 2004). Neues *et al.* (2011), and Pokroy, (2015) mentioned calcium as major minerals in organisms, usually present as crystalline of calcite, calcium phosphates and carbonates in vertebrates as skeletons, although metastable vaterite and amorphous calcium carbonate can also be important, either structurally, or as intermediate phases in bio-mineralization (Jacob *et al.*, 2017; and Mass *et al.*, 2017). Phosphorus as well, as another major bio-mineral in skeleton, is distributed as hydroxyapatite, the most widely calcium phosphate ($Ca_{10}(PO_4)_6(OH)_2$), which is a primary constituent of bone, teeth, and fish scales (Onozato, 1979).

Porter *et al.* (2006) indicated that vertebral column in fishes is relatively dense, with calcified tissue in terms of material stiffness and strength to be increased with bio-mineralization for faster swimming, to indicate that specific morphological peculiarities of skeletal structure may respond for evolution, during the developmental stages (Kubo & Asano, 1987; Desse *et al.*, 1989), and because of the stiff anatomical and functional relationships with the axial musculature (Le Danois, 1958; Lindsey, 1978; Vronskii & Nikolaitchouck, 1989).

Stiffness is related positively with: **(i)** function; in varying locomotion degrees (Macesic and Summers, 2012), **(ii)** cross-sectional shape of cartilage; in appendicular and axial skeletal elements, and **(iii)** the loading regime; which is functionally similar to adapting of bones with loading regime, then it remains to determine how cartilage is adapting to load, and whether the response is dynamic, such as bone modeling (Goldstein, 1987).

Structures of these bio-composite materials are controlled highly between nanometer and macroscopic level, to result a complex architecture, with multifunctional properties (Vinn, 2013). Range of control over minerals is desirable for materials engineering applications, by understanding and elucidating the mechanisms of biologically-controlled bio-mineralization (Boskey, 1998; and Sarikaya, 1999).

A range of diversity of sharks, skates, rays and stingrays is varied in the Mediterranean (Bradai *et al.*, 2012; Buzaid & El-Mor, 2015). However, most of their fisheries are not recorded by a database to indicate the important economic species in the Mediterranean, including the Libyan eastern region, where contain unique breeding grounds for species such as the Tope Sharks *Galeorhinus galeus* (Linnaeus, 1758), Round fantail stingray *Taeniura grabata* (E. Geoffroy Saint-Hilaire, 1817), Common guitarfishes *Rhinobatos rhinobatos* (Linnaeus, 1758), *Squalus acanthias* (Linnaeus, 1758) (Spiny dogfish) and the Thornback ray *Raja clavata* (Linnaeus, 1758) as well.

Galeorhinus galeus are large hound shark sized nearly 200cm (Compagno, 1984; Al-Huni and Al-Kabier, 1991; and Ben-Abdalla *et al.*, 2012). Active and strong swimmers, founded near shallow bays (Al-Huni and Al-Kabier, 1991; Serena, 2005; Iglésias, 2006; and Golani *et al.*, 2006), between 0-1100 m depth (Cox and Francis, 1997). Their recorded migrations reach 16,000 km in Atlantic, extending up to 1,610 km from the coast (Camhi *et al.*, 1998), and in the Mediterranean (Dehaas and Knorr, 1979; Serena, 2005; Iglésias, 2006; Walker *et al.*, 2006; and Buzaid, 2017).

About *Taeniura grabata*, tail is no longer than the disk length and bears one or more stinging spines on the upper surface (McEachran and Capape, 1989), these spines sized to 50 mm long in males and 66 mm in females, and have a central groove and 29–45 lateral serrations (Dehaas and Knorr, 1979). Sharp serrated spine on tail grows in front of primary spine and causes painful wounds (Golani *et al.*, 2006; and Summit, 2012). Distributed from the Southern Iberian Peninsula to Angola and southern Mediterranean (Whitehead et al. 1984; Basusta et al. 1998; Serena 2005 and Froese and Pauly, 2009). These neritic species (Reiner, 1996), often found solitary (Summit, 2012).

McEachran and Capapé (1984) recognized *Rhinobatos rhinobatos* by their rostral ridges widely separated; anterior nasal lobe reaching to inner corner of nostril, distance between nostral ridges equals to eye diameter (Golani *et al.*, 2006), growing till 40–150 cm (Bauchot, 1987; and Golani *et al.*, 2006). Distributed among the Eastern Atlantic and the Mediterranean coasts (Bauchot, 1987). Absent in Black sea (Serena, 2005). Living in the sandy, muddy beds, and rocky reefs till about 100 m (Michael, 1993). Swimming over seafloor or partially buried (Last *et al.*, 2016). According to Golani *et al.* (2006) using their snout to dig the substrate to expose preys of benthic invertebrates.

Al-Huni and Alkabier (1995), Golani *et al.* (2006), and White *et al.* (2007) identified *Squalus acanthias* distinctively this moderately-sized species by a short spine in front of short first dorsal fin. According to Riede (2004) cosmopolitan schools of this oceano-dromous species were observed in the Mediterranean and Black Sea, southern Greenland, Iceland and Murmansk coasts south to Madeira, Morocco and Canary Islands, western Atlantic and North Pacific (Serena, 2005).

Capapé & Desoutter (1981) described *Raja clavata* with rub-rhomboid shape, pointed wing-tips; and bluntly pointed snout, with light and dark crossbars, upper surface always wholly prickly in juveniles, adults with large thorns (Serena, 2005). Inhabit the muddy, sandy and gravel shelfs in 10-300 m depth (Wheeler & Stebbing, 1978; Stehmann, 1990; Brito, 1991;

Walker *et al*., 1997; Muus & Nielsen, 1999; and Golani *et al*., 2006). Serena (2005) mentioned their distribution in the Mediterranean and west of the Black Sea, from Iceland and Norway to Madeira and Morocco, extending to South Africa, Southwestern Indian Ocean and Madagascar (Golani *et al*., 2006).

The Calcified Cartilage system in elasmobranches plays a very important mechanical role in fish movement (Learm, 1976; Lindsey, 1978; and Weihs, 1989). Average coefficients of variation within parts were relatively homogenous for them (Carrier *et al*., 2004). Enult *et al*. (2016) indicated that the skeleton of Chondrichthyes is notoriously difficult to study. Literature of this subject is extremely rare comparing to other vertebrates.

Raoult *et al*. (2016) mentioned that the physiological growth deposition makes symmetrical patterns are expected in the vertebrae bindings, especially the distribution of crystalline phosphate and carbonate salts distribution were asymmetrical with calcium. Therefore, studying bio-mineralization is useful to clarify banding within vertebrae, to determine stiffness, locomotion and ages in vertebrates, including cartilaginous fishes (Summers, 2000).

Various aspects of bio-mineralization in fish have not been established to a satisfactory degree. Therefore, there is a significant interest in understanding and elucidating different aspects of bio-mineralization (Boskey, 1998; and Sarikaya, 1999).

The importance of this work is studying skeletal specimens in research collections, using a few ways to study, meaning nano-technique of X-Ray fluorescence (XRF), the particularly well-suited application for analyzing of Major oxides (Ali, 2016), in skeletons of five elasmobranches from Susah and Dernah coasts; and to estimate a first steps to create a cell of their biological database in the Libyan eastern coast to prelude for the regulation the fishing of cartilaginous fishes in the future.

2. Materials and Methods

2.1. Locations

Shown in Figure (1.A-B) (Reynolds *et al*., 1995; MBRC, 2005; and Abu-Madinah, 2008):

2.1.1. Susah Harbour **(31° 54' 18''N, 21° 58' 00''E):**

About 25 km north of Albayda. Offshore harbor, with poor shelter by old jetty, permanent landing site.

2.1.2. Dernah Harbour **(32° 16' 00''N, 22° 39' 12''E):**

Located at a narrow coastal plain, where the mountain edge provides protection from the southern winds.

2.2. Species Identification:

Samples had been identified according to some reference collection (Whitehead *et al*., 1984; Serena, 2005; Golani, 2006; Iglésias, 2006; Abdallah, 2007; Corke, 2012; and Ben-Abdalla *et al*., 2012), in Marine Sciences Department, Faculty of Science, Omar Al-Mukhtar University, Albayda, Libya.

2.3. Skeletons Preparation

Following to Parker (1981); Hildebrand (1968), and Enault *et al*. (2016), specimens of study had been measured, gutted and roughly fleshed and skinned. Most of the perichondrium had been removed as well, then transferred into 1:1 solution of [50% ethanol and Hydrogen peroxide (concentration 30%)] for 72 hrs. (Figure 1.F-H).

2.4. X-Ray Fluorescence Scanning (XRF) Analyze

According to (La-Tour, 1989; Vengadesan & Kumar, 2016; and Ali, 2016) skeletons were dried on 85ºC (48 hrs.), grounded to homogenous-textured powders, then compressed into steel rings to put in the XRF scanner (S2 Ranger 2008-Bruker Company), in chemical lab, technician sector, the Libyan Co-operative Company (LCC) in Dernah, Libya (Figure 1.J-K)

2.5. Statically Analyze

MS Excel 2010 was a method to elaborate results of XRF analysis.

Figure 1. (A-B) Harbor of Susa and Dernah (Google earth, 2017), **(F-H)** preparing skeletal samples, **(I)** drying and **(J-K)** preparing for **(L)** XRF scanning with Ranger S2 2008.

3. Results & Discussion

Table (1) and Figure (2) showed that *G. galeus* peaked its CaO (71.11%), compare to the nadir value of *T. grabata* (69.03%), the same species had a significant value of SO_3 (16.2%), which dropped into 9.21% in *R. clavata*, which had the same number as zenith of Phosphates (P_2O_5), that was lower than 8% in *G. galeus*, as lower as Silicates (SiO_2) (7.80%). Also, *R. clavate* had maximized values of Magnesium, Aluminum, Iron, and Manganese oxide in this work till 0.32, 0.26, 0.42, and 0.89% respectively.

Minerals of Tope shark (37.39%) significantly lower than in Dogfish's skeleton (39.95%), these values were lower than that recorded by Porter *et al*. (2006). According to Macesic and Summers (2012) dry skeletal weight was estimated to be 39% mineral and 61% other material, based on composition data from several species of batoid fish (rays, skates, and relatives). Using these data, a value of 2.34×10^2 kg of mineral per kg of shark (i.e. 39% mineral×skeletal mass) was estimated.

The essential presence of minerals, it could be striking difference between elasmobranch's cartilage and others. In shark's vertebral cartilage, the mineral content can be between 54% in trabecular bone, and 94% in compact bone (Currey, 2002). According to McCalden *et al*. (1997), Currey (1999), and Porter *et al*. (2006) mineral content is a great predictor of stiffness and strength in any organism's skeletons. Take an example, a 20% loss in mineral content corresponded to a 35% diminish in strength, and a 60% mineral loss means 75% weaker (Shah *et al*., 1995). Furthermore, Porter *et al*. (2006) suggested that the mineralized structures dominate the compressive properties of elasmobranch vertebrae.

Also, *S. acanthias* recorded the significant Ca/P ratio (9.53: 1.00), compare to *R. clavata* (7.70: 1.00) (Table 1). Urist (1961) reported a Ca/P ratio of 1.5 for elasmobranch skeletal mineral. Hypothetically, if the mineral is only calcium and phosphate (as HPO_4^{2-}), the ratio and molecular weight of Ca^{2+} and HPO_4^{2-} were used to estimate the Ca mass in mineral of elasmobranch's skeleton (0.385 g Ca/g mineral). Estimating of 2.34×10^2 kg of mineral/kg shark, this represents 9.01×10^3 kg of calcium ($0.385\times2.34\times10^{-2}$), and 1.44×10^2 kg of HPO_4^{2-} in skeletal mineral / kg of shark.

These results lend structural support, it is thought to exert control over this process, where calcium carbonate, calcium phosphate and hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] can be distinguished by other nano-techniques easily. Also, bio-mineralization is useful to recognize different protein content what they intake (Bonucci, 1992; Bahrololoom *et al*., 2009; and Vengadesan & Kumar, 2016).

Differences in bio-mineralization in elasmobranches' vertebra should have implications for the spine's ability to resist deformation from the loads imposed by swimming. Vertebral centra consist of a double cone of mineral are varying, according for several factors (Ridewood, 1921). According to Porter *et al.* (2006) hydroxyapatite (calcium phosphate) is distributed in web-like patterns of varying density throughout the vertebral centra to make the mineralization 'areolar' in cartilage of elasmobranch (Moss, 1977). Per contra, the 'tesselated' part of skeleton consists of mineralized tesserae in tiny blocks, occurring in multiple layers, on the surface of a hyaline cartilage skeletal element mainly (Dean and Summers, 2006; and Porter *et al.*, 2006).

Physiologically, calcitonin is produced in the glandular ultimobranchial bodies of fishes (Copp *et al.*, 1967). However, Louw *et al*. (1969) suggested that the role of this hormone in fish remained unknown. Pang and Pickford, (1967) were unable to elicit hypocalcemia in bony fish using thyrocalcitonin. According to Milhaud *et al.* (1977), dissociation of the two responses by propranolol suggests that they result from a direct effect on gill epithelium rather than from a hemodynamic effect. Phosphate influx is also increased by catecholamine, but the actual amounts are very small; because concentration of phosphate is low in seawater [about 1/1000 that of calcium]. Mainly, calcitonin in fish is regulating incoming calcium from seawater, which decreases calcium influx through the gill, affects the bone-blood equilibrium.

Eventually, this work would be: (a) motive for more studies of chondrichthyes in Libya, (b) beneficial to facilitating a physiological [especially regulating hormones for skeletal mineralization, for medical uses] and (c) first step to study migration, by studying chemistry of water of covering ranges, to admit these strategies for optimum use of their fisheries.

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Table 1. The results of minerals (using XRF analyzing) in five elasmobranches' skeletons from Susah and Dernah coasts, Libya, December 2018 till April 2019.

Item	Tope Shark		Dogfish		Violin-fish		Thornback Ray		Common Sting-Ray	
Total length (cm)	90.4		86.6		71.2		74.6		67.8	
Total weight (gm)	4058.45		3287.55		3100.11		3574.68		3620.23	
Skeleton weight (WM) (gm)	348.12		389.26		275.77		266.45		289.19	
Skeleton weight (DM) (gm)	130.16		155.51		105.75		100.11		110.29	
Major Oxides	**XRF**	**%**	**XRF**	**%**	**XRF**	**%**	**XRF**	**%**	**XRF**	**%**
CaO	26.07	69.72	28.40	71.11	27.50	71.71	27.00	72.20	26.33	69.03
P_2O_5	2.79	7.45	2.98	7.45	2.94	7.67	3.49	9.21	3.01	7.88
SO_3	5.26	14.07	4.90	12.26	5.11	13.33	3.49	9.21	6.15	16.12
SiO_2	2.92	7.80	3.28	8.20	2.50	6.51	2.97	7.85	2.37	6.22
MgO	0.12	0.32	0.090	0.22	0.05	0.13	0.016	0.04	0.02	0.04
Fe_2O_3	0.02	0.09	0.015	0.04	0.02	0.23	0.16	0.42	0.01	0.42
Cl	0.10	0.05	0.005	0.01	0.09	0.04	0.002	0.01	0.09	0.01
Al_2O_3	0.08	0.26	0.080	0.19	0.05	0.23	0.089	0.23	0.01	0.23
MnO	0.01	0.22	0.200	0.51	0.01	0.13	0.34	0.89	0.01	0.03
Na_2O	0.03	0.05	0.003	0.01	0.09	0.03	0.008	0.02	0.16	0.03
SUM	37.39	100.0	39.95	100	38.35	100.0	37.57	100.1	38.14	100.0
Ca / P	9.34 : 1.00		9.53 : 1.00		9.35 : 1.00		7.70 : 1.00		8.74 : 1.00	

* DM: Dry mass, WM: Wet mass

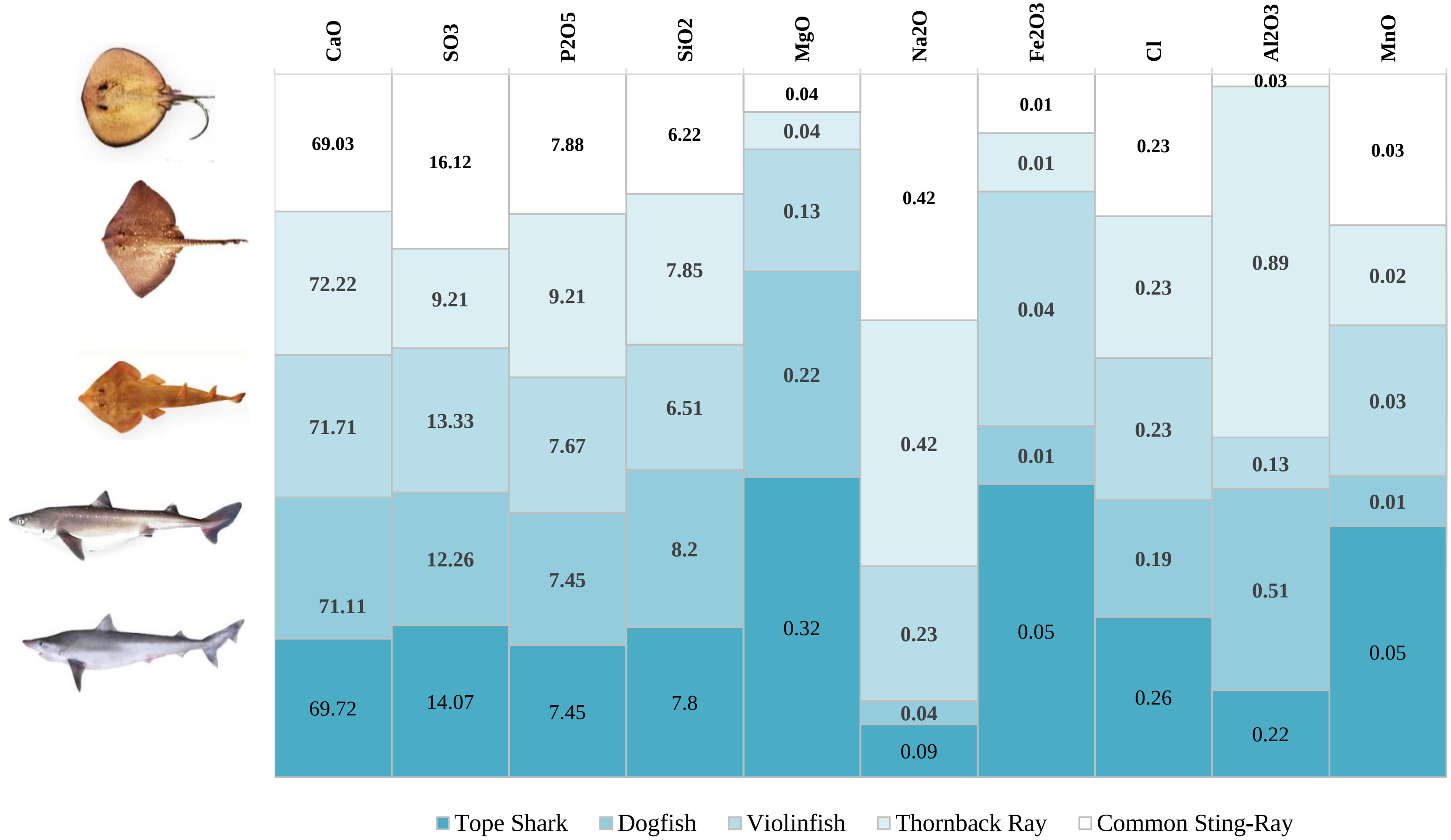

Figure 2. The mineral oxides (using XRF analyzing) in five elasmobranches' skeletons from Susah and Dernah coasts, Libya, December 2018 till April 2019.

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