ECOLOGICAL FACTORS AFFECTING THE WHALE SHARK OCCURRENCE IN DJIBOUTI AND PRESENCE OF CONTAMINANTS IN THE TROPHIC WEB

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Chapter II: Boldrocchi, G., Omar, Y. M., Rowat, D., & Bettinetti, R. (2018). First results on zooplankton community composition and contamination by some persistent organic pollutants in the Gulf of Tadjoura (Djibouti). Science of the Total Environment, 627, 812-821.

Chapter III: Boldrocchi, G., Omar, Y. M., Azzola, A., & Bettinetti, R. (2019). The ecology of the whale shark in Djibouti. Aquatic Ecology (under reviw).

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Chapter V: Boldrocchi, G., Monticelli, D., Butti, L., & Bettinetti, R. (2019). First concurrent assessment of elemental- and organic-contaminant loads in skin biopsies of whale sharks in Djibouti. Science of The Total Environment (under review).

Chapter VI: Boldrocchi, G., Monticelli, D., Omar, Y. M., & Bettinetti, R. (2019). Trace elements and POPs in two commercial shark species from Djibouti: Implications for human exposure. Science of The Total Environment, 669, 637-648.

CHAPTER I. GENERAL INTRODUCTION

1.1. INTRODUCTION

1.1.1. The whale shark

The whale shark (*Rhincodon typus* Smith, 1828) is a pelagic filter feeder, distributed in all oceans from tropical to temperate latitudes (Stevens, 2007). Although the species is the largest fish on earth, reaching up to 18.8 m total length (TL) (McClain et al., 2015), until recently, little was known on the main aspects of its biology and ecology (Rowat and Brooks, 2012). With the worldwide discovery of several near coastal aggregations and the increase in marine technology, the number of research on whale sharks rapidly increased (Colman, 1997; Rowat and Brooks, 2012; Cagua et al., 2015).

The whale shark is the only member of the family Rhincodontidae and belongs to the order Orectolobiformes, which includes 42 species, mostly benthic and bottom-feeders (Rowat and Brooks, 2012). Within the Orectolobiformes, the whale shark is the only pelagic species (Rowat and Brooks, 2012), that shares several morphological and anatomical similarities with other families within the order, including skeletal anatomy, tooth and dermal denticle morphology, fin placement and barbell morphology (Compagno, 1973; Rowat and Brooks, 2012; Robinson, 2016). This species is characterized by a broad flattened head, a very large terminal mouth, three conspicuous longitudinal ridges along its upper flanks, a large first dorsal fin, a semi-lunate caudal fin and a unique body marking (Fig. 1.1) (Compagno, 2001). Indeed, the pattern of light dots and stripes on a dark background serves as camouflage: the spots and stripes being disruptive coloration, while the lighter ventral surface is termed counter-shading (Wilson and Martin, 2003). The pattern bears evidence to its phyletic relationship with orectolobiform carpet sharks (Colman, 1997) and might serve as defense, especially during their early life stages to hide from predators (Rowat and Brooks, 2012). The spot pattern on the whale shark skin allows for the individual identification; for this purpose, the area posterior to the fifth gill slit is used for mark-recapture methods based on photo-identification (Arzoumanian et al., 2005; Speed et al., 2007). Indeed, with the photo-identification technique, individuals can be uniquely identified and their re-sighting in subsequent years can be used to determine population abundance estimates (Brooks et al., 2010). On a regional scale, comparisons between different sites can provide an indirect evaluation of small and large movements and potentially enable broad-scale population estimates (Brooks et al., 2010).

Fig. 1.1 A whale shark specimen from the Gulf of Tadjoura (Photo credit: Emilio Mancuso)

Despite an increasing interest in whale shark studies since the past 20 years, information about its reproduction, development and growth is still very limited (Colman, 1997; Rowat and Brooks, 2012). The species is an aplacental viviparous and the most fecund elasmobranch, with a single female found bearing 304 developing embryos (Joung et al., 1996). Indeed, the capture of this pregnant specimen in Taiwan in 1995 provided insights into reproduction of the species (Joung et al., 1996). A total of 237 embryos were sexed, comprising 123 females and 114 males, reflecting a 50:50 sex ratio at birth (Joung et al., 1996). Paternity analyses on 29 of those embryos suggested that the same male sired the entire litter and that a form of sperm storage was utilized (Schmidt et al., 2010). The largest neonates fell within a size range of 58-64 cm, suggesting that whale sharks are born at this length size. However, a 94 cm not a fully developed pup was reported from Gujarat coast (India) with an external yolk sac attached (Manojkumar 2003). Other four pups of 60-100 cm have been recorded from the same area (Premjothi et al., 2016). While, a free-swimming pup of 46 cm was found in the Philippines with a fully absorbed yolk sac and healed vitelline scar (Aca and Schmidt, 2011). These findings suggest that whale shark pups may vary greatly in length at birth (Rowat and Brooks, 2012).

Information about size at sexual maturity and longevity is scarce as well (Colman, 1997). Whale sharks probably reach the maturity at 8-9 m TL (Colman, 1997). Indeed, in South Africa, three stranded males of 9.03 m, 9.22 m and 9.45 m TL were reported as mature individuals, while other three of 8.66 m, 9.03 m and 9.10 m TL as immatures (Wintner, 2000). No mature sharks were identified from seven stranded females of 4.8-8.7 m TL in South Africa (Beckley et al., 1997), while at Ningaloo Reef, it has been estimated that 95% of male whale sharks were mature at 9.1 m TL, while 50% at 8.1 m TL (Norman and Stevens, 2007). Overall, these findings suggest that males mature at ≥9 m TL and females at the same or even larger size (Rowat and Brooks, 2012).

Whale sharks, along with megamouth shark *Megachasma pelagios* (Taylor, Compagno and Struhsaker, 1983) and the basking shark *Cetorhinus maximus* (Gunnerus, 1765) are filter feeders and prey on a variety of nektonic and planktonic organisms (Compagno 2001; Rohner et al., 2013). Whale sharks filter water through five sets of porous pads before passing through its gills and external gill slits (Motta et al., 2010). They possess spherical and moderately large olfactory capsules that provide responses to chemosensory cues (Martin, 2007; Dove, 2015). Indeed, when exposed to plumes of dimethyl sulfide, released by phytoplankton, and homogenized krill, whale sharks displayed foraging behavior responses by changing swimming speed, and visiting multiple times the patch of plume (Dove, 2015). Chemosensory cues might be used to navigate to high prey densities (Dove, 2015).

The distribution and abundance of this species appeared to be linked to oceanographic processes such as upwelling, coastal currents, and fronts, which increase the biological productivity, enhancing high concentrations of food (Wilson et al., 2001; Duffy, 2002; Cárdenas-Palomo et al., 2010; Sleeman et al., 2010; Sequeira et al., 2012; Hacohen-Domené et al., 2015). Marine species are often associated with specific physical or biological conditions, and there is a growing interest in understanding how such drivers shape the animal distribution and abundance patterns (Elith et al., 2011). Whale sharks require high-density food patches to sustain their energy demands, since they are consuming preys that are many order of magnitude smaller than their own body (Marcus et al., 2016; Rohner et al., 2017). To enhance cost-efficiency, whale sharks form aggregations in coastal habitats highly productive to feed on dense patches of zooplankton organisms (Colman, 1997; Rowat and Brooks 2012; Rohner et al., 2015; Marcus et al., 2016). Evidence from direct observations, faecal and stomach content analysis have identified a wide range of organisms as their target prey, which includes copepods, krill, crab larvae, sergestids, gelatinous zooplankton, chaetognaths, fish eggs and small fish (Clark and Nelson, 1997; Wilson et al., 2001; Heyman et al., 2002; Rowat 2007; Meekan et al., 2009; Rohner et al., 2015). Recent studies has indicated that whale sharks feed also on meso- and bathypelagic sources (Duffy, 2002; Borrell et al., 2011; Rohner et al., 2013; Marcus et al., 2016). However, despite such wide range of target organisms reported worldwide, many of these aggregations appear to target specific prey items. Nevertheless, a detailed understanding of the zooplankton community and its spatiotemporal distribution in term of biomass is scarce from most of these locations (Cárdenas-Palomo et al., 2015; Rohner et al., 2015). Information on feeding habits could significantly improve our ecological understanding of the underlying drivers of the whale shark movements and could improve strategies for sustainable management of the aggregation areas.

The whale shark is highly vulnerable to overexploitation due to its migratory nature, small population size and K-selected life history traits, such as slow growth rate, late sexual maturity, low fecundity and long life expectancy (Stevens et al., 2000; Rowat and Brooks, 2012; Dulvy et al., 2014). The species is listed as endangered by the International Union for Conservation of Nature (IUCN) Red List and included on Appendix II of the Convention of Migratory Species of Wild Animals (CMS) and Appendix II of the Convention of Trade in Endangered Animals since 2002 (CITES, 2002).

Indeed, the slow growth rate, late maturation and extended longevity make the whale shark particularly slow to recover from exploitation or habitat disruption, and recent evidences indicate that whale sharks are declining in number (Stevens et al., 2000; Rowat and Brooks, 2012; Dulvy et al., 2014). Historically, whale sharks were exploited by several countries, including India (Colman, 1997), Pakistan (Silas, 1986; Hanfee, 1997), Iran (Fowler, 2000), the Maldives (Anderson and Ahmed, 1993) the Philippines (Trono, 1996), and traditionally harvested for their meat and liver oil used to waterproof traditional boats (Rowat and Brooks, 2012). Today, national conservation measures have developed in several countries worldwide, including Australia, Belize, Djibouti, Honduras, India, Maldives, Mexico, Philippines, Seychelles, Taiwan, Thailand and U.S.A. (Rowat and Brooks, 2012), however the growing market for shark products, targeted fisheries, bycatch as well as vessel strikes and inappropriate tourism are still considered important threats for this species (Hsu et al., 2012; Capietto et al., 2014; Dulvy et al., 2014; Pierce and Norman, 2016).

1.1.2. Whale sharks in Djibouti

Within the Gulf of Aden, the Gulf of Tadjoura (Djibouti) has long been known as a place visited by whale sharks (Rowat et al., 2007), that aggregate on a regular basis from October to February (Rezzolla and Storai, 2010; Rowat et al., 2011). However, despite the importance of the site for this species, only few studies has been carried out (Rowat et al., 2007; Rezzolla and Storai, 2010; Rowat et al., 2011). Indeed, three short-term monitoring programs carried out in 2006, 2009 and 2010 and some monitoring carried out from 2003 to 2008, allowed to photo-identified 297 individuals (Rowat et al., 2007, 2011). The whale sharks identified in Djibouti were reported to be smaller than other coastal aggregations worldwide, with a mean length of 3.7 m (Rowat et al., 2007), and appeared to be mostly juvenile males (Rowat et al., 2007; Rowat et al., 2011). Qualitative and anecdotal observations reported that whale sharks in Djibouti were feeding on dense swarms of chaetognaths (Rowat et al., 2007). However, a detailed study on the whale shark ecology is still missing, leaving a knowledge gap on its feeding habits, distribution, habitat preference and environmental drivers for its occurrence in Djibouti. Furthermore, although Djibouti has adopted protection measures for the whale shark protection, and capture and/or trading is forbidden (Rowat and Brooks, 2012), major concerns comes from human activity nearby the foraging ground of this species in the Gulf of Tadjoura. Indeed, the increasing human pressure in Djibouti has given rise to organic and inorganic contamination from industrial activities, wastewater discharge, vessels, agriculture run-off and organic as well as solid waste (Ahmed et al., 2017). Despite the growing scientific attention on the ecotoxicology of large filter feeding animals, considered as an early warning of the presence of a mixture of contaminants in the marine food web (Fossi et al., 2014, 2017), nothing is known on the contamination status of the Djibouti marine ecosystem, including the status of contamination of the whale shark.

1.1.3. Elemental and organic contaminants

Contamination of marine environment has been linked to increasing lethal and sub-lethal effects to individuals, populations, and ecosystems (Todd et al., 2010; Ley-Quiñónez et al., 2013). Organochlorine compounds (OCs), such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT), as well as trace elements are still present in the marine environment since they are persistent, and because of their bioaccumulative nature and toxicity, they cause negative effects on the organisms health, including shark species (Storelli and Marcotrigiano, 2001; Storelli et al., 2005).

PCBs are organic chemicals, produced since the 1930s for industrial applications and composed by a biphenyl nucleus with 1-10 chlorine atoms, having a chemical formula of $C_{12}H_{10-n}Cl_n$ (Fig. 1.2) (Wiegel and Wu, 2000).

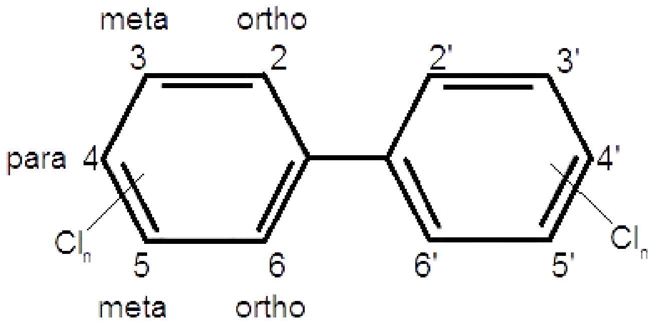

Fig. 1.2 Structural formula of PCB (Wiegel and Wu, 2000)

Their manufacture produced a complex mixture of multiple isomers with different degrees of chlorination yielding up to 209 products called congeners, marketed worldwide (UNEP, 1999). The widespread industrial application of PCBs was related to their excellent physical and chemical properties, such as chemical stability, dielectric properties, low inflammability, and miscibility with organic solvents (Borja et al., 2005). Therefore, PCBs were used extensively as heat transfer fluids, hydraulic lubricants, dielectric fluids for transformers and capacitors, plasticizers, wax extenders, adhesives, organic diluents, pesticide extenders, cutting oils, carbonless reproducing paper and flame retardants (Safe, 1992). Indeed, over 600 million kilograms of PCBs were used between 1930 and 1979 in the North America alone (Laukers, 1986).

DDT was first synthesized in 1874, but its insecticidal properties were discovered only in the 1940s (Smith, 1991), and since then, it has been used to control malaria and typhus. The low price contributed to its worldwide distribution and in the early 1960s, about 400,000 tons of DDT were used annually worldwide for agriculture purposes (IARC, 1974; Smith, 1991). Indeed, DDT was widespread used for the control of agricultural and forest pests (Turusov et al., 2002). DDT co-occurs with its principal metabolites, the dichlorodiphenyldichloroethylene (DDE) and the dichlorodiphenyldichloroethane (DDD). Once released in the environment, DDT undergoes to processes of chemical or microbial transformations that leads to the formation of DDE, when aerobic oxidative dehydrochlorination occurs, and DDD, in case of anaerobic reductive dechlorination (Fig.

1.3) (Ricking and Schwarzbauer, 2012). DDT and its principal metabolite DDE pervade the environment, including water, air and soil as well as the biota (Leber and Benya, 1994; Spear, 1999).

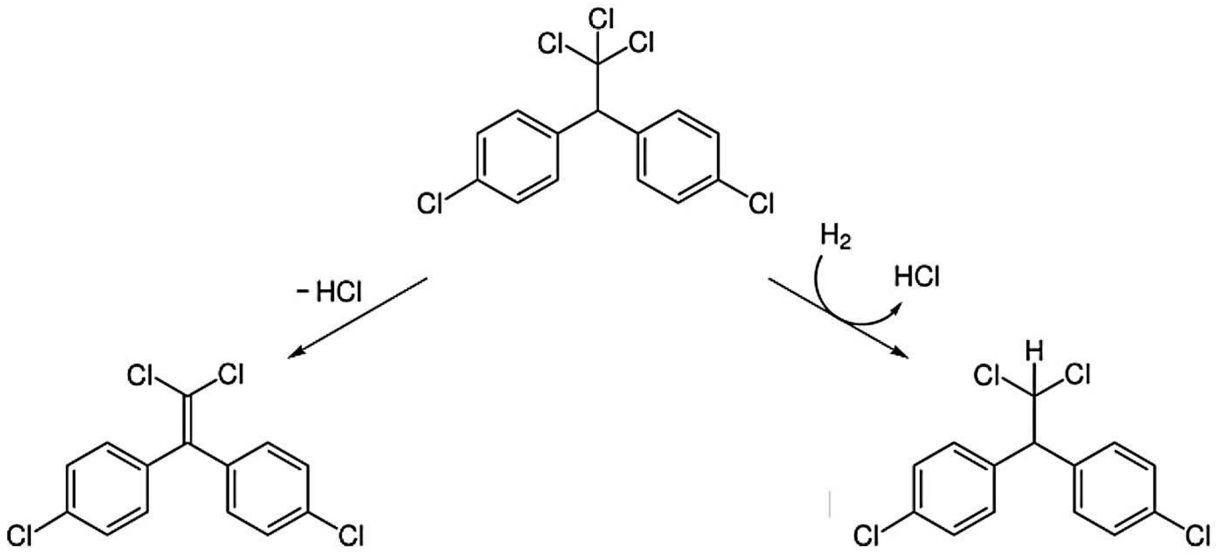

Fig. 1.3 Structure of DDT and its main metabolites, DDD and DDE (Ricking and Schwarzbauer, 2012)

Because of their persistent, semivolatile and bioaccumulative nature, long-range transport and high toxicity, Sweden was the first country to ban the use of both PCBs and DDT in 1970 (IARC, 1991). Concerned over the impact on the environment and their persistence, shortly thereafter, most of the production and uses of both OCs were banned in most countries (AMAP, 1998), and globally in 2001 under the Stockholm Convention on Persistent Organic Pollutants. Despite of bans, they are still found all over the world. The uncontrolled use of both PCBs and DDT coupled with improper disposal practices has resulted in their worldwide diffusion into the environment and, once released, they can travel long distances in the air and settles in areas far away from source of emission (ATSDR, 1993). Moreover, due to their resistance to degradation, they remain in soils and water bodies for several time, and due to their lipophilicity, they bioaccumulate in cells and then in the food chain (Safe, 1992; Borgå et al., 2011). Biomagnification is one of the cause of bioaccumulation, described as the progressive increase of the concentration of a substance from one trophic level to the next one, due to ingestion of food, resulting in higher concentrations in top predators compared with those found in their prey (Rasmussen et al., 1990; Borgå et al., 2004; Johnson-Restrepo et al., 2005).

Both PCBs and DDT are stored in all tissues, with the highest accumulation in lipid-rich tissues due to their lipophilic nature (Smith, 1991). Once in the body, they cause a variety of adverse health effects depending on the route of exposure, age, sex, and area of the body where they are concentrated (Borja, 2005). Nowadays they still represent a global problem and are among the most widely identified OCs found in extracts from almost all environmental samples. Indeed, today no living organism can be considered OCs-free (Turusov et al., 2002).

In contrast to organochlorine compounds, trace elements occur naturally in the environment, but their release can increase due to anthropogenic causes (Duruibe et al., 2007). Natural phenomena includes weathering and volcanic eruptions, however most contamination and human exposure result from a wide range of anthropogenic sources, such as mining and smelting, domestic and agricultural use of metals as well as in refineries, fuel and petroleum combustion, coal burning in power plants, nuclear power stations and high tension lines, plastics, textiles, microelectronics, wood preservation and paper processing plants (Duruibe et al., 2007; Jakimska et al., 2011).

The term ‘heavy metals’ is usually applied to a group of metals and metalloids that are toxic, and, therefore, are considered environmental contaminants (Duffus, 2002). They are also defined as trace elements because of their presence in trace concentrations (from ppb range to less than 10 ppm) (Kabata-Pendia, 2001). Trace elements can create adverse effects due capability of bioaccumulation in various biota compartments. Moreover, some of them, as mercury (Hg), can biomagnify along the food web and produce toxic effect to humans as well, through fish consumption (Escobar-Sánchez et al., 2011). However, not all trace elements are hazardous and toxic. Indeed, some are actually essential to maintain the body metabolism, such as cobalt (Co), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se) and zinc (Zn), and are required for various biochemical and physiological functions (WHO/FAO/IAEA, 1996). Nevertheless, some of them become toxic at higher concentrations, such as chromium (Cr) and copper (Cu) (Chang et al., 1996; Tchounwou et al., 2008). Metals such as Hg, lead (Pb), nickel (Ni), cadmium (Cd) and arsenic (As) are generally related to the environmental pollution and are toxic to living organisms at quite low concentrations (Bosch et al., 2016).

Trace elements enter into the marine ecosystems through various pathways including atmospheric deposition, transport by rivers, leaching from agricultural land, weathering of rocks and soils (Valavanidis and Vlachogianni, 2010). Some are available for organism uptake from solution as free ions, while others are transported over biological membranes as inorganic complexes. Once in the organism, some trace elements, such as Pb, Hg and Cd, show high affinity to sulfur and sulfhydryl groups of proteins that are ubiquitous in organisms, and binding to them, inhibit their correct functioning, altering metabolic processes (Corsolini et al., 2014). Therefore, bioaccumulation occurs preferentially in proteinaceous tissues contrarily to OCs, which are highly lipophilic and bioaccumulate in lipids. The bioaccumulation processes are species-dependent and related to the mechanisms of detoxification and metabolism of each organisms (Storelli et al., 2002). Moreover, the degree to which metals are bioaccumulated varies greatly depending both on the element involved and on the organ or tissue targeted. As consequence, different organisms from the same environment may show different levels of trace elements bioaccumulation (Ritterhoff and Zauke, 1997; Kahle and Zauke, 2003).

1.1.4. Elemental and organic contaminants in African marine ecosystems

While OCs have been banned or restricted in the developed countries since 1970s, several studies have found that pollution levels in Africa are increasing, instead of declining or remaining stable (Klanova et al., 2009; Ndiaye et al., 2012; Adu-Kumi et al., 2012; Ssebugere et al., 2013; Gioia et al., 2014). The most serious environmental problem the African countries face is an issue of stocks and reservoirs of obsolete and banned chemicals that in practice are still used without control (Klanova et al., 2009). Moreover, although one of the main aims of the Stockholm Convention on Persistent Organic Pollutants is to reduce and ultimately eliminate DDT, the Convention still allows the production and use of DDT for disease vector control, in accordance with WHO recommendations (van den Berg et al., 2017). Therefore, DDT continues to be used in some indoor residual spraying (IRS) programs in Africa countries (Ranson, 2019), including Mozambique, South Africa, Botswana, Gambia, Namibia, Swaziland and Zimbabwe (WHO, 2009, 2016; Chanda et al., 2015; Chihanga et al., 2016). It is worth to add that, although the reported progress across the globe in prohibiting or restricting DDT is encouraging, illegal trade in DDT, and actual or suspected use of DDT outside of the health sector have been mentioned in several countries (van den Berg et al., 2017).

Even trace elements levels have been recently increasing compared to their natural background conditions, exceeding international limits (Yabe et al., 2010). Indeed, the increasing industrialization, population, and urbanization have given rise to an increasing environmental pollution (Gioia et al., 2011). Open burning of metals and plastics, metals extraction in acid baths, ship wreckages, biomass burning and the exhaust from obsolete cars, industrial engines, unregulated and inadequate waste treatment and disposal, as well as a lack of pollution legislations are important contributors of the current situation (Lammel et al., 2013; Luzardo et al., 2014). Consequence of the growing land-based activities result in an increased load of contaminants that end up in the marine environment, affecting on the ecosystem and human health (Van der Oost et al., 2003; Klánová et al., 2011; Rumisha et al., 2012). For instance, a trace elements contamination has been already reported from the Red Sea and the surrounding areas (Kress et al., 1999; Ahmed et al., 2017).

Although the growing evidences of the massive release of pollutants in Africa, relatively few studies had focused on contamination levels in coastal environments that represent an essential source of proteins and income for millions of African people (Béné and Heck, 2005). Contaminants enter in the marine environment throughout direct disposal, urban and industrial waste, contaminated rivers inflow, agricultural run-off and deposition from air (Storelli and Storelli, 2005; Ahmed et al., 2017). Once in the ocean, they are readily absorbed by organic matter and incorporated by planktonic organims at the bottom of marine trophic chains (Elfes et al., 2010). Zooplankton not only serve as direct food source for whale sharks and other marine organisms, but it is also responsible for transferring pollutants through food webs (Bettinetti et al., 2010). Indeed, once assimilated by zooplankton, due to the biomagnification effect, contaminants accumulate in higher trophic level species. By occupying lower positions of the trophic web and responding promptly to pollution changes, zooplankton can be considered as an early warning of the presence of a mixture of contaminants in the marine habitat (Bettinetti et al., 2012).

Bioaccumulation of pollutants is cause for concern for aquatic organisms, particularly for K-selected life history predators, characterized by large size, slow growth rate, late maturation, production of few offspring and extended longevity (Elfes et al., 2010). Indeed, several studies have showed that sharks bioaccumulate and biomagnify elemental and organic contaminants in their body (Fisk et al., 2002; Newman et al., 2011; Delshad et al., 2012; McKinney et al., 2016). Chronic or intermittent exposure to OCs and trace elements results in severe effects on aquatic organisms at different levels (Thophon et al., 2003; Jepson et al., 2005; Ylitalo et al., 2005; Gelsleichter et al., 2006). Evidence of pathological effects on kidneys, liver and gills (Battaglini et al., 1993; Thophon et al., 2004), as well as impairment of reproduction and growth (Cross, 1988; Hose et al., 1989), and possible immune suppression (Lahvis et al., 1995; Jepson et al., 2005; Ylitalo et al., 2005; Gelsleichter et al., 2006) have been already described in several organisms. Given that sharks are particularly susceptible of biaccumulating significant levels of pollutants in their tissues, they result key indicators of the environmental contamination status (Marcovecchio et al., 1991; Vas, 1991). Nevertheless, despite an increasing number of research, primary information on the contaminant loads in most elasmobranch species as well as the possible impact of pollutants on their health are lacking and mainly focus on few areas of the world or tends to evaluate only a limited number of species (Mull et al., 2012; Tarassoli et al., 2012; Lee et al., 2015).

Within the Gulf of Aden, including Djibouti, elasmobranchs are an important component of small-scale fisheries and they are caught to be exported or sold for local consumption (Gladstone et al., 2003; Bonfil, 2003; Henderson et al., 2007; Alabsi and Komatsu, 2014; Spaet and Berumen, 2015; Jabado and Spaet, 2017). Indeed, in Djibouti, shark resources are heavily fished and an unknown amount of illegal shark fishing regularly occurs by unlicensed national and foreign boats for production of fins for the east-Asian shark-fin market (PERSGA/ALECSO, 2003). Even the endangered whale shark, which is protected in Djibouti (Rowat and Brooks, 2012), in near-by countries are sought out by fishermen (Li et al., 2012). Although pollution has not yet considered the main threat to shark survival, the negative effects on the organism health may worsen the already alarming situation (Camhi, 1998). Moreover, marine pollution is an issue of concern that extends beyond the marine food web: seafood consumption has been described as one of the major route of human exposure to a variety of elemental and organic contaminants (Llobet et al., 2003; Storelli et al., 2005), especially within coastal communities. Consumption of shark muscle tissue and shark products is believed to represent a major dietary source of trace elements for human consumers (Adams and McMichael, 1999; Pethybridge et al., 2010; FSANZ, 2011) and since the 1950s, the human demand for shark products has increased dramatically (Dulvy et al., 2014; Dent and Clarke, 2015).

Within the northern Indian Ocean, elasmobranch research has been very limited and little biological and ecological information exists on most species involved in fisheries (Henderson et al., 2007). Although several shark species are regularly fished in the Gulf of Aden, few of them form the bulk of these landings, which include the scalloped hammerhead shark *Sphyrna lewini* (Griffith & Smith, 1834) and the milk shark *Rhizoprionodon acutus* (Ruppell, 1837) (Bonfil, 2003; Henderson et al., 2007; Spaet and Berumen, 2015).

The scalloped hammerhead shark is a coastal-pelagic species distributed worldwide throughout tropical and temperate oceans and form large aggregations of adults around oceanic islands (Klimley, 1983, 1987; Compagno, 1984; Baum et al., 2009). Currently, *Sphyrna lewini* is listed as Endangered by the IUCN Red List (Baum et al., 2009), due to the reduction in population sizes over multiple areas, its high mortality in longline fisheries and low population rebound potential (Smith et al., 1998; Morgan and Burgess, 2007; Hayes et al., 2009). Scalloped hammerhead sharks feed on a mixture of fish and cephalopods, the proportions of which largely depend on prey availability (Klimley, 1983). This species reaches a maximum size of 370-420 cm, matures at around 140-212 cm and produces litters of up to 47 embryos, as reported for a pregnant female of 310 cm TL landed in Qusayar (Bonfil, 2003). In Djibouti, early juvenile scalloped hammerhead sharks inhabit the coastal areas and they are often landed, suggesting the existence of nursery areas in the proximity of Djibouti city (Bonfil, 2003).

The milk shark occurs primarily throughout the coastal regions of the Indian Ocean, Indo-Pacific and the western coast of Africa (Compagno, 1984). This species mainly feeds on fish, cephalopods and brachyuran crabs (Salini et al., 1990). The maximum size at maturity for males is at 68-70 cm and at 70-81 cm for females; the litter size ranges from 1 to 8 pups (Compagno, 1984; Henderson et al., 2006).

In contrast to the scalloped hammerhead shark, the milk shark has a generation time much shorter with rapid sexual maturity and a maximum lifespan of 8 years that provides resilience to exploitation (Simpfendorfer, 2003). *Rhizoprionodon acutus* is listed as Least Concern by the IUCN. However, due to the coastal distribution and accessibility in shallow bays and continental shelves of both species, the milk shark and the scalloped hammerhead shark comprise a large proportion of elasmobranch catches (Chen et al., 1988; Castro, 1993; Simpfendorfer, 2003). Furthermore, although all life-stages of this species can be found in great numbers in landings within the Gulf of Aden, the catches are heavily centered on juveniles and newborns, highlighting concerns of failure of recruitment to the breeding stock in the near future (Bonfil, 2003). Moreover, even if both sharks are commonly harvested for local consumption in Djibouti, little scientific information exists on the contamination load of these species in the area. High levels of contaminants in sharks sold for consumption pose a potential risk to human health (Holtcamp, 2012; Man et al., 2014; Lee et al., 2015).

1.2. DESCRIPTION OF THE STUDY AREA

1.2.1. Djibouti

The Republic of Djibouti (latitude 11°35'20.44"N, longitude 43°08'42.11"E) is located at the southern entrance to the Red Sea, at the border with Eritrea, Ethiopia and Somalia. The Djibouti coastline extends for approximately 370 km; a third of this faces the narrow straits of the Bab el Mandeb. The remainder of the coastline lies along the Gulf of Tadjoura between Obock in the north and the main port of Djibouti to the south (Fig. 1.4) (PERSGA/ALECSO, 2003).

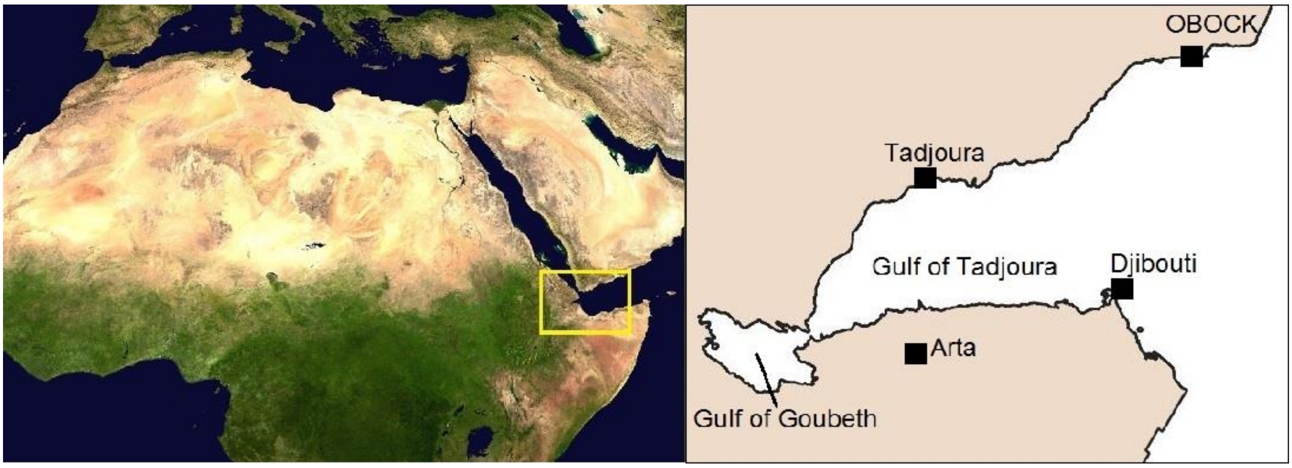

Fig. 1.4 Location of Djibouti within Africa (left image) and the Gulf of Tadjoura (right image)

1.2.2. The Gulf of Tadjoura

The study was carried out in the Gulf of Tadjoura, a westward prolongation of the Gulf of Aden Ridge, between Arabia and Somalia, within the East African Rift System, that transects Djibouti, Ethiopia and Kenya (PERSGA/GEF, 2003; Bosworth et al., 2005). This narrow semi-enclosed sea is surrounded by Djiboutian lands, except for the south-eastern part which extends into Somalia, and represents a unique marine ecosystem, due to its specific geomorphological characteristics that differentiate it from the Gulf of Aden (Bosworth et al., 2005, Omar et al., 2016).

The climate is mainly governed by the Asian monsoon regime; indeed the alternation of the north-eastern (NE) and south-western (SW) monsoon winds govern the sea surface circulation (Omar et al., 2016). The monsoon induces a biannual reversal of the current regime due to changes in the wind direction. During winter months, the north-eastern winds generate a northern anticlockwise gyre (Molinary et al., 1990), inducing surface water flow toward the Gulf of Tadjoura (Fig. 1.5). This system is replaced during summer through October by the SW monsoon, which creates a wide-scale clockwise current system that induces strong upwelling in the Arabian Sea (Brock et al., 1992; Rixen et al., 1996). Indeed, in July-August the SW monsoon drives surface water from the Gulf of Tadjoura to the Gulf of Aden and thus induces westward intrusion of the high salinity thermocline water (> 38 psu) from the Gulf of Aden (Fig. 1.5). Therefore, the thermocline water is injected into the gulf by advection in the northeastern part and then propagates toward the west. In the extreme west, upwelling brings the thermocline water to the surface where it mixes with the surface water (Fig. 1.5). The intrusion of high salinity water from the east is possibly due to the mixing with Red Sea water before entering into the Gulf of Tadjoura (Omar et al., 2016). The maximum CHL-*a* concentration recorded in the west part of the gulf, is consistent with the vertical supplying of nutrients from deep water and it is an indicator of biological activity enhanced by the upwelled nutrient rich waters (Omar et al., 2016). Overall, the SW monsoon induces eastward movement of surface water, enhances upwelling particularly at the extreme west and southeast part of the gulf.

In contrast, the deepening of the mixed layer and the thermocline in February, caused by NE monsoon winds, induces surface water flow toward the Gulf of Tadjoura (Fig. 1.5). By February, temperature in the gulf has decreased by about 5˚C (26˚C - 26.5˚C) and the salinity is greatly reduced (37.2 psu) (Omar et al., 2016).

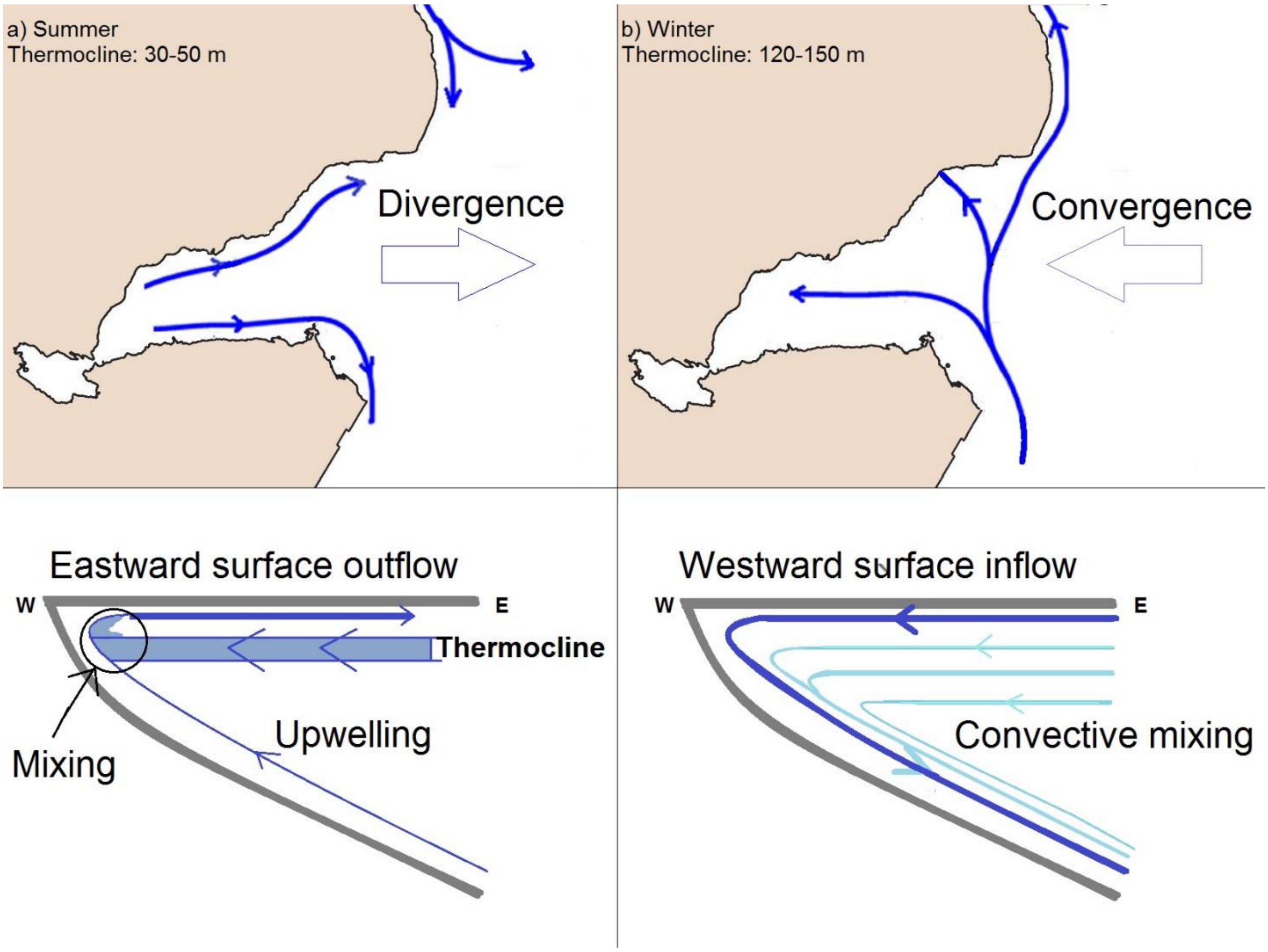

Fig. 1.5 Diagram shows surface and sub-surface circulation patters in the Gulf of Tadjoura during summer (left panels) and winter (right panels). Image modified from Omar et al. (2016)

From a biological point of view, Djibouti constitutes a unique marine ecosystem (Omar et al., 2016). It hosts coral reef formations, with an average coral cover of 52%, which at the upper end of the range reported for the Red Sea of 30-50% (PERSGA, 2010; Cowburn et al., 2019). The corals of Djibouti are a unique assemblage of species due to the confluence of several biogeographic zones including the tropical warm water biota of the Indian Ocean and Red Sea and species common to cold water upwelling habitats of the Somali and Arabian regions. Indeed, one hundred and sixty-seven species of corals have been recorded from Djibouti, including three species of black coral (PERSGA, 2006), and the highest diversity was recorded in the Gulf of Tadjoura at Arta Plage (93 species) (Fig. 1.4).

Coral cover is dominated by *Acropora hemprichii*, *Echinopora fruticulosa* and *Porites nodifera* (PERSGA, 2006).

Ocean productivity has been linked to high fish biomass (Williams et al., 2015; Samoilys et al., 2019) and the high nutrient levels in the Gulf of Aden may be contributing to the exceptionally high fish biomass found on the Djibouti reefs (Cowburn et al., 2019). However, these estimates are lower than those reported in the Red Sea (Sudan and Saudi Arabia), which includes top predators such as sharks and large Carangidae (Kattan et al., 2017), that are not recorded in Djibouti. Indeed, the illegal shark fishery supplying the oriental shark fin market has resulted in a decline in shark stocks. There is also a large bycatch of turtles, manta rays, dolphins, dugongs and finfish, and damage to reefs (PERSGA/ALECSO, 2003). Indeed, Djibouti, and in particular the Gulf of Tadjoura, suffers from many sources of impact including domestic tourism, sewage discharges, shipping and associated spills and pollution, with pressure particularly high around the capital city (PERSGA, 2004). Shipping is an important commercial sector in Djibouti, which is the major port for Ethiopia. Anchor, boating and tourism damage is increasing, with little increase in environmental awareness (PERSGA, 2010). The main fishing areas are in the north and south of the Gulf of Tadjoura. The northern area is most productive and least exploited because of a lack of cold storage, shore facilities and its distance from the city of Djibouti (PERSGA, 2006). Djibouti has developed several marine protected areas (MPAs), however there is no effective management (PERSGA/ALECSO, 2003), despite the fact that conservation of living marine resources are of prime importance for Djibouti (PERSGA/ALECSO, 2003). Indeed, from both social and economic perspectives, the Gulf of Tadjoura plays a crucial role as a local fishing area (Omar et al., 2016). Surprisingly, the oceanographic and biological data are scarce and the area remains poorly studied (Omar et al., 2016).

1.3. THESIS OBJECTIVES

Given the importance of Djibouti for whale sharks and the lack of studies carried out in its marine ecosystem, the main outcome from this study is to determine the environmental conditions that influence the presence and distribution of whale sharks. Successful conservation of a species of concern, as the endangered whale shark, requires a robust understanding of its spatiotemporal distribution in order to assess potential interactions with human activities. Indeed, although the whale shark is protected in Djibouti, human activities, including vessels passage, boat speed and tourisms are not regulated and no code of conduct is applied when approaching this species (Rowat et al., 2007). Therefore, this study aims to provide useful information for developing new management strategies for the protection of the whale shark in the Gulf of Tadjoura.

In addition, despite the massive release of pollutants in African marine environments and the deleterious effects on marine biota and humans, at present almost nothing is known about the contamination status in the Gulf of Aden, including Djibouti. The scarcity of studies on the bioaccumulation levels and the extent of contamination includes all trophic levels (Deribe et al., 2011). Therefore, with a bottom-up approach, starting from zooplankton and whale sharks to get to predatory shark species, this study aims to characterize the trophic chain from an ecotoxicology point of view. With global decline in shark numbers, evaluating the extent of contaminant exposure is an urgent aspect, since anthropogenic pollution may exasperate the current situation, especially for endangered species (Camhi et al., 1998). Furthermore, since elasmobranchs are a component of artisanal fishery in Djibouti, and seafood consumption is as an important source of contaminants exposure for humans, this study aims to estimate for the first time the exposure health risks derived from the consumption of shark species, including the whale shark.

Specifically, this study addresses the following aims:

Aim 1. To provide a detailed qualitative and quantitative description of the zooplankton community during the whale shark aggregation season and to evaluate for the first time the presence of organic pollutants (PCBs and DDT) in zooplankton samples.

This aim is achieved in **Chapter 2**. Indeed, **Chapter 2** provides a first assessment of the zooplankton composition and its spatiotemporal distribution in the Gulf of Tadjoura during the whale shark aggregation. Indeed, zooplankton is an essential food source for numerous species and its fluctuations in the spatiotemporal distribution might influence the biodiversity trends of various marine organisms (Nybakken, 1997), including the whale shark. Although the evident ecological role of zooplankton in functioning marine ecosystems, a detailed characterization of its community is still lacking and the temporal variation in zooplankton biomass in the Gulf of Tadjoura has not been yet well described, mainly due of the scarcity of adequate in situ data (Gittings et al., 2017). Moreover, zooplankton is an entry point for contaminants into the food web (Day, 1990; Chiuchiolo et al., 2004; Bettinetti et al., 2010; Bettinetti et al., 2012; Bettinetti and Manca, 2013). Therefore, **Chapter 2** provides also baseline information on the pollution status at the bottom of trophic web in the marine ecosystem of Djibouti.

Aim 2. To investigate the environmental conditions controlling the whale shark aggregation in the Gulf of Tadjoura.

This aim is fully described in **Chapter 3** that focuses on multiple aspects of the whale shark ecology in Djibouti. Indeed, this chapter evaluates the feeding ecology of the whale shark by quantitatively analyzing previous qualitative and anecdotal observations. Secondly, it fully describes how spatiotemporal distribution patterns of whale sharks are related to the variability of zooplankton conditions in Djibouti. Third, in **Chapter 3** a general picture of the human-shark interactions in the Gulf of Tadjoura is also provided. Finally, possible animal movements outside the aggregation period are investigated by analyzing the carbon and nitrogen signatures in whale shark skin tissues.

Aim 3. To investigate the feeding ecology outside the aggregation season. This is presented in **Chapter 4** that describes an alternative feeding strategy used by whale sharks when dense patches of zooplankton are not available in the Gulf of Tadjoura. Indeed, for the first time in this area, seven juvenile whale sharks were filmed feeding on a school of baitfish (anchovies).

Aim 4. To redress the knowledge gap on pollutant loads in the whale shark, and especially in Africa. In particular, in **Chapter 5** the presence of elemental and organic contaminants in skin tissues of twenty whale sharks are described for the first time, providing baseline information on the contamination status of this species in Djibouti. Furthermore, this study estimates the biomagnification factor of trace elements in whale sharks and the possible exposure health risks derived from the consumption of this species.

Aim 5. To redress the lack of scientific information on pollutant loads in predatory shark species from the Gulf of Tadjoura.

This is achieved in **Chapter 6** that focuses on two commonly targeted species: the scalloped hammerhead shark, *Sphyrna lewini* and the milk shark, *Rhizoprionodon acutus*. **Chapter 6** evaluates for the first time the presence of legacy contaminants (PCBs and DDT) and trace elements in these two commercial shark species. Since no studies have been carried out in Djibouti, and very few in the wider Gulf of Aden, this study provides baseline information to be used for comparison with the results described in Chapter 5. Furthermore, in this chapter, species-specific accumulation of these contaminants and the distribution of pollutants in different tissue types (fin, muscle and liver) are investigated in the milk shark and scalloped hammerhead shark, as well as potential health risks for humans resulted from the consumption of both species, by the target hazard quotient and the carcinogenic risk.

Chapter 7 is the final chapter of this document and consists of the main conclusions, impacts and conservation recommendations to be applied to future research and conservation strategies in Djibouti.

Each chapter of this thesis corresponds to a specific publication, in particular:

Chapter 2 was published in the Science of the Total Environment journal in 2018 and is titled: "*First results on zooplankton community composition and contamination by some persistent organic pollutants in the Gulf of Tadjoura (Djibouti)*" (Boldrocchi et al., 2018).

Chapter 3 was submitted to Aquatic Ecology journal on September 2019 and is titled "*The ecology of the whale shark in Djibouti*".

Chapter 4 was published in the Marine Biodiversity journal in 2019 and was titled "*Whale shark foraging on baitfish off Djibouti*" (Boldrocchi and Bettinetti, 2019).

Chapter 5 was submitted to the Science of the Total Environment journal in October 2019 and is titled: "*First concurrent assessment of elemental- and organic-contaminant loads in skin biopsies of whale sharks in Djibouti*".

Chapter 6 was published in the Science of the Total Environment journal in 2019 and is titled: "*Trace elements and POPs in two commercial shark species from Djibouti: Implications for human exposure*" (Boldrocchi et al., 2019).

CHAPTER II. First results on zooplankton community composition and contamination by some persistent organic pollutants in the Gulf of Tadjoura (Djibouti)

First results on zooplankton community composition and contamination by some persistent organic pollutants in the Gulf of Tadjoura (Djibouti)

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ABSTRACT

The Gulf of Tadjoura is located in the Horn of Africa and is widely recognized as an important site where the zooplanktivorous whale sharks seasonally aggregate from October to February. The surface zooplankton community (0-3 m) was weekly sampled from November 2016 to February 2017 in two sites during the whale shark aggregation period. A total of 12 phyla were identified. Copepoda represented the most abundant and diverse group with 29 different genera, and contributed with an average of 82% of the mean zooplankton density of approximately 6600 ind m^{-3}. During the sampling period, copepods were dominated numerically by Calanoida (3600 ind m^{-3}), followed by Poicilostomatatoida (1300 ind m^{-3}). Within the copepods, Paracalanidae, Calanidae, Oncaeidae and Miraciidae were the most common families. The temporal trend in zooplankton biomass at both stations revealed the highest peak in December (41.3 ± 36.4 mg m^{-3}), and the lowest in February (6.6 ± 3.3 mg m^{-3}). As no information are available on the occurrence of legacy contaminants use and release in this area, our analysis revealed the consistent presence of both DDTs and PCBs residues in zooplankton samples in the Gulf of Tadjoura. PCBs ranged from approximately 110 to 637 ng g^{-1} dw, while DDTs from 21 to 80 ng g^{-1} dw The proportion of primary DDT in the total residue was higher than DDE and DDD, which strongly suggests that the area might actually be subjected to DDT inputs of the parent compound.

2.1. INTRODUCTION

Zooplankton constitutes the largest ecological group of organisms in the sea and, even if regarded as a homogeneous constituent, it is composed by organisms which differ substantially from each other, not only in their taxonomy but also in body size, metabolic rates and ecological roles (Bettinetti and Manca, 2013; Goswami et al., 2000). Zooplankton plays an important role in regulating patterns and mechanisms through which matter and energy are transferred from the base to the upper levels of food webs (Bettinetti and Manca, 2013). They serve as a primary food source for fish, fish larvae, cephalopods and other zooplanktivorous organisms (Benli et al., 2001). Zooplankton, being an important trophic link between primary producers and higher trophic levels, represent a key route in transferring persistent contaminants entering the marine environment to the higher trophic levels since it accumulates pollutants both from water and from food (Day, 1990; Elfes et al., 2010).

Although the oceans cover around the 70% of the Earth’s surface, our knowledge of biodiversity patterns in marine phyto- and zooplankton is quite limited compared to that of the terrestrial world (Irigoien et al., 2004). Whole community studies of zooplankton are limited; this is especially true in “politically complicated” areas such as the Red Sea (Raitsos et al., 2013), some oceanic islands and many of the rim countries of the Indian Ocean, which have been poorly examined in depth (Conway, in preparation). The Gulf of Tadjoura, located in the Gulf of Aden, is another area whose information is still scarce. This semi-enclosed sea is almost completely surrounded by the Djibouti land masses, except for the south eastern portion which extends into Somalia and has specific geomorphological features that distinguish it from the Gulf of Aden (Bosworth et al., 2005, Youssouf et al., 2016). In the southeast, the continental shelf is wider and includes two islands (Mucha and Maskali) and many sandbanks are scattered throughout the entrance of the Gulf of Tadjoura. In the north, the Gulf of Tadjoura is crossed by a large tectonic rift extending westward toward the Ghoubet El Kharab Bay and the Assal Lake (Bosworth et al., 2005). The climate is mainly governed by the Asian monsoon regime; alternation of the north-eastern (NE) and south-western (SW) monsoon winds regulates surface circulation (Youssouf et al., 2016). The monsoon, driven by the atmospheric pressure difference between land and ocean, induces a biannual reversal of the current regime due to changes in monsoon wind directions. During winter months, between December and February, the NE winds generate a northern anticlockwise gyre (Molinary et al., 1990). This system is replaced during the boreal summer, between May-June through October, by the SW monsoon, which forms a large-scale clockwise current system causing strong upwelling in the Arabian Sea (Brocket al., 1992; Rixen et al., 1996). Considering these aspects, the Gulf of Tadjoura constitutes a unique marine ecosystem (Youssouf et al., 2016); however, despite this peculiarity, information on zooplankton community, species composition, and spatio-temporal changes in biomass and densities is extremely scarce. At present, only Rezzolla and Storai (2010) briefly reported a list of 21 taxa related to holoplankton and various components of meroplankton referred as Polychaeta larvae, Lamellibranch larvae, Decapod larvae, Echinoderm larvae, fish eggs and larvae and collected in the Gulf of Tadjoura during an ecotourism expedition in search of whale sharks, *Rhincodon typus* Smith 1828. Zooplankton plays, in fact, a crucial role as a direct food source for whale sharks, which are filter-feeders fish, listed by the IUCN as Endangered and added in the Appendix II of the Convention on International Trade in Endangered Species (CITES). To date, whale sharks aggregate on regular basis in the Gulf of Tadjoura from October to February (Rowat et al., 2007, 2011; Rezzolla and Storai, 2010) and it has been speculated that their occurrence could be related to the upwelling phenomenon caused by the Asian monsoon regime (Rezzolla and Storai, 2010). Phyto- and zooplankton provide an essential food source for numerous species and their fluctuations in the spatio-temporal distribution might influence the biodiversity trends of various marine organisms (Nybakken, 1997), including whale sharks. Despite the evident importance of zooplankton in the function of marine ecosystems, a detailed study of its community is still lacking and the seasonal succession of zooplankton biomass in the Gulf of Aden has not yet well established, mainly because of the lack of adequate in situ data (Gittings et al., 2017). A recent study has investigated the seasonal succession of phytoplankton biomass in the Gulf of Aden by analyzing 15 years of remotely-sensed chlorophyll-a data (Gittings et al., 2017). The study revealed a distinct phytoplankton growth during summer in the western part of the gulf, when the prevailing southwesterlies causes upwelling. During autumn, the prevailing northeasterlies enables upwelling along the southern coastline (Somalia) causing local nutrient enrichment in the euphotic zone, leading to higher levels of phytoplankton biomass along the coastline and in the lower central gulf (Gittings et al., 2017).

Giving the lack of information on the Djiboutian marine ecosystem, the aim of this study is to provide a first assessment of the zooplankton community in this area. Specifically, the objective of this paper is to present a detailed description of the zooplankton composition and the spatio-temporal variations in biomass and density in the Gulf of Tadjoura during the sampling period. It was speculated that zooplankton density and biomass would be consistent to the whale shark presence and might show a decreasing trend in proximity of the end of the aggregation period (February). Moreover, considering the paucity of studies carried out, not only in Djibouti, but also in the wider Gulf of Aden, and the importance of this area for the endangered whale shark, the second objective of this study is to evaluate for the first time the presence of some persistent organic pollutants (POPs) in zooplankton samples. Zooplankton, in fact, not only can represent a direct source of food for whale sharks, but it also plays an important role in transferring contaminants through the trophic chain (Bettinetti et al., 2010). Contaminants entering the marine environment are readily absorbed by organic matter and taken up and adsorbed by plankton at the base of marine food webs (Elfes et al., 2010). Several studies have showed that sharks bioaccumulate and biomagnify certain metals and metalloids in their tissues (Newman et al., 2011; Delshad et al., 2012; Mckinney et al., 2016) as well as organochlorine contaminants (Fisk et al., 2002). Bioaccumulation of pollutants is cause for concern, particularly for long-lived, top-level predators (Elfes et al., 2010). The whale shark has a k-selected life history that makes it vulnerable to exploitation such as large size, slow growth, late maturation, production of few offspring and extended longevity (Rowat and Brooks, 2012). This species is still taken in fisheries either as the result of targeted fisheries or as bycatch (Li et al., 2012). Whale sharks may be also particularly vulnerable to environmental contaminants since the increasing human activity in their aggregation sites gives rise to chemical pollution from urban wastewaters, vessels, agriculture and waste (Fossi et al., 2017). Even if banned since the ‘70s, large quantities of POPs have been released into the environment in the past and due to their propensity for long-range transport, high environmental persistence, bioaccumulation potential and inherent toxicity, nowadays they represent a global problem. DDTs and PCBs, for instance, are still found in countries of the northern hemisphere where they were banned long time ago. Even if at present almost nothing is known about the existence of these pesticides in the Gulf of Aden, this region of Africa faces several threats such as land-based pollution from agriculture, industry and domestic waste (Hariri et al., 2000). Agricultural run-off, containing sediment, pesticides, trace metals and fertilizers, is increasing and there is a constant threat of oil spills of varying magnitude along the coastline in Djibouti (Hariri et al., 2000). In lieu of this general situation, it was expected to record these compounds also in the Djiboutian marine ecosystem.

2.2. MATERIALS AND METHODS

The study has been carrying out in the Gulf of Tadjoura (Djibouti, latitude 11 40°N, longitude 43 00°E) at the southern entrance to the Red Sea, an inlet of the Indian Ocean created by the fault line of the northerly end of the East African Rift Valley that transects Djibouti, Ethiopia and Kenya. The seabed shelves steeply from the coast of the Gulf dropping to 100 m depth around 2 km offshore and to around 450 m depth in the center of the Gulf of Tadjoura (Dauteuil et al., 2001).

Fieldwork was carried out from 13 November 2016 to 15 February 2017 on board a sailing vessel, the 29 m M/Y “Elegante”, motorized Iveco with two 400 hp motors. For monitoring activities two Zodiacs of 5 m motorized with 40 hp, offshore motors have been used. Each week two stations were sampled S1, Ras Eiro and S2, Ras Korali, which are approximately 7 km apart (Fig. 2.1). These stations were chosen based on local information in regards to the whale shark presence in the Gulf of Tadjoura: S1 was considered as a control station, while S2 as a hotspot for this species. Samples of zooplankton were collected vertically at a depth of 3 m from surface using a 200 μm mesh zooplankton net with (mouth 35 cm diameter). A part of samples was immediately fixed in alcohol for taxonomical analyses, while the rest was frozen for ecotoxicology investigation. 44 zooplankton samples were collected, 8 were in S1 and 36 in S2. Biomass was calculated for all of them, by drying each sample at 60 °C and weighting it, using an analytical balance with a readability of 0.1 mg (Gibertini, Italy), until no weight variations were detected. The calculated biomass was expressed as mg m^{-3}. Zooplankton samples were analyzed by microscopy for taxonomy characterization and zooplankton was identified to the lowest possible taxa using standard keys. Of 44 zooplankton samples, 19 were analyzed for taxonomy characterization, 5 in S1 and 14 in S2. In S1 only 5 of 8 samples were in good conditions for organisms recognition, while for S2, 2 samples for week were chosen to cover all the sampling period. For each of these samples, organisms were recognized and counted; their densities expressed as number of individuals m^{-3}. For density assessment, each sample was resuspended in water, with varying volumes depending on the zooplankton concentration. For all samples, the analysis was performed on subsamples of 5 ml each, taken after gently mixing the initial volume (V), so that the organisms were uniformly distributed (Zunini Sertorio, 1990b). The number of organisms in the original sample (Nc) was quantified by the total number of organisms in the sub-samples (Nsc) and the number of ml examined in the sub-sample (v) by the following formula:

$$Nc = \frac{Nsc}{v} \times V$$

V = initial volume.

Finally, the density of individuals was calculated by dividing Nc by the sampled volume of filtered water (m^3) (Zunini Sartorio, 1990b).

Once in laboratory, four frozen samples were first lyophilized and then analyzed to investigate the presence of contaminants (DDTs and PCBs) following Bettinetti et al. (2016). For each sample, a subsample of approximately 0.5 g dw (dry weight) was extracted with 50 ml of an acetone/n-hexane v/v mixture. The gravimetric determination of lipids was carried out by weighting each sample, until no weight variation was detected. Acetone/n-hexane mixture of zooplankton samples was evaporated using nitrogen. Lipids were then suspended in 2 ml of n-hexane and digested with 5 ml of H_2SO_4 (98%, Carlo Erba, Italy). The organochlorine compounds were then recovered by several n-hexane washings. The n-hexane extracts were concentrated and cleaned on a Florisil column. The resulting samples were concentrated to 0.5 ml and analyzed by gas chromatography (GC Carlo Erba, Top 8000) coupled with 63Ni electron capture detector (Carlo Erba ECD 80) using 1 µL of an on-column injection system. The column was a WCOT fused silica CP-Sil-8 CB (50 m×0.25 mm I.D., film thickness 0.25 µm, Varian, USA), and the temperature program used was from 60 °C to 190 °C at 20 °C min^{-1}, followed by a run from 190 °C to 250 °C at 1.5 °C min^{-1} and a further run from 250 °C to 270 °C at 3 °C min^{-1} and a final isotherma at 270 °C for 17 min, with helium as carrier gas (1 ml min^{-1}) and nitrogen as auxiliary gas (30 ml min^{-1}). Sample quantification was performed using external reference standards of pp′DDT, pp′DDE and pp′DDD (Pestanal, Sigma-Aldrich, Germany) in iso-octane (Carlo Erba, pesticide analysis grade). Arochlor 1260 (Alltech, IL, USA) with the addition of PCB 28, and 118, was used for PCB quantification. The analysed congeners consisted of: PCB 18, 28, 31, 44, 101, 118, 138, 149, 153, 170, 180, 194 and 209. The detection limit for each OC was 0.1 ng g^{-1} dw (dry weight).

Zooplankton samples were carefully collected and preserved in order to avoid any cross-contamination even in the field. Once in the lab, good laboratory practices were tested on standard reference materials BCR-598 and BCR 349 (Community Bureau of Reference –BCR Brussels) for DDT and PCB residues, respectively. Reference samples were analysed with the same method used for collected zooplankton in triplicate. The percentage recoveries were: DDE 107.5% ± 4%, pp'DDD 106.2% ± 4%, pp'DDT 106.2% ± 3%., while those for PCB ranged between 91.3% (± 1.1%) and 102.2 (± 1.6%).

Zooplankton samples were analyzed for spatial and temporal distribution. After ensuring that the assumptions for parametric tests were not violated, an independent-samples t-test was conducted to compare the zooplankton density between S1 and S2. Differences with $p<0.05$ were considered significant. An independent-samples t-test was also used to compare the density of Copepoda and the four major copepod orders between the hotspot and the control station. After ensuring that the assumptions for parametric tests were not violated, a one-way ANOVA was used to investigate the temporal variation in the mean zooplankton density along the sampling period. To investigate the spatial difference in biomass between the two stations a Wilcoxon Rank Sums test was used, since the assumptions for parametric tests were violated. A one-way ANOVA was used to investigate the temporal trend in biomass concentration and the post-hoc Tukey test was used for pairwise comparisons with log-transformed data. A simple linear regression was used to investigate the prediction of biomass based on density. Statistical analysis was performed using JMP® Pro 13.

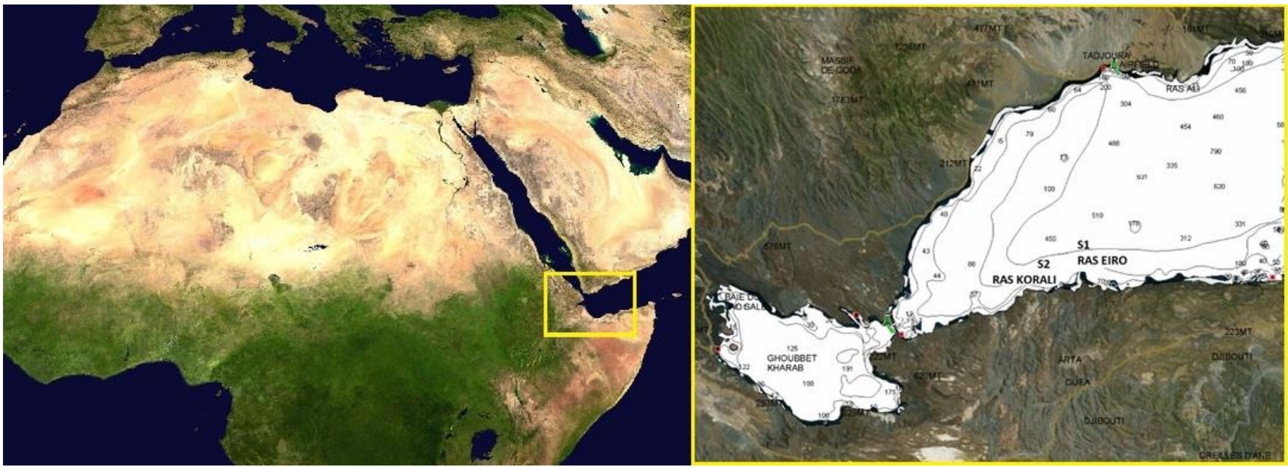

Fig. 2.1 The Gulf of Tadjoura (left image) and locations of station 1 (S1) Ras Eiro and station 2 (S2) Ras Korali

2.3. RESULTS

In the Gulf of Tadjoura, the average sea surface temperature varies from a minimum of approximately 25-26 °C between December and February to a maximum of 31-32 °C achieved in May and September (Youssouf et al., 2016). The mean salinity recorded in the first 50 m depth in September was 37.6 ppt, while the dissolved oxygen 4.5 mg/l. In February the mean salinity was 37.3 ppt and the dissolved oxygen 6 mg/l (Dr Moussa personal communication).

The zooplankton collected in the Gulf of Tadjoura was comprised by 12 different phyla (Table 1.1) and mean density was 6661.1 ± 1204.2 ind m^{-3}. Considering only groups exceeding 2% of the whole mean density, Arthropoda were the most abundant taxon (85.5%), followed by Sarcomastigophora and Chaetognata (3%), Chordata (2.6%), Mollusca (2.4%) and gelatinous plankton (2.2%). Among all taxa, copepods represented the most abundant and diverse group with 29 different genera and contributed an average of 82% (5489.8 ± 1122.2 ind m^{-3}) of the mean zooplankton density. Within the Copepoda, Calanoida were the most abundant taxa (65%, 3567.2 ± 981.6 ind m^{-3}), followed by Poicilostomatatoida (24%, 1318.6 ± 220.9 ind m^{-3}), Harpacticoida (9%, 489.5 ± 309.6 ind m^{-3}) and Cyclopoida (1%, 71.5 ± 22.8 ind m^{-3}). The remaining 1% were undefined species and juvenile stages. The most common Families were: Paracalanidae, comprising 32.4% (1548.7 ± 262.4 ind m^{-3}) of the mean density of all the identified copepods, Calanidae 20.3% (969.6 ± 708.8 ind m^{-3}), Oncaeidae 17.5% (838.9 ± 150.1 ind m^{-3}), Miraciidae 10.2% (489.4 ± 309.6 ind m^{-3}), Corycaeidae 9.8% (468.8 ± 81.3 ind m^{-3}), Pontellidae 2% (95.2 ± 32.1 ind m^{-3}) and Acartiidae 1.9% (93.2 ± 25.4 ind m^{-3}). Besides Copepoda, other taxa which occurred in both stations were: Appendicularia and Sagittoidea that represented 8.8% and 2.9%, respectively.

Table 2.1 Taxonomic classification of zooplankton collected in the Gulf of Tadjoura

Taxonomic Classification							Life stage(s) observed
Phylum	**Dinoflagellata**						
Phylum	**Sarcomastigophora**	(Radiolaria, Acantharia, Foraminifera)					
Phylum	**Ochrophyta**	**Bacyllariophycae**					
Phylum	**Annelida**	**Polychaeta**					Larvae and adults
Phylum	**Plathelminthes**						Adults
Phylum	**Arthropoda**						
	Subphylum **Crustacea**	**Branchiopoda**		**Cladocera**			Adults
		Thecostraca	**Cirripedia**				Nauplius, cyprids and adults
		Ostracoda					
		Copepoda					Nauplius, copepodid and adults
				Calanoida			
					Acartiidae	*Acartia* spp *Acartiella* spp	
					Candaciidae	*Candacia* spp	
					Centropagidae	*Centropages* spp *Centropages furcatus*	
					Pontellidae	*Calanopia* spp *Labidocera* spp *Pontellopsis* spp	
					Calanidae	*Canthocalanus pauper* *Undinula vulgaris* *Cosmocalanus* *Mesocalanus* spp *Nannocalanus minor*	
					Paracalanidae	*Acrocalanus* spp *Paracalanus* spp	
					Clausocalanidae	*Clausocalanus* spp	
					Temoridae	*Temora* spp	
					Eucalanidae	*Eucalanus* spp *Subeucalanus* spp	
					Euchaetidae	*Euchaeta* spp	
					Tortanidae	*Tortanus* spp	
					Scolecitrichidae	*Scolecitricella* spp	
					Rhincalanidae	*Rhincalanus* spp	
				Cyclopoida			

Taxonomic Classification							Life stage(s) observed
					Oithona	*Oithona* spp	
				Harpacticoida			
					Miraciidae	*Macrosetella gracilis*	
				Poicilostomatatoida			
					Oncaeidae	*Oncaea* spp	
					Sapphirinidae	*Copilia* spp	
						Sapphirina spp	
					Corycaeidae	*Corycaeus* spp	
		Malacostraca		**Stomatopoda**			
				Amphipoda			
			Eucarida				
				Euphausiacea			Larvae stages
				Decapoda			
				Dendrobranchiata			Zoea stages, megalopae
					Luciferidae	*Lucifer* sp	
				Pleocyemata			
				Caridea			
				Anomura			Zoea stages, megalopae
				Brachyura			Zoea stages, megalopae
Phylum	**Mollusca**	**Gastropoda**					
		Bivalva					
Phylum	**Echinodermata**	**Echinoidea**					Larvae
		Ophiuroidea					
Phylum	**Chaetognata**	**Sagittoidea**					Adults
Phylum	**Chordata**						
	Subphylum: Urochordata	**Appendicularia**					Adults
		Thaliacea				*Doliolum* spp	
	Subphylum: Vertebrata	**Osteichthyes**					Eggs, larvae
Phylum	**Ctenophora**						
Phylum	**Cnidaria**						

A total of 19 zooplankton samples were analyzed for density determination, of which 5 were from S1 and 14 from S2. Station S1 was represented mainly by Arthropoda (90.1%), Chordata (2.4%) and Sarcomastigophora (2.4%). Copepoda represented the most abundant taxa reaching 88% of the whole mean density of S1 (Table 1.2). Within copepods, Calanoida represented 58.6% of all density, followed by Poicilostomatatoida (20.2%), Harpacticoida (18.4%) and Cyclopoida (2%). The remaining percentage corresponded to the undefined copepods (Table 1.2). In Station S2, 83.3% were Arthropoda, 4.1% Chaetognata, 3.3% Sarcomastigophora, 2.9% Mollusca, 2.7% Chordata and 2.6% gelatinous plankton. The remaining 1.1% comprised uncommon taxa. Copepoda represented 79.9% of the mean density in Ras Korali (Table 1.2). Calanoida was the most abundant taxa with 68.2% of all copepods, followed by Poicilostomatatoida (26%), Harpacticoida (4%) and Cyclopoida (1%). The undefined copepods comprised 0.7% (Table 1.2). Besides Copepoda, Appendicularia contributed with 14% (868.1 ± 708.2 ind m^{-3}) of the mean zooplankton density, Sagittoidea represented 4.1% (253.7 ± 78.7 ind m^{-3}) and Ostracoda 3.1% (191.2 ± 173.6 ind m^{-3}).

An independent-samples t-test was conducted to compare the zooplankton density between S1 and S2. Results did not show any statistical difference between the two stations (t(17)=-0.952, p=0.3545). When comparing density of Copepoda, which are the most relevant group of organisms, between S1 and S2, no statistical differences were found (t(10.7)=-1.385, p=0.1943). Similarly, no differences were found between copepod orders: Harpacticoida (t(5.4)=-0.396, p=0.7074); Calanoida (t(8.1)= -0.641, p=0.5392); Cyclopoida (t(4.8)=-1.084, p=0.3297) and Poicilostomatatoida (t(17)=-0.954, p=0.3531) (Table 1.2).

Table 2.2 Zooplankton mean density (± SE) in S1 and S2

	Mean Density (ind m^{-3})		**P value**
Station	**Ras Eiro (S1)**	**Ras Korali (S2)**	
Zooplankton	7950 ± 1650	6201 ± 1538	p=0.3545
Copepoda	6992 ± 1694	4953 ± 1403	p= 0.1943
Calanoida	4098 ± 1694	3377 ± 1219	p=0.5392
Cyclopoida	138 ± 76	48 ± 14	p=0.3297
Harpacticoida	1286 ± 203	205 ± 76	p=0.7074
Poicilostomatatoida	1410 ± 630	1286 ± 298	p=0.3531

When investigating any possible variations in the mean zooplankton density along the sampling period, a temporal trend was found, having its maximum peak in December (7791.7 ± 6173.8 ind m^{-3}) and the lowest in February (1539.6 ± 1246 ind m^{-3}) (Fig. 2.2). A One-Way ANOVA did not show any significant difference between mean density across the sampling period (p=0.5269).

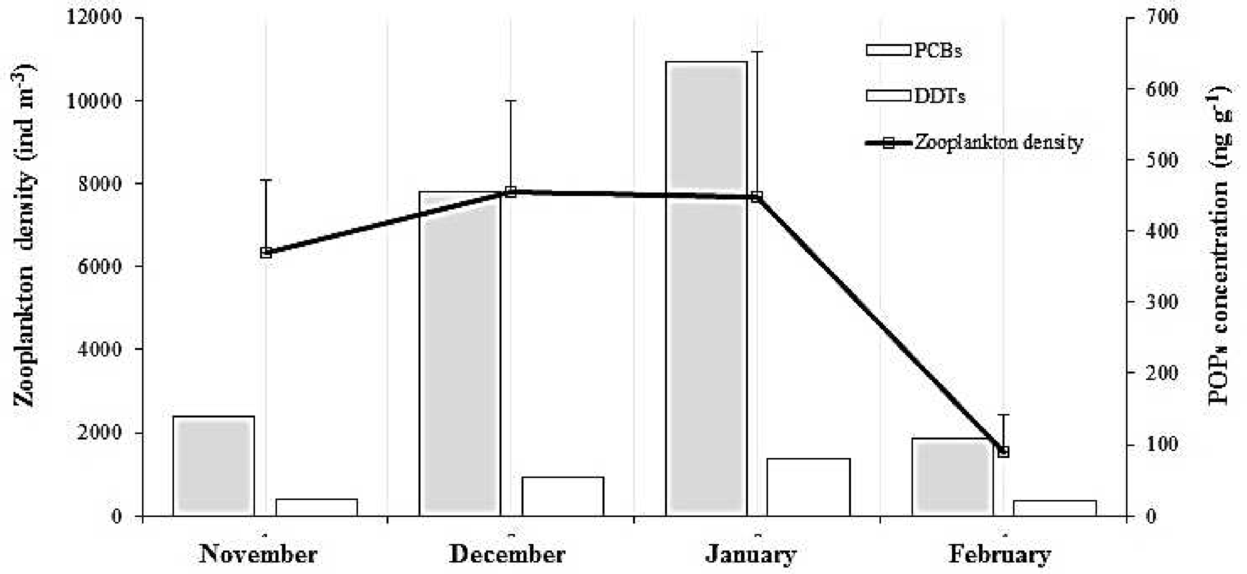

Fig. 2.2 Temporal trend in zooplankton density, represented by the line chart, combined with PCBs (grey histograms) and DDTs (white histograms) concentrations along the sampling period

Considering both stations, the biomass average was 30.7 ± 4.2 mg m^{-3} dw. Zooplankton biomass in Ras Eiro had a mean value of 34 ± 21.7 mg m^{-3} dw, while in Ras Korali was 33.5 ± 37.6 mg m^{-3} dw. The Wilcoxon Rank Sums Test did not show any statistical differences between stations (Z=0.694, p=0.4875).

A temporal trend in biomass concentration was investigated along the sampling period (Fig. 2.3). November had an average value of 27.3 ± 14.9 mg m^{-3} dw that increased to 41.3 ± 36.4 mg m^{-3} dw. in December, when the zooplankton biomass reached its maximum peak. A decreasing trend started from December, leading to the lowest biomass in February (6.6 ± 3.3 mg m^{-3} dw) (Fig. 2.3). One-Way ANOVA has shown a statistical difference in the mean biomass along the sampling period (F (3,40)=6.067, p=0.0017). The post-hoc Tukey test showed that the zooplankton mean biomass in February (6.6 ± 3.3 mg m^{-3} dw) was statistically lower than November (27.3 ± 14.9 mg m^{-3} dw; p=0.0054), December (41.3 ± 36.4 mg m^{-3} dw; p=0.0008) and January (25.4 ± 19.4 mg m^{-3} dw; p=0.0243) (Fig. 2.3).

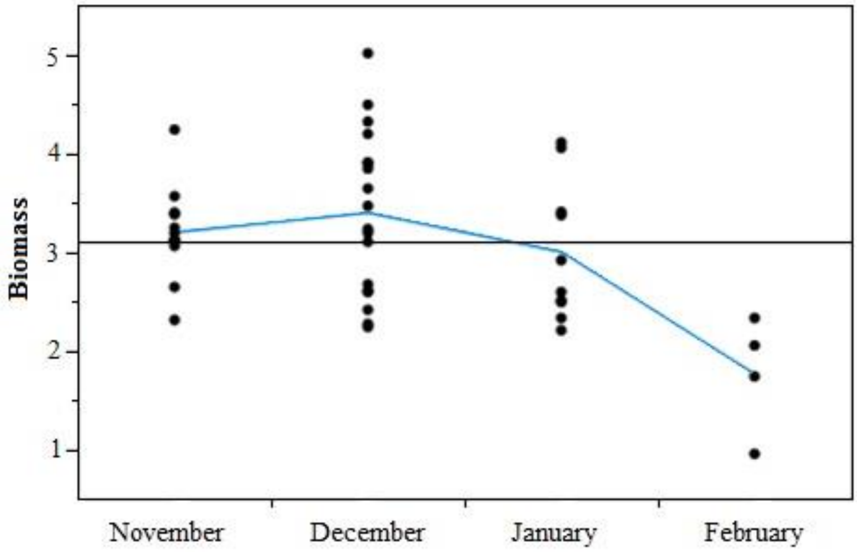

Fig. 2.3 Temporal change in zooplankton biomass (mg m^{-3} dw) along the sampling period. Biomass values are presented on a logarithmic scale

A simple linear regression was used to investigate the prediction of biomass based on density. A significant regression equation was found (F(1,16)=34.508, $p<0.0001$), with an R^2 of 0.683 (Fig. 2.4).

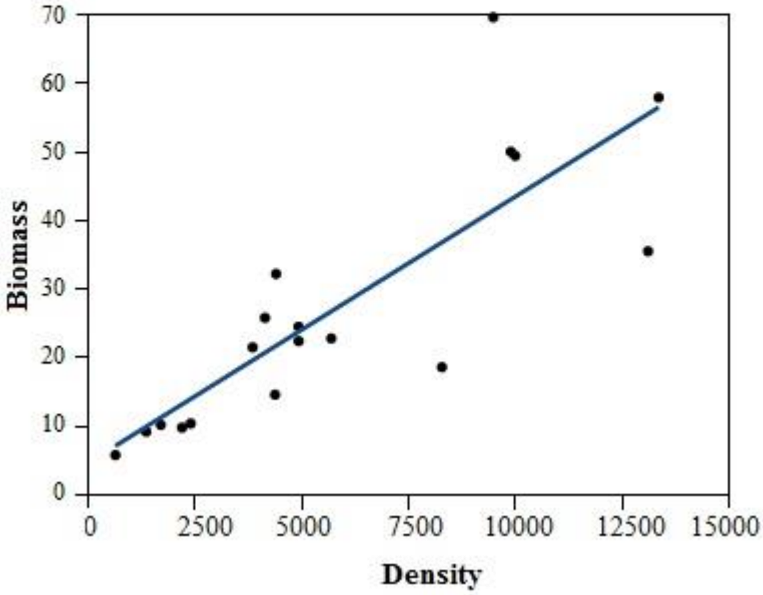

Fig. 2.4 Relationship between zooplankton density (ind m^{-3}) and biomass (mg m^{-3} dw)

Concerning the results from the analyses of persistent organic pollutants, PCB and DDT in zooplankton showed a mean concentration of 335.6 ± 254.3 ng g^{-1} dw and 44.7 ± 27.4 ng g^{-1} dw, respectively (Table 1.3). Total PCB concentration varied substantially between samples from a minimum value of 109.7 ng g^{-1} dw up to 636.1 ng g^{-1} dw. Similarly, DDT showed a minimum concentration of 21.4 ng g^{-1} dw to a maximum of 79.2 ng g^{-1} dw (Table 1.3). The presence of 13 PCBs congeners have been investigated: PCB 44, 118, 153, 180 and 209 were found in all samples, while the less common congeners were PCB 194 and 149 (Table 1.3). Zooplankton was dominated by higher chlorinated PCB congeners (hepta-CB, 118.2±120.5 ng g^{-1}) (Fig. 2.5).

p-p' DDT was the dominant in the first sample (08/12/2016), representing 71% of all metabolites, a week later (16/12/2016) both o-p' DDE and p-p' DDT were in higher concentration (28.3% and 25.7%, respectively) and a month later (16/01/2017) p-p' DDT was again the major compound (48.4% of all metabolites). The fourth sample (17/01/2017), collected with a day lag, was similar in composition and had p-p’ DDT as dominant metabolite (43.2%). Considering the whole sampling period (approximately 40 days), DDT (both isomers) was the major compound (47.8%), followed by DDE (32.7%) and DDD (19.5%).

Table 2.3 Concentrations of PCBs and DDTs (ng g^{-1} dw) in zooplankton samples collected in the Gulf of Tadjoura

	08/12/16	**16/12/16**	**16/01/17**	**17/01/17**	**Mean ± Sd.**	**Min-Max**
Lipid content (% dw)	5.7	6.8	10.6	5.4	8.9 ± 4.5	5.4-10.6
Total DDT	23.5	53.7	79.2	21.4	44.7 ± 27.4	21.42-79.2
o-p’ DDD	3.5	13.5	2.3	5.4	6.2 ± 5	2.3-13.5
p-p’ DDD		2.6	5.3	2.1	3.3 ± 1.7	2.1-5.3
o-p’ DDE		15.2	10.9	0.2	8.8 ± 7.7	0.2-15.2
p-p’ DDE	3.3	6.9	18.5	3.2	8 ± 7.2	3.2-18.5
o-p’ DDT		1.7	3.8	1.4	2.3 ± 1.3	1.4-3.8
p-p’ DDT	16.7	13.8	38.4	9.3	19.5 ± 12.9	9.3-38.4
Total PCB	140.1	455.6	636.1	109.7	335.6 ± 254.3	109.7-636.1
PCB 18		39.7	54.9	7.9	34.2 ± 23.9	7.9-54.9
PCB 28+31		32.2	5.4		18.8 ± 19	5.4-32.2
PCB 44	49.3	79	104	22.3	63.7 ± 35.5	22.3-104
PCB 101	16.2	9	42.4		22.5 ± 17.6	9-42.4
PCB 149				32.6	32.6	32.6-32.6
PCB 118	12.4	47.1	84.6		48 ± 36.1	12.4-84.6
PCB 153	37.2	13.8	60	10.9	30.5 ± 23	10.9-60
PCB 138		14.4		10.5	12.5 ± 2.7	10.5-14.4
PCB 180	13.9	173.9	252.2	19.2	114.9 ± 118.1	13.9-252.7
PCB 170		3,6	6.7	2.8	4.3 ± 2	2.8-6.7
PCB 194	9.4				9.4	9.4-9.4
PCB 209	2.3	43	26.	3.40	18.7 ± 19.5	2.3-43

* Blank boxes: concentration was less than the limits of detection and therefore was not reported in the manuscript.

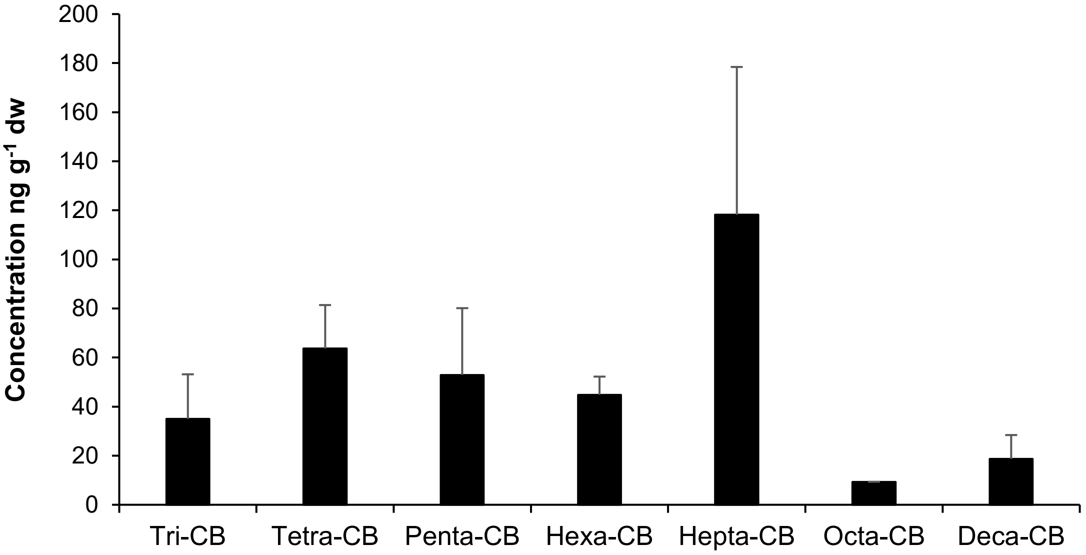

Fig. 2.5 Mean (± S.E.) of PCBs, grouped according to the chlorine content of each PCB congener of all samples

2.4. DISCUSSION

Phyto- and zooplankton of the Gulf of Aden and Southern Red Sea have been investigated by several authors (e.g. Gradinger et al., 1992; Baars et al., 1998; Wiebinga et al., 1997). However, knowledge on large-scale biological processes in the area is limited due to lack of adequate data. Until 2017 the temporal trend in phytoplankton biomass across the Gulf of Aden was still unknown (Gittings et al., 2017). In lieu of this lack of information, our work is the first study on the zooplankton community in the area of Djibouti and provides preliminary baseline information about the zooplankton composition, biomass and density as well as ecotoxicology analysis in this biota.

Zooplankton abundance in the current study reflects high diversity and abundance within the zooplankton community. The majority of organisms in the Gulf of Tadjoura were copepods comprising 82% of the whole zooplankton mean density, in particular Calanoida and Poicilostomatatoida. At genus level, the most abundant organisms were *Acartia*, *Paracalanus*, *Canthocalanus*, *Nannocalanus*, *Macrosetella*, *Oncaea* and *Corycaeus*. Besides copepods, at class level, Appendicularia and Sagittoidea were also common, as well as gelatinous plankton. However, due to their decomposition in normal preservation (Harbison et al., 1978; Larson, 1986), it was not possible to taxonomically characterize these organisms. This deficiency in our analysis is believed to be the reason why no statistically differences have been found in the density and biomass between the control and hotspot stations. From personal observations, S2 was characterized by a higher density of gelatinous zooplankton, in particular Ctenophora, unfortunately in this study it was not possible to make a proper density estimate. Furthermore, the high content in water of these organisms could not be taken in consideration in the biomass analysis, since biomass of each sample was calculated as dry weight. Alternative analysis will be carried out to overcome this methodology limitation. The zooplankton taxonomy seems similar to those reported by Rezzolla and Storai (2010) in the Gulf of Tadjoura and by Al-Aidaroos et al. (2016) in the Southern Red Sea. The latter reported a zooplankton composition mainly dominated by holoplanktonic forms, predominant with copepods followed by chaetognaths and urochordates. Similarly, Kidwai and Amjad (2000) reported a zooplankton composition in the Arabian Sea made up mainly by copepods that reached 74.9% of mean density. Cheatognaths were numerically the next most abundant group. Al-Aidaroos et al. (2016) calculated a zooplankton standing crop that fluctuated between 1,058 and 25,787 individuals m^{-3} in the Southern Red Sea, which is comparable to the measurements reported in this study. Zooplankton density in the Gulf of Tadjoura varied between 658 to 21,474 ind m^{-3}, with an average of approximately 6600 ind m^{-3}.

In the Gulf of Tadjoura the mean biomass calculated in this study during the whale shark aggregation season was 30.7 ± 4.2 mg m^{-3} dw. Similarly, in Tanzania Rohner et al. (2015) reported a biomass average of 25.2 ± 22.8 mg m^{-3} dw when the whale sharks were present in the sampling area. Results from this study showed an increasing trend from November to December, when the zooplankton biomass reached its maximum peak, and then a decreasing tendency that lead to the lowest biomass measurements in February. The Gulf of Aden is influenced by the Indian Monsoon (Almogi-Labin et al., 2000) so that during winter months, between December and February, the northeast winds generate a northern anticlockwise gyre (Wyrtki, 1973; Molinary et al., 1990). This system is replaced during the summer from May/June through October by the Southwest monsoon (Almogi-Labin et al., 2000), which cause strong phytoplankton bloom off the western part of the Gulf of Aden (Gittings et al., 2017). The phytoplankton bloom terminates in September, just before the intermonsoon period. It is possible that the decrease in Chl-a concentration in this area could be caused by an increase grazing pressure following a rise in zooplankton abundance (Goericke, 2002; Gittings et al., 2017). As reported by Ashjian et al. (2002) in the Arabian Sea, the area of the highest concentration of zooplankton coincided spatially with the area of low chlorophyll-a fluorescence. The authors found a strong negative correlation between Chl-a fluorescence and the upper-ocean zooplankton biomass, similarly to that observed off Somalia (Smith, 1982; Baars and Oosterhuis, 1997). In this study, we could not provide data on zooplankton biomass during summer and investigate the role of zooplankton in modulating the Gulf of Aden phytoplankton dynamics. However, considering a time lag between phytoplankton and zooplankton bloom and a decrease in Chl-a concentration at the end of September, it is possible that the zooplankton population increases at the end of summer, reaching its maximum in December, as reported by our results. The arrival of NE monsoon in winter causes a decrease in Chl-a along the gulf (Bower et al., 2002; Gittings et al., 2017) and a subsequent decline in zooplankton bloom, which is consistent by the low zooplankton biomass measured in February. This hypothesis not only is supported by our results, but could explain the whale sharks presence between October-February. However, additional data is needed to investigate the occurrence of whale sharks in the Gulf of Tadjoura during those months, since the aggregation could be linked to multiple factors rather than the only zooplankton biomass. Alternatively, the peak in zooplankton biomass recorded in December could be induced by an autumn phytoplankton bloom, similar to the one identified along the southern coastline of the Gulf of Aden, which is induced by the seasonal reversal in wind direction at the beginning of the winter monsoon (Gittings et al., 2017). Giving the lack of information regarding the role of zooplankton in modulating the Gulf of Aden phytoplankton dynamics, future studies should be carried out.

Our study, by determining DDTs and PCBs residues in zooplankton for the first time in the Gulf of Tadjoura, provides preliminary baselines on the pollution status at the base of trophic chain in the coastal area of Djibouti. Zooplankton plays also an important role in transferring contaminants to the higher trophic levels as an entry point for water-borne POPs into the food web (Day, 1990; Chiuchiolo et al., 2004; Bettinetti and Manca, 2013). Polychlorinated biphenyls (PCBs) and dichloro-diphenyl-trichloroethane (DDTs) are well known persistent organic pollutants (POPs) that, although banned in most countries of the northern hemisphere in the 1970s, are found in biota all over the world, including remote locations far from sources (Bidleman et al., 1989; Auman et al., 1997; Chiuchiolo et al., 2004; Wania, 2003). Damage to marine organisms by organochlorine pesticides is well-documented (Swart et al., 1995; Muir et al., 1999; Islam and Tanaka, 2004; Jepson et al., 2005).

Typically, the POPs released in the marine environment are easily assimilated by organisms and, due to the biomagnification effect, tend to accumulate in higher trophic levels. Since whale sharks feed primarily upon low trophic level organisms, they should be expected to have low concentrations of POPs (De Moura et al., 2015). However, Fossi et al. (2014) reported high levels of both DDT (1648-2639 ng g^{-1} lipid basis) and PCBs (1576-1970 ng g^{-1} lipid basis) in the large filter feeder basking shark, Cetorhinus maximus, accidentally captured in Mediterranean waters. These high values of POPs may be the consequence of the constant presence of the basking shark in the Mediterranean Sea, which is particularly affected by different contaminants (Fossi et al., 2003). The Gulf of Aden faces several threats that include poor management of waste water: the disposal of solid waste causes localized problems for coastal habitats in all countries and also contaminated runoff arising from pesticide use (PERSGA, 2006). In Djibouti industrial activities concentrated around Djibouti City result in chronic release of industrial pollutants, mostly untreated (PERSGA/GEF, 2001). A particular concern surrounds the risk of wash-out of pesticides and organic chemical wastes dumped inland in streams (PERSGA/UNEP, 2008). Our results showed the presence of both DDTs and PCBs residues in zooplankton samples in the Gulf of Tadjoura and, as consequence, it could be speculated that whale sharks, which show site fidelity to the study site, might be exposed to organochlorine pesticides as well. At present, apart from the results presented in this study, almost nothing is known about the existence of these pesticides in the Red Sea and in the Gulf of Aden (Al-Shwafi et al., 2009). Therefore, it is difficult to compare the present results with those from other studies since little information is available, especially in the Gulf of Aden. Moreover, a lack of uniformity in the expression of data (e.g. fresh, dry or lipid weight), makes the comparison even more complicated (Joiris and Overloop, 1991). Al-Shwafi et al. (2009) provided for the first time data on the bioaccumulation of PCBs and DDTs in different fish and shell fish species collected from the Gulf of Aden. The authors reported ranges of DDTs extended from 0.3 to 6.3 ng g^{-1} dw with an average of 2.9 ng g^{-1} and of PCBs extended from 0.1 to 0.9 ng g^{-1} dw with an average of 0.4 ng g^{-1}. In the northern

Arabian Sea, Shailaja and Nair (1997) found a total DDTs levels in zooplankton that ranged from 3.36 to 38.8 ng g^{-1} wet weight (0.73 to 8.81 µg g^{-1}, lipid weight) before the southwest monsoon, while in September these values were 5 times greater. Our results showed that in the Gulf of Tadjoura PCBs ranged from approximately 110 to 637 ng g^{-1} dw, with an average of 336 ng g^{-1} dw Comparable results were described in the zooplankton of the subtropical zone of the Indian sector (Southern Ocean), where the PCBs mean value was 370 ng g^{-1} dw (Joiris and Overloop, 1991). Organochlorine concentrations in zooplankton varied among species and appear to be related, in part, to a combination of trophic position and body size (Braune et al., 2005). In Arctic marine zooplankton, Fisk et al. (2001) found PCB congener profiles in Calanus hyperboreus to be dominated by lower chlorinated congeners, while higher chlorinated PCBs such as CB 180 were rarely detected. In our study, the homologue composition was on average mainly composed by hepta (35.2%), tetra (19%) and penta (15.8%) -CBs (Fig. 2.5). Elimination to water is more efficient for less hydrophobic PCB congeners, implying that higher concentration would be expected to increase with hydrophobicity. The highest concentration of hepta-CBs found in the zooplankton of the Gulf of Tadjoura, could be mainly explained by their molecular structure, responsible for their high lipophilicity, stability and persistence in the environment, and by the past use of these PCB congeners in Aroclor mixture (Quadroni and Bettinetti, 2017).

As regards to DDT analyses, we found the proportion of primary DDT in the total residue to be higher than DDE and DDD, which strongly suggests that the area might actually be subject to new DDT inputs. The DDE/DDT ratio was in fact lower than 1 (n=0.68), which suggests that recent inputs of DDT have occurred. For more than 40 years, the use of chemical pesticides has been developing in plenty countries through sub-Saharan Africa (Bon et al., 2014). Most of pesticides are chemicals used since the 1950s, and their frequency of application is quite high (Bon et al., 2014). Although the use of POPs has been banned or restricted, developing countries still use them for agricultural and health purposes, such as the use of DDT in combating malaria, and they can be still found in aquatic

(Yohannes et al., 2013a) and terrestrial ecosystems (Gebremichael et al., 2013). In sub-Saharan Africa pesticide choice, quality, and frequency of application and use are not hardly controlled. Quite often, pesticides not licensed for use on food crops end up in the food chain (Bon et al., 2014). In addition, many African countries are burdened by obsolete pesticide stocks (Haylamicheal and Dalvie, 2009). According to the FAO's obsolete pesticide inventory report (FAO, 1999), African countries were estimated to have more than 1000 tonnes of obsolete pesticide stocks. Ethiopia, for instance, has accumulated obsolete pesticide stocks since pesticides were first imported in the 1960s. The stocks reached over 1500 tonnes in 1999 and included pesticides such as chlordane, DDT, dieldrin and lindane that are banned or restricted in most countries (Haylamicheal and Dalvie, 2009). However, in countries like Ethiopia, banned pesticides are being sold in open markets (EPA and SEG, 2003). Considering the general situation in the sub-Saharan Africa, zooplankton, occupying the lowest position of the food web and responding promptly to pollution changes, could be considered as an early warning bioindicator of hydrophobic pollutants in the aquatic ecosystems (Bettinetti et al., 2012). Therefore, analysis on the presence and temporal changes of contaminants in this component should be further investigated in this area.

2.5. CONCLUSIONS

During the whale shark aggregation season (October to February), the Gulf of Tadjoura was characterized by copepods (82% of the mean zooplankton density), in particular Calanoida and Poicilostomatatoida. The overall zooplankton composition and density was comparable to those reported in the Southern Red Sea and in the Arabian Sea. In the Gulf of Tadjoura, during the whale shark aggregation season, the mean biomass was slightly higher than that measured in Mafia Island (Tanzania) in presence of this species. Results from this study support the hypothesis of an increase in zooplankton biomass due to the SE monsoon upwelling. However, data on zooplankton biomass in relation to the NE winter monsoon should be collected as well, to obtain solid conclusions. The biomass trend described in this study supports the hypothesis that this might be the primary driver for whale sharks use of the Gulf of Tadjoura as a feeding ground during those months.

The activities concentrated around Djibouti City, such as industries, sea traffic, slaughterhouses and the domestic wastewater, result in chronic release of pollutants, mostly untreated. Our results confirm for the first time the presence of some of these pollutants in the zooplankton of the Gulf of Tadjoura. Considering the role of zooplankton in transferring contaminants to the higher trophic levels and its importance as an early warning bioindicator of regional pollution, further studies will be carried out to investigate the presence of POPs along the food webs.

2.6. ACKNOWLEDGMENTS

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CHAPTER III: The Ecology of the Whale Shark in Djibouti

The Ecology of the Whale Shark in Djibouti

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ABSTRACT

The Gulf of Tadjoura (Djibouti) is an important site where the whale shark (*Rhincodon typus* Smith 1828) aggregates seasonally, however, only few studies have been carried out in the area. From 2015 to 2018, whale shark photo-id, skin biopsies and zooplankton samples were collected to investigate the ecology of this species in Djibouti. A total of 190 individuals were identified, with a majority of males, confirming a significant male bias aggregation ($X^2 = 65$, df = 1, $p < 0.0001$). Within each season, the maximum number of times an individual was re-sighted was 11, while the inter-annual re-sighting rates varied between 11 and 32%. Animals were feeding on surface zooplankton, consisting primarily of copepods (85%), however Chaetognatha and Ctenophora appeared to be important taxa, since the areas most visited were also those with higher concentration of these organisms. Feeding zooplankton patches had a mean biomass of 42.2 ± 31.9 mg m^{-3}, almost 1.5 higher than control stations, indicating that the abundance of this species was positively related to food availability. Furthermore, whale sharks exhibited a clear temporal distribution associated with the variation in zooplankton biomass due to the monsoon winds. Sharks aggregated in October-February, with a peak in sightings in November, and disperse outside the upwelling season. $\delta^{13}C$ values suggested that whale sharks might migrate to other inshore habitats or shift their activities to deeper areas outside the aggregation phase. Little intra-specific variation in $\delta^{15}N$ and $\delta^{13}C$ was found between animals, suggesting a similar habitat use.

3.1. INTRODUCTION

Marine species are often associated with specific physical or biological habitats, and there is growing interest in understanding the role of environmental conditions as drivers of distribution and abundance patterns (Elith et al., 2011). Large planktivores, such as the whale shark (*Rhincodon typus* Smith 1828), require high-density prey patches to sustain their energy demands, while consuming organisms that are many order of magnitude smaller than their own body (Marcus et al., 2016; Rohner et al., 2017). To enhance cost-efficiency, whale sharks form aggregations in areas associated with dense aggregations of preys (Colman, 1997; Rowat and Brooks, 2012; Rohner et al., 2015; Marcus et al., 2016). Indeed, the distribution and abundance of whale sharks appeared to be influenced by oceanographic processes, which increase productivity in the local environment and enhance high concentrations of food (Colman, 1997; Wilson et al., 2001; Duffy 2002; Cárdenas-Palomo et al., 2010; Sleeman et al., 2010; Sequeira et al., 2012; Hacohen-Domené et al., 2015). Evidence from direct observations, faecal and stomach content analysis have identified a wide range of planktonic and nektonic organisms as their target prey, including copepods, krill, crab larvae, sergestids, gelatinous zooplankton, chaetognaths, fish eggs and small fish (Clark and Nelson, 1997; Wilson et al., 2001; Heyman et al., 2002; Rowat et al., 2007; Meekan et al., 2009; Rohner et al., 2015; Boldrocchi and Bettinetti, 2019). Recent studies indicate that whale sharks feed also on meso- and bathypelagic sources (Rohner et al., 2013; Marcus et al., 2016). Despite the wide range of prey, many of these aggregations appear to target specific prey items. However, a detailed understanding of the zooplankton community as well as spatiotemporal distribution in biomass are scarce from most of these locations (Cárdenas-Palomo et al., 2015; Rohner et al., 2015). Information about whale shark feeding habits can improve ecological understanding of the underlying drivers of its movements and support strategies for sustainable management of the aggregation areas.

Within the Gulf of Aden, the Gulf of Tadjoura (Djibouti) has long been known as a place frequented by whale sharks (Rowat et al., 2007). Sharks gathering off the coast of Djibouti appeared to be mostly juvenile males and to target dense concentrations of zooplankton organisms (Rowat et al., 2007; Rowat et al., 2011). Despite the area is widely recognized as an important site for this species, only few studies has been carried out, mainly focused on the demography and membership of the Djibouti aggregation (Rowat et al., 2007; Rezzolla and Storai, 2010; Rowat et al., 2011). Boldrocchi et al. (2018) provided for the first time a description of the zooplankton composition and speculated that an increase in zooplankton biomass might be the primary driver for whale sharks use of the Gulf of Tadjoura in October-February. However, a detailed study on the whale shark ecology is still missing, leaving a knowledge gap on feeding habits, distribution, habitat preference and environmental factors affecting the whale shark distribution. Given the importance of this area for whale sharks, it is necessary to determine the environmental conditions that shape their distribution. Successful conservation of a species of concern, as the endangered whale shark, requires a robust understanding of their spatiotemporal distribution in order to assess potential interactions with human activities (McClellan et al., 2014). Although this species is protected in Djibouti, whale sharks are particularly vulnerable to boat strike, since vessels passage and speed, including tourism activities, are not regulated and can cause physical damage to sharks (Rowat et al., 2007). Analysis of scarring patterns may provide an insight into the conservation status of this species in the Gulf of Tadjoura and highlight the need of formulating new initiatives for conservation, including a code of conduct when approaching this species (Speed et al., 2008).

This study, therefore, aims to address this general knowledge gap by 1) evaluating the feeding ecology of the whale shark by quantitatively analyzing previous qualitative and anecdotal observations 2) understanding how spatiotemporal distribution patterns of whale sharks are related to the variability of zooplankton conditions in Djibouti 3) providing an insight of the human-shark interactions in the Gulf of Tadjoura. Finally, 4) Investigating possible animal movements outside the aggregation period, by analyzing the carbon and nitrogen signatures in whale shark skin tissues.

Overall, this study aims to provide useful information for developing new management strategies for the protection of this species in the Gulf of Tadjoura.

3.2. MATERIALS AND METHODS

3.2.1. Study site and sampling design

The study has been carrying out in the Gulf of Tadjoura (Djibouti, latitude 11 40°N, longitude 43 00°E) at the southern entrance to the Red Sea (Fig. 3.1). Fieldwork was conducted in 2015-2018 on board of a sailing vessel. In November-December 2015 (season I), two weeks of photo-survey for shark identification were carried out, while in November-February 2016-2017 (season II), the fieldwork included also zooplankton samples collection. The same data were collected also in 2017-2018 (season III) and 2018-2019 (season IV) from October to January. Each cruise started and ended in Djibouti city and lasted a week, maintaining the same route and schedule every week. Within the Gulf of Tadjoura, the boat route visited four bays: Ras Eiro (S1), Ras Korali (S2), Star Bay (S3) and La Faille (S4) (Fig. 3.1). Stations S2 and S4 were considered feeding hotspot for whale sharks, while S1 and S3 control stations. Within the hotspot S2, a control station was created at the eastern side of the bay, opposite to the feeding area, where the sailing boat anchored each time.

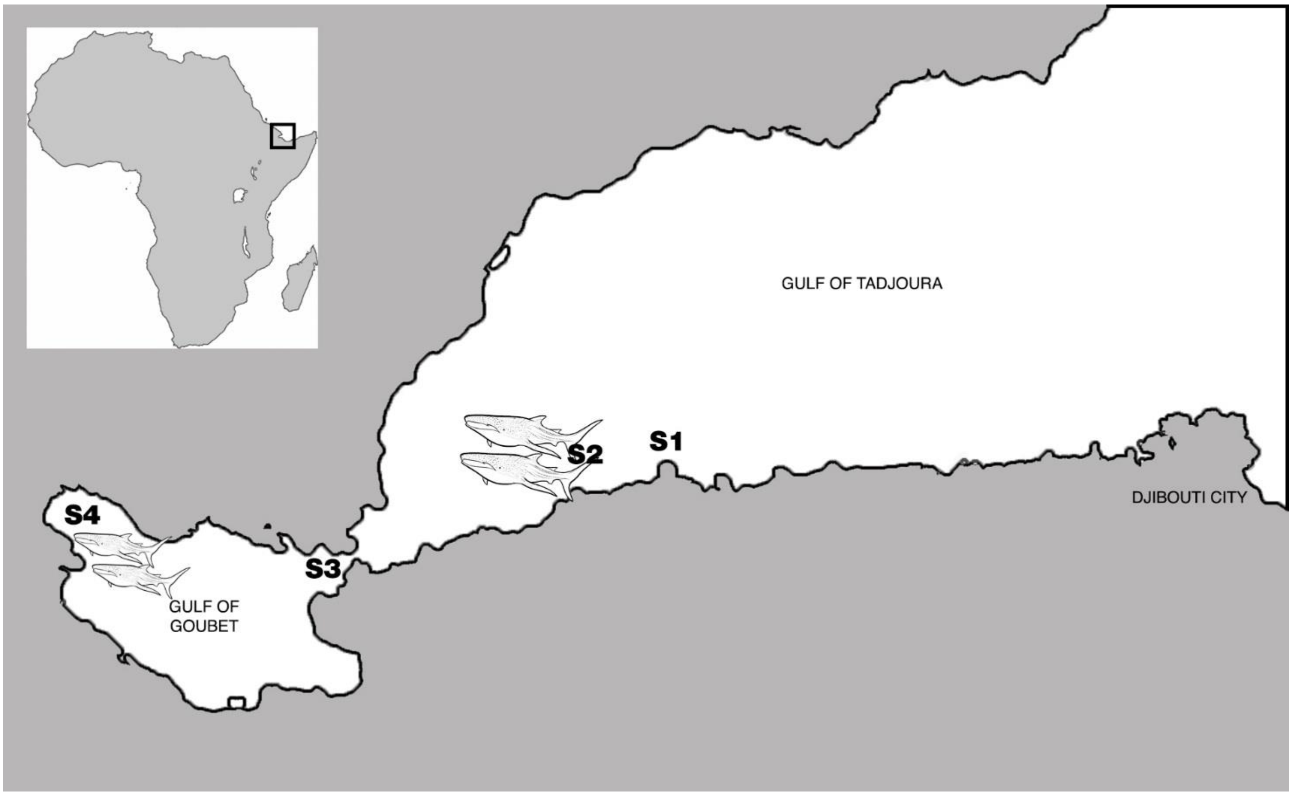

Fig. 3.1 The Gulf of Tadjoura (Djibouti) and the four sampling stations: Ras Eiro (S1), Ras Korali (S2), Star Bay (S3) and La Faille (S4)

3.2.2. Whale shark observations

Once anchored, two zodiacs were used to monitor the presence of whale sharks. Once a specimen was spot, photographs and videos of its left and right flanks, genitals and scars were collected. The sex of each shark was determined visually by examining the pelvic fins for the presence of claspers in males or their absence in females. Photographs were loaded into I^3S (Interactive Individual Identification System) pattern-recognition software to evaluate matches among photographs (Van Tienhoven et al., 2007).

For each encountered shark, information on the number, type and position of wounds was recorded. Following Speed et al. (2008), scars were subdivided into 7 categories (Fig. 3.2) and for each of them, a probable origin was also attributed (Speed et al., 2008). When an injured shark was re-captured, a photographic comparison was carried out to observe the healing process over the time.

Skin biopsies of 9 individuals were collected in November-December 2016-2018 with a biopsy punch (30 mm sampling tips) on a pole spear (Ceta-Dart, Copenhagen, Denmark) for stable isotope analyses.

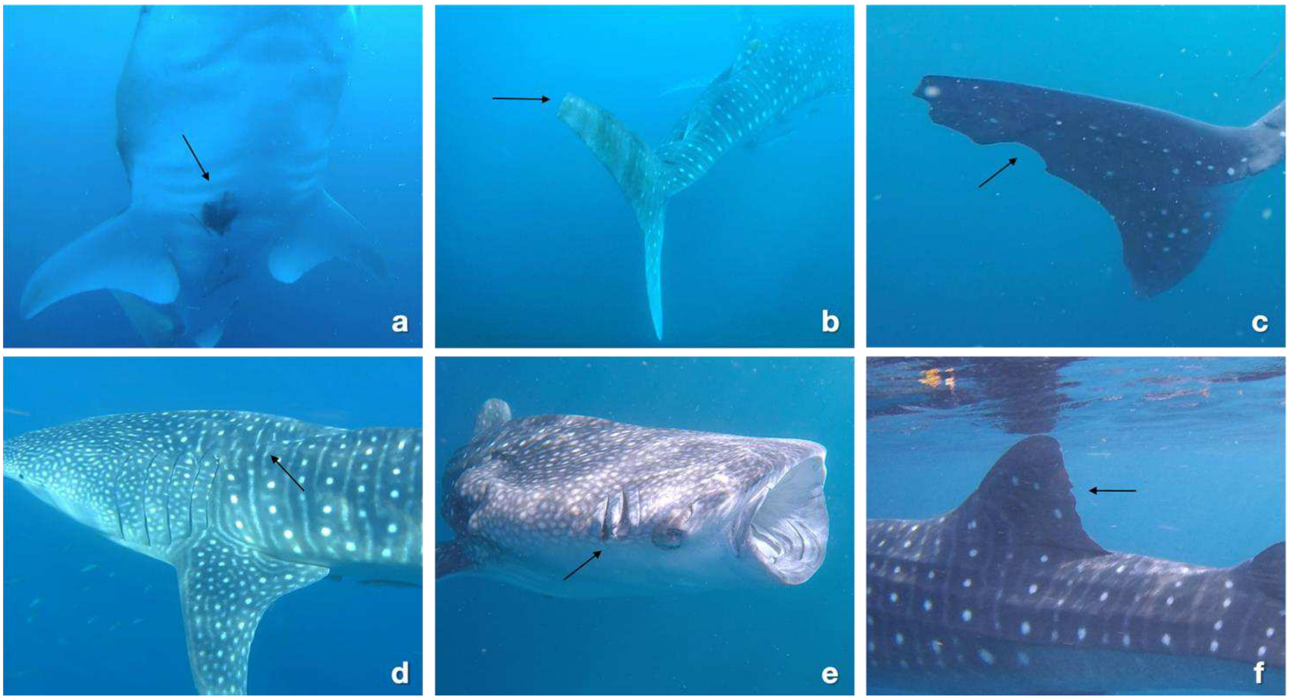

Fig. 3.2 Scarring categories: (a) abrasion, (b) amputation, (c) bite, (d) blunt trauma, (e) laceration, (f) nicks

3.2.3. Zooplankton collection and analyses

Samples of zooplankton were collected vertically at a depth of 3 m from surface using a 200 µm mesh net and immediately fixed in alcohol for taxonomical analyses. Biomass was calculated for 54 samples collected in season II, 68 in season III and 43 in season IV, by drying each sample at 60 °C and weighting it, using an analytical balance with a readability of 0.1 mg (Gibertini, Italy), until no weight variations were detected. The calculated biomass was expressed as mg m^{-3}. To take into account the presence of gelatinous zooplankton, in particular ctenophores, wet weights of zooplankton samples were measured in situ and again in laboratory, to measure possible differences in water loss from organism's destruction.

A total of 4 zooplankton samples collected in S2 were analyzed by microscopy for taxonomy characterization. For each of these samples, organisms were recognized, counted and their densities expressed as number of individuals per m^3. Density assessment followed the same methodology described in Boldrocchi et al. (2018). Results from 14 zooplankton samples from S1 and S2 (7 during feeding event and 7 in control areas) from Boldrocchi et al. (2018) were used together with 4 samples collected in the present study to characterize the zooplankton composition during feeding event and to evaluate possible differences between control and hotspot stations.

3.2.4. Chlorophyll-*a* Data

Annual variation of chlorophyll-a in the Gulf of Tadjoura was retrieved from Moussa (2016) (Fig. 3.3). Satellite derived monthly estimates of chlorophyll-*a* ($mg\ m^{-3}$) were also extracted for November-January 2017, October-January 2018 and November-January 2019. Monthly mean chlorophyll-*a* was derived from the Globcolour observation program from the Moderate Resolution Imaging Spectroradiometer (MODIS) with a 4x4 km spatial grid resolution. The time range corresponded to those of zooplankton sampling period. Data were downloaded from http://www.globcolour.info/

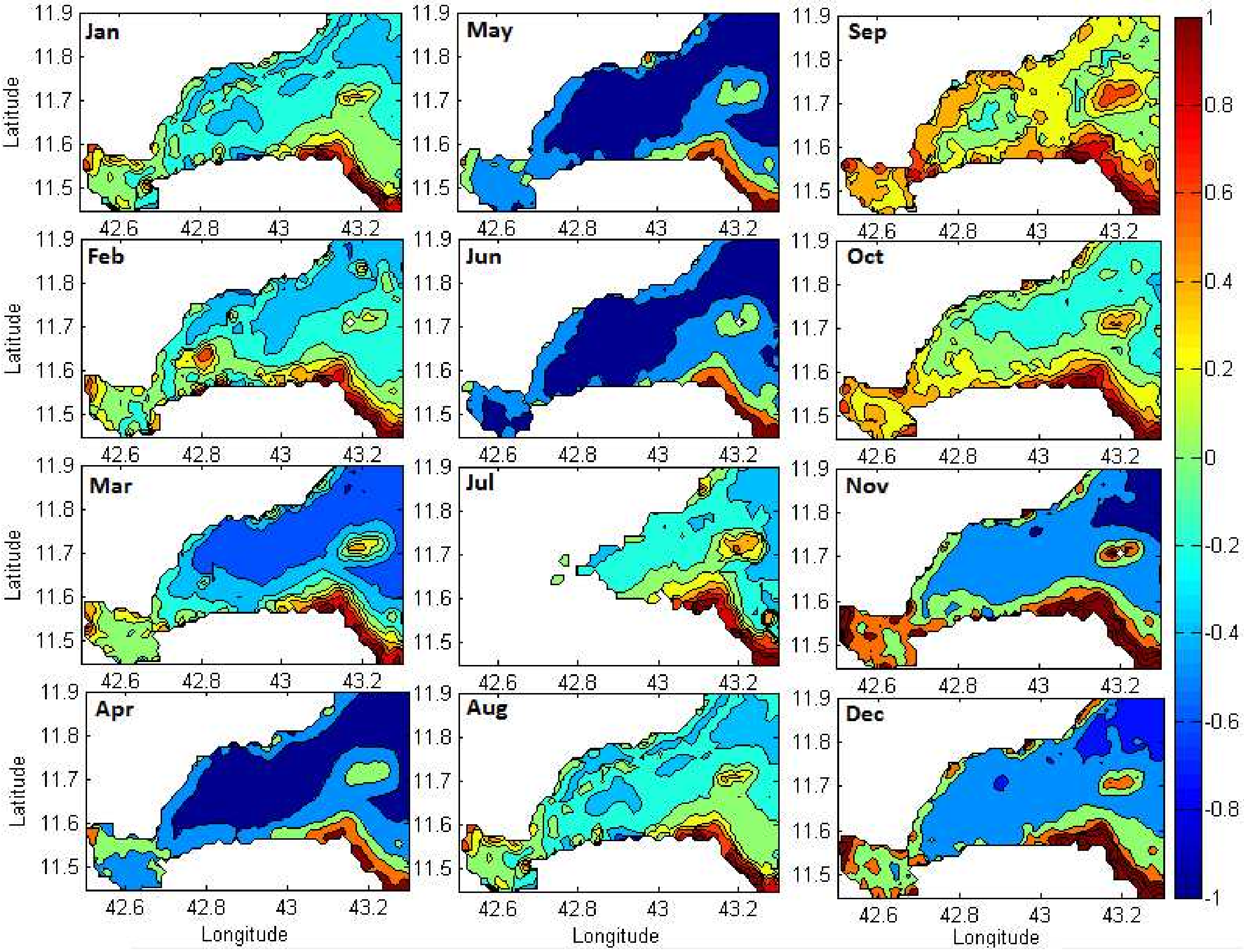

Fig. 3.3 Monthly chlorophyll-*a* variation in the Gulf of Tadjoura. Image was taken from Moussa (2016) with the author's permission

3.2.5. Spatial Analyses

All whale shark encounter locations reported through the sampling seasons were input to QGIS version 3.4.4 (QGIS Development Team 2017). The "kernel density tool" was used to calculate occurrence magnitude per km^2. The Kernel density estimates represents values for the estimated number of sightings per unit effort (sightings per hour of searching effort during boat surveys) per km^2 that are likely to occur within each grid cell. From these values, utilization distribution within the study area was defined as the minimum area in which an individual had a 95% probability of being located (Worton 1995). Hereafter, the 95% utilization distribution was referred to as home range within the study area, while the 50% was used to reveal core portions of coastline where whale sharks concentrated their activity.

3.2.6. Stable Isotope Analyses

To determine C and N isotopic compositions of zooplankton (N = 3) and whale shark skin tissues (N = 9), approximately 1 mg of freeze-dried samples was weighed in aluminum capsules (5 × 9 mm) and sent to Isotope Laboratory (Department of Biosciences University of Oslo, Norway). The analytical precision of analysis (i.e. standard deviation), based on laboratory internal standard, was 0.08‰ for carbon and 0.09‰ for nitrogen. Isotope ratios were calculated according to the following equation:

$$\delta^{13}C \text{ and } \delta^{15}N = [(R_{sample}/R_{standard}) - 1] * 1000$$

where, R equaled $^{13}C/^{12}C$ for $\delta^{13}C$ and $^{15}N/^{14}N$ for $\delta^{15}N$ and standards were Vienna PeeDee Belemnite and atmospheric N_2 for C and N respectively.

3.2.7. Statistical analyses

Statistical significance was tested at $p = 0.05$. Pearson's chi-squared test was performed to determine any bias among sex-identified whale sharks from an assumed 1:1 male to female ratio. To test any statistical differences in zooplankton composition between control and hotspot stations, a T-test was performed, after verified that assumptions of normality and homogeneity of variance were respected. To take into account the presence of gelatinous zooplankton, Wilcoxon signed rank test was used to test any differences in wet weight measured in situ to those in laboratory, since data were not normally distributed and homogeneity of variance was violated. Similarly, Wilcoxon/Kruskal-Wallis test was performed to test any statistical differences in zooplankton biomass between control and hotspot stations. A T-test for independent data was used to evaluate any statistical differences in term of zooplankton biomass between the western and the eastern side of hotspot S2. One-way Anova and the post-hoc Tukey test for pairwise comparisons were performed to evaluate any difference in zooplankton biomass between the 4 stations for each sampling year. Log transformation was used to normalize data for 2017.

The same tests were used to observe differences in the zooplankton biomass between the analyzed months (October-February). Log transformation was used to normalize data. Considering only S2 and S4, Wilcoxon/Kruskal-Wallis and Steel-Dwass tests were performed to evaluate any statistical differences in the whale shark sightings per unit effort between the analyzed months. Simple linear regression was used to test whether zooplankton biomass was positively correlated with the number of individual whale sharks encountered. Data were [log10 (x + 1)] transformed. The relationship between chlorophyll-a concentration and zooplankton biomass was tested using linear regression model, after a [log10 (x + 1)] transformation of chlorophyll-a data. The statistical analyses were conducted using JMP 14.1 software.

3.3. RESULTS

3.3.1. Photo-identification

A total of 190 individuals were photo-identified from 2015 to 2018. Of the 136 individuals for which sex was determined, 115 were males (84.6%) and 21 females (15.4%). Overall, the number of males was statistically greater than females ($X^2 = 65.0$, df = 1, $p < 0.0001$), however, comparing the 4 years of sampling, in 2018 the number of females was statistically higher than those measured in 2015-2017 ($X^2 = 15.0$, df = 3, $p = 0.0009$).

Of the 190 individuals identified, 21 were encountered in the first season, 80 during season II, 57 in season III and 70 in season IV (Fig. S1 in Supplementary Material I). Among the 80 whale sharks identified in season II, 68 were new individuals, while 12 re-sightings (Table 3.1). Within the season, 70% of sharks were encountered only one time, while 30% multiple times. During season III, 57 individuals were photo-identified of which 39 were new specimens and 18 recaptures (Table 3.1). Overall 49.1% of whale sharks were encountered only once and 50.9% multiple times. Among the 70 whale sharks identified in season IV, 62 were new individuals and 8 re-sightings from previous seasons (Table 3.1). Within the aggregation period, 52.9% of sharks were encountered only one time, while 47.1% multiple times.

Table 3.1 Inter-annual re-sight rates for individual sharks first identified in the Djibouti aggregation from 2015 to 2018

Year	Identified shark	2015 re-sight rate (%)	2016 re-sight rate (%)	2017 re-sight rate (%)	2018 re-sight rate (%)	Total re-sight rate (%)
2015	21	NA	NA	NA	NA	NA
2016	80	15	NA	NA	NA	15
2017	57	12.3	19.3	NA	NA	31.6
2018	70	1.4	1.4	8.6	NA	11.4

Within each sampling season, the maximum number of times an individual was re-sighted was 11 in 2017, 7 in both 2017 and 2018, and 4 in 2015 (Fig. 3.4). Excluding the single-sighting sharks, 62.8% (N = 54) of individuals were observed in one season, 29.1% (N = 25) in two and 8.1% (N = 7) in three years. None individual was encountered in all consecutive seasons.

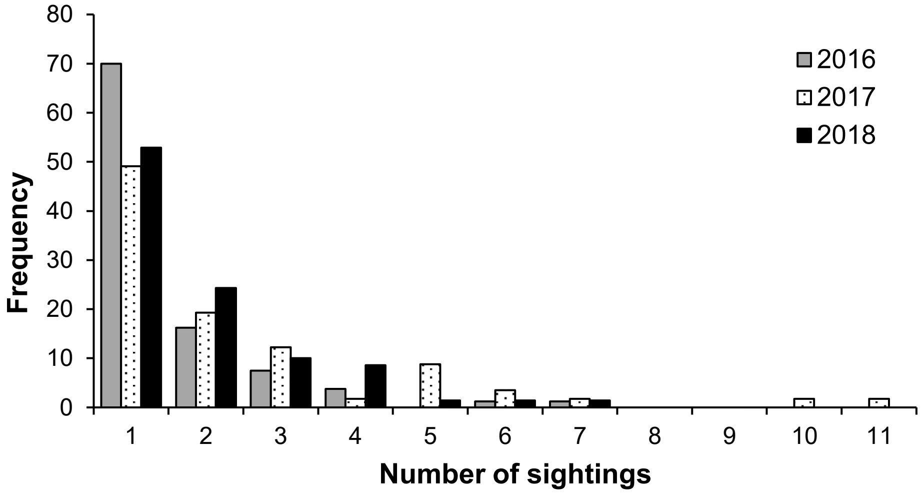

Fig. 3.4 Histogram represents the frequency distribution of whale sharks based on the number of sightings for each sampling season in Djibouti

A total of 93 whale sharks (49%) encountered during the whole study had scarring. In 2015 the percentage of wounded sharks was 48% (N = 10), in 2016 45% (N = 36), while in 2017 the scarred individuals increased to 72% (N = 41). In 2018, the percentage decreased to 34% (N = 24). Overall, nicks were the most abundant scar category, followed by abrasions and lacerations (Table 3.2). The most affected areas of the whale shark's body were the caudal and pectoral fins with a mean percentage of 31% and 24% (Table 3.2). Among the scarred individuals, 60 sharks were recaptured along the study. Photographic comparison of wounds showed a complete recovery in 10% of sharks (N = 6). In addition, a partial recovery was observed in the laceration of an individual between two consecutive sampling seasons (Fig. S2 a-b in Supplementary Material I), while a short term recapture (a month distance) of another shark showed an evident improvement of a severe wound caused by a boat propeller (Fig. S2 c-d in Supplementary Material I).

Table 3.2 Percentages of injured whale sharks, according to scar type and body area, in Djibouti in 2015-2018

Scars and Wounds		**2015**	**2016**	**2017**	**2018**	**Mean ± sd**
Type of scar	Nicks	38%	33%	49%	81%	50 ± 22
	Abrasion	19%	29%	29%	11%	22 ± 8
	Laceration	19%	24%	7%	3%	13 ± 9
	Blunt Trauma	12%	6%	7%	3%	7 ± 3
	Amputation	6%	7%	4%	2%	5 ± 2
	Bites	6%	2%	3%	0%	3 ± 3
	Others	0%	0%	1%	0%	0 ± 1
Body area	Caudal fin	25%	25%	28%	46%	31 ± 10
	Pectoral fins	31%	25%	16%	22%	24 ± 5
	Dorsal fin	6%	20%	27%	21%	19 ± 9
	Back side	19%	6%	7%	3%	9 ± 7
	Head	13%	6%	9%	5%	8 ± 4
	Side	6%	14%	12%	3%	9 ± 5
	Ventral side	0%	4%	1%	0%	1 ± 2

3.3.2. Feeding ecology

Analyses of 11 zooplankton samples from 2016 and 2017 collected in presence of ≥ 2 whale sharks showed a taxonomic composition mainly composed by copepods (85%), with more than half of all specimens identified were calanoids (53.9%) and poecilostomatoids (26.9%). The most representative families were: Paracalanidae (25.9%), Oncaeidae (15.5%), Corycaeidae (11.3%), Calanidae (10.8%) and Acartiidae (5.9%) (Table S1 in Supplementary Material I). Among non-copepods (15%), Chaetognata (4.9%), Appendicularia (2.4%) and Luciferidae (2.2%) were the most abundant taxa (Table S1 in Supplementary Material I). Similarly, control station samples were mainly characterized by calanoids (51.8%), poecilostomatoids (18.6%) and harpacticoids (16.8%). Among non-copepods (10.4%), Appendicularia represented 3.3%, while Luciferidae and Chaetognata approximately 1%, respectively. Indeed, the mean density of Chaetognatha was statistically higher in presence of feeding whale sharks than in control areas (t = 4.23; df = 17; p = 0.0005) (Fig. S3 in Supplementary Material I).

To take into account the presence of gelatinous zooplankton, in particular Ctenophora, possibly *Bolinopsis spp.* (Fig. S4 in Supplementary Material I), wet weights of zooplankton samples measured in situ were compared with those in laboratory. A statistically difference was found in the wet weights measurements of samples collected in the hotspot ($Z = -31.5$, $p < 0.05$), but not in those collected in absence of whale sharks ($p > 0.05$).

3.3.3. Spatial analyses

A total of 503 encounters were reported from the Gulf of Tadjoura. Ras Korali was the area with the highest number of sightings per unit effort were (N = 308) (Fig. 3.5), followed by La Faille with 166 encounters. Other areas, Devil Island, Boutres Island, Ras Eiro and Arta Beach, comprised all together 5.8% of total encounters. The home range of the whale shark community was 41.3 km^2 (95% kernel) and comprised two main areas, one in the La Faille area (17.9 km^2) and the second one between Ras Korali and Boutres Island (23.4 km^2) (Fig. 3.5). The core area was 1.98 km^2 in Ras Korali (50% kernel, Fig. 3.5).

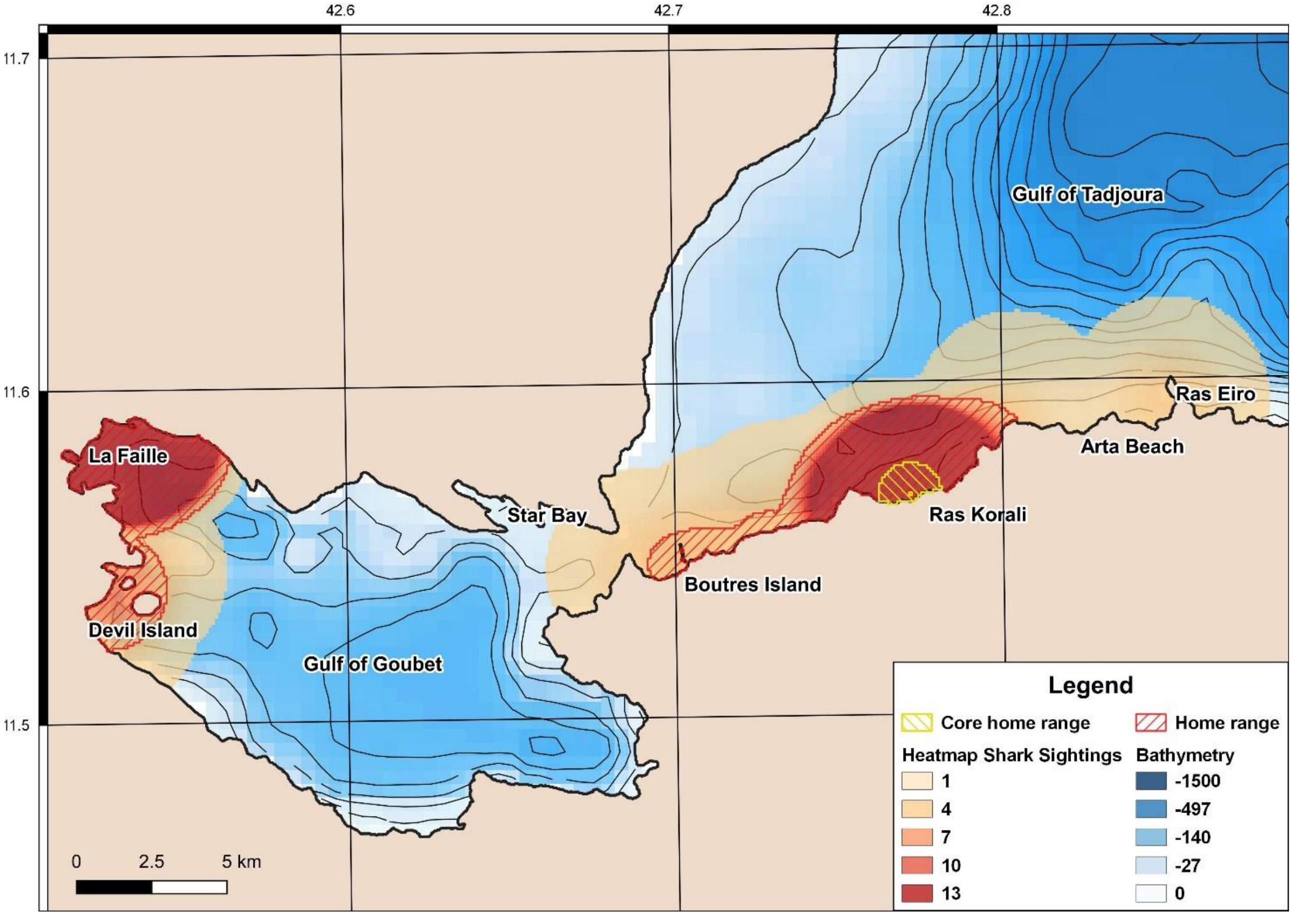

Fig. 3.5 Kernel density analysis of effort weighted whale shark sightings (N = 503) in the Gulf of Tadjoura in 2015-2018, showing the home range (dashed red) and core area (dashed yellow)

Mean zooplankton biomass calculated as dry mass from control stations was 27.3 ± 21.4 mg m^{-3} (N = 40), with the lowest mean biomass at S3, (25 ± 14.4 mg m^{-3}, N =16) and the highest at S1 (28.9 ± 25.2 mg m^{-3}, N = 24). Mean zooplankton biomass in whale shark feeding samples was 52.5 ± 33.3 mg m^{-3} (N =31) in S4 and 37.8 ± 30.5 mg m^{-3} (N =73) in S2. Analyses aimed to evaluate any differences in zooplankton biomass between control (27.3 ± 21.4 mg m^{-3}) and hotspot stations (42.2 ± 31.9 mg m^{-3}), showed that bays with whale sharks were statistically higher in term of biomass (Z = -2.85; p = 0.0043) (Fig. 3.6).

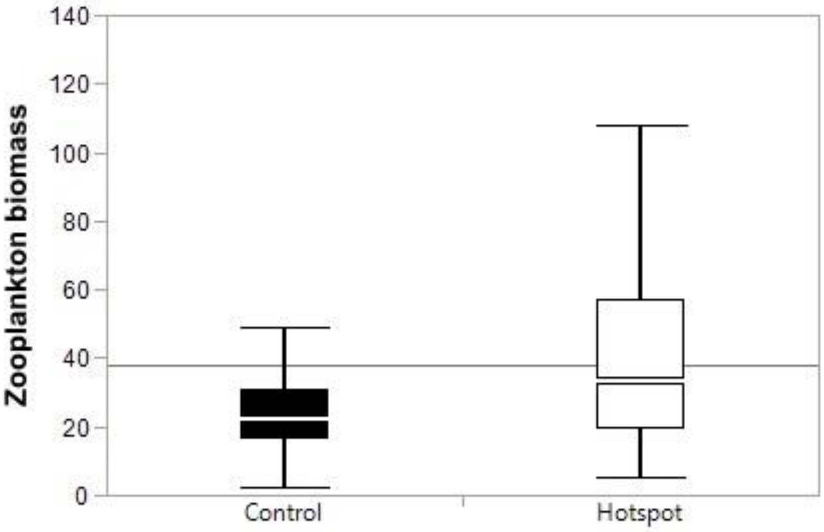

Fig. 3.6 Box plots showed the mean zooplankton biomass (mg m^{-3}) at control (black boxplot) and hotspot (white boxplot) stations collected in Djibouti in 2016-2018

Within the hotspot S2, zooplankton samples collected in the western side of the bay, where whale sharks aggregate to feed, showed a statistically higher biomass than the eastern side (t = 3.08; df = 14.5; p = 0.0079) (Fig. S5 in Supplementary Material I).

Considering each year separately and only November-January, in 2016 zooplankton samples collected from each station did not statistically differed from each other in term of biomass (F (3, 44) = 1,707, p = 0.1794). In 2017, the hotspot station S4 (83.9 ± 39.4 mg m^{-3}) showed a zooplankton biomass statistically higher than control stations S1 (38.2 ± 32.5 mg m^{-3}) and S3 (33.5 ± 18.4 mg m^{-3}) (F (3, 48) = 4.057, p = 0.0120). Similarly, in 2018 the hotspot S4 (39.6 ± 22.5 mg m^{-3}) showed a zooplankton biomass statistically higher than S1 (13.7 ± 6.9 mg m^{-3}) and S2 (19.7 ± 14.2 mg m^{-3}) (F (3, 34) = 4.708, p = 0.0075).

3.3.4. Temporal analyses

A temporal trend in zooplankton biomass was found in the Gulf of Tadjoura, with an increase in biomass from October (21.8 ± 11.9 mg m^{-3}) to December (49.6 ± 46.1 mg m^{-3}) and decrease up to February (6.6 ± 3.3 mg m^{-3}) (Fig. 3.7). One-way Anova showed a statistical difference in the zooplankton mean biomass between the analyzed months (F (4, 158) = 7.323, p = <0.0001). The Post-Hoc Tukey test revealed a statistical difference between biomasses measured in November-January with those in February ($p<0.05$).

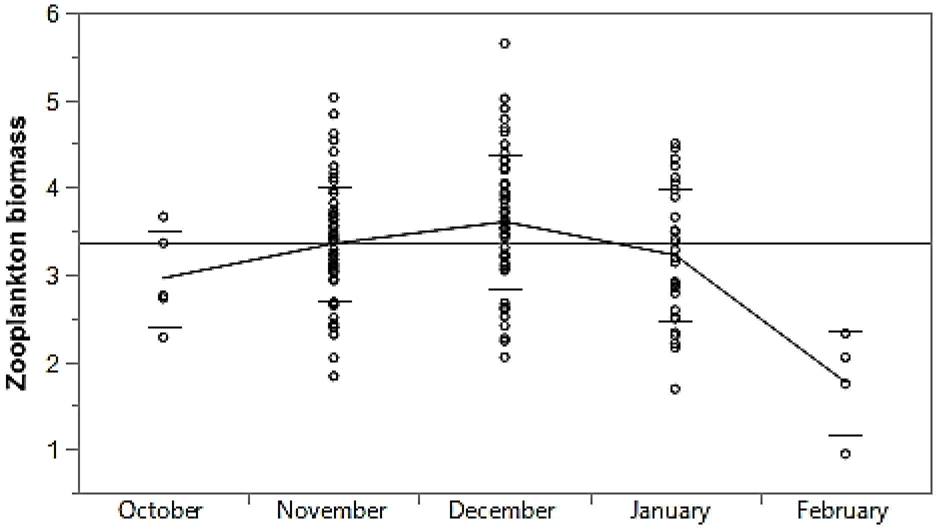

Fig. 3.7 Temporal variation in zooplankton biomass (mg m^{-3}) along the sampling period. Biomass values were presented on a logarithmic scale. Black line represents the average values

Similarly, a temporal trend in whale shark sightings was found in the Gulf of Tadjoura, with an increase from October to November and decrease up to February (Fig. S6 in Supplementary Material I). Wilcoxon/Kruskal-Wallis showed a statistical difference in the number of whale shark sightings between the analyzed months (X^2 = 25.93, df = 4, p = <0.0001). The number of sightings measured in November and December were higher than those in January and February ($p<0.05$).

Weekly whale shark sightings per unit effort correlated significantly with mean zooplankton biomass over the sampling period (r = 0.65; N = 22; p = 0.0012) (Fig. S7 in Supplementary Material I). The relationship was more evident in January, when zooplankton biomass had already started to decrease ($R^2 = 0.6$; N = 22, P < 0.0001; Fig. 3.8).

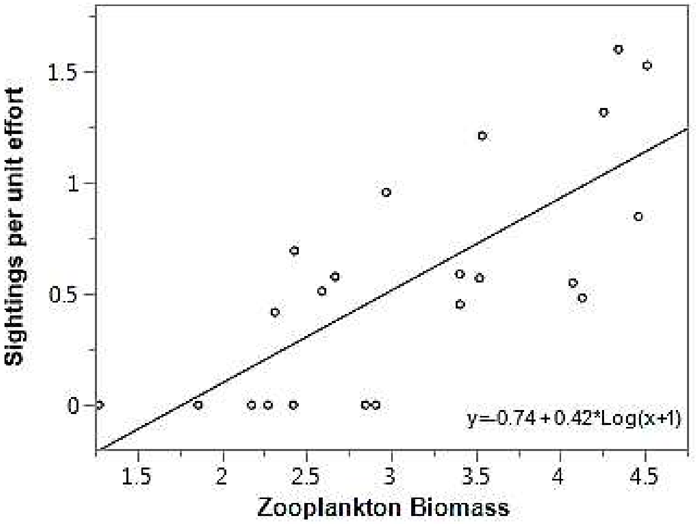

Fig. 3.8 The relationship between zooplankton biomass and total sightings per unit effort [log10 (x + 1)] recorded off Djibouti area in January ($R^2 = 0.6$, N = 22, P < 0.0001)

In the present study, a positive relationship was found between monthly chlorophyll-a concentration (mg m^{-3}) and zooplankton biomass collected during the whale shark aggregation period ($R^2 = 0.62$; N = 11; p = 0.0042) (Fig. 3.9).

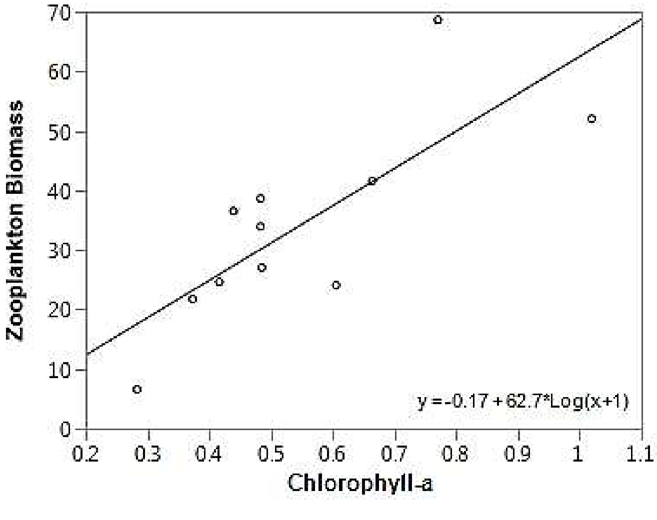

Fig. 3.9 Relationship between chlorophyll-a [log10 (x + 1)] concentration and zooplankton biomass recorded off Djibouti area in January ($R^2 = 0.62$; N = 11; p = 0.0042)

3.3.5. Stable Isotope Analyses

Stable isotope values for whale shark (N = 9) ranged from -19.4‰ to -14.6‰ (-15.8 ± 1.5‰) for $\delta^{13}C$ and from 7.8‰ to 11.8‰ (10 ± 1.1‰) for $\delta^{15}N$ (Fig. 3.10). No lipid normalization model was applied to $\delta^{13}C$ values as the C: N ratio of all samples was <3.5. Zooplankton samples (N = 3) were depleted in $\delta^{13}C$ and $\delta^{15}N$ compared with whale shark, indeed the mean $\delta^{13}C$ and $\delta^{15}N$ values were -18.2 ± 0.7‰ and 8.1 ± 0.9‰ respectively (Fig. 3.10). On average, whale shark was enriched in $\delta^{13}C$ by 2.4‰ and in $\delta^{15}N$ by 1.9‰ relative to the zooplankton samples.

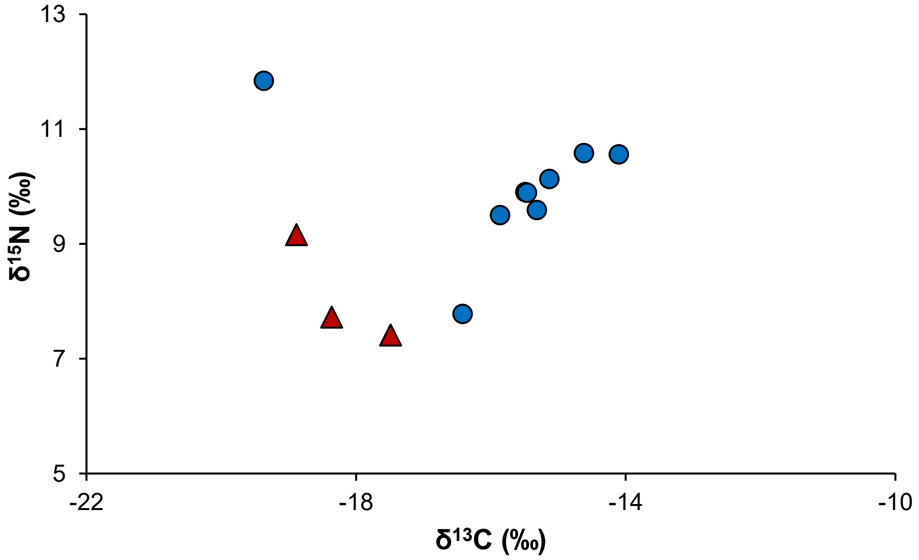

Fig. 3.10 Carbon and nitrogen isotopic signatures of zooplankton organisms (triangle) and whale sharks (circle) in Djibouti

3.4. DISCUSSION

A large number of whale sharks frequented the Gulf of Tadjoura in 2015-2018. If compared with other nearby aggregation sites, such as the Maldives, where 64 individuals were identified over 3 years (Riley et al., 2009), Shib Habil (Saudi Arabia) where 136 individuals were identified in 2010-2015 (Cochran et al., 2016) and Seychelles with 443 sharks identified over 9 years (Rowat et al., 2011), Djibouti hosts a considerable abundance of whale sharks. Rowat et al. (2011) reported 297 individuals identified in 2003-2010, which suggests that a greater number of animals might regularly aggregates in Djibouti. The identified individuals were mostly juvenile males, which is congruent to what previously reported by Rowat et al. (2011), confirming Djibouti as a male-based aggregation. However, the frequency of females was not constant over the sampling years, showing that in some seasons the percentage of females can increase significantly.

Forty-five percent of whale sharks identified in the study area were re-sighted at least once, with a maximum number of sightings of 11 within the same season. Furthermore, some sharks were regularly observed over each sampling season and among years, revealing that at least some individuals have prolonged residency in this region.

A considerable number of whale sharks encountered in the Gulf of Tadjoura were injured. Whale sharks spent a lot of time on surface and, consequently, were exposed to boat collisions and floating fishing gears. The lower percentage recorded in 2018 should not be understood as an improvement of protective measurements, since no effective conservation strategies aimed to reduce anthropogenic impact on animals have ever applied in the region. Whale sharks identified in the latter season were mostly small individuals (personal observation) recorded for the first time, which might explain why the percentage was lower than those recorded in previous seasons.

Whale shark distribution has been documented in coastal areas and has been associated with periods of high food availability (Wilson et al., 2001; Heyman et al., 2002; de la Parra Venegas et al., 2011; Rowat et al., 2011); however, this is the first study to spatially and temporally quantify the areas of highest suitability and the potential biotic drivers associated with the whale shark presence in Djibouti. Our study showed that whale sharks exhibited a clear temporal distribution over the Gulf of Tadjoura associated with the temporal variation in zooplankton biomass recorded in October-February. In this area, the monsoon winds and their seasonal reversal are the most important forces that govern surface circulation (Omar et al., 2016). From July to September (Fig. 3.3), the southwest monsoon induces eastward movement of surface water and enhances upwelling in the Gulf of Tadjoura (Omar et al., 2016), representing an important driver for the presence and abundance of whale sharks in the area. In line with data on chlorophyll-a concentration, results presented here showed that zooplankton population increased at the end of summer, reaching its maximum peak in December (Fig. 3.7). In winter, the arrival of northeast monsoon winds induce surface water flow toward the Gulf of Tadjoura (convergence), preventing the upwelling phenomenon (Fig. 3.3) (Omar et al., 2016). This caused a decrease in Chl-a along the gulf (Fig. 3.3), a subsequent decline in zooplankton bloom (Fig. 3.7), and the disappearance of whale sharks (Fig. S6 in Supplementary Material I). Indeed, results from this study suggest a strong positive relationship between the zooplankton biomass and abundance of whale sharks in the Gulf of Tadjoura, which was particularly evident when zooplankton biomass started to decrease (Fig. 3.8). Even if annual variation in zooplankton biomass was not provided, satellite derived data from the Gulf of Tadjoura clearly showed a decrease in the chlorophyll-a concentration from January to July (Fig. 3.3).

In regards to spatial distribution of whale sharks in the Gulf of Tadjoura, some bays resulted as preferred habitats, supporting the hypothesis that the probability of occurrence for whale sharks is higher in some areas, as environmental conditions are appropriate for the species and/or food availability (Hacohen-Domené et al., 2015). Kernel density analysis identified Ras Korali as the core area mainly utilized by whale sharks in October-February. While, an area of 41.3 km^2 was identified as the home range, comprising La Faille (S4) and the area between Ras Korali and Boutres Island (Fig. 3.5). Indeed, the southwest monsoon enhances upwelling preferentially at the extreme west and southeast part of the gulf (Omar et al., 2016). Therefore, some areas are expected to retain more nutrients than others. Both S2 and S4 stations are entrenched area, protected from the wind, and therefore phytoplanktons accumulate after their transport by sea currents. Indeed, the affinity to these main hotspots were strongly linked to their zooplankton biomass values. Sites with highest zooplankton biomass coincided with those where whale shark abundance was greatest, indicating that the abundance of this species in Djibouti was positively related to food availability. Whale sharks were feeding in zooplankton patches with a mean of 42.2 ± 31.9 mg m^{-3}, almost 1.5 higher than the biomass found in control samples. This agrees with reports for other aggregation areas (Clark and Nelson, 1997; Colman, 1997; Wilson et al., 2001; Ketchum et al., 2013; Robinson et al., 2013; Cárdenas-Palomo et al., 2015).

In addition to biomass, zooplankton composition might play an important role as well. Rowat et al. (2007) reported that in the Gulf of Tadjoura, chaetognaths formed dense swarms in surface waters and the zooplankton on which the sharks were feeding were dense concentrations of them. Results from this study confirmed a potential preference of whale sharks for these organisms. Indeed, in feeding samples the mean density of Chaetognatha was statistically higher than those in control areas. Chaetognatha plays a key role in pelagic food webs as the dominant predators of copepods and as a significant food source for a wide variety of larger organisms (Øresland, 1990, 1995). They are larger zooplanktonic organisms than copepods and are probably energetically richer (Postel et al., 2000); therefore, whale sharks might demonstrate a degree of preference for these groups since they can meet their energy requirements over a shorter time. Besides Chaetognatha, gelatinous zooplankton blooms, in particular Ctenophora, could be another important driver for the whale shark's presence in some bays. From personal observations, peaks in whale shark sightings coincided with those of Ctenophora (Fig. S4 in Supplementary Material I), however, sampling gelatinous zooplankton remains a major challenge for biological oceanography (Weisse et al., 2002). One reason for discrepancy between the results in net collection and in situ observations is that some species are considerably fragile that even a slight current is sufficient to destroy them or are usually broken into fragments when collected in plankton nets (Harbison et al., 1978). Of those that can be collected, most are destroyed by the subsequent preservation of samples (Harbison et al., 1978). Therefore, in this study, the contributions of gelatinous zooplankton to the calculated zooplankton biomass could not be quantified. This discrepancy could be the reason why the peak in zooplankton biomass in November did not corresponds to that of whale shark sightings (Fig. S6 in Supplementary Material I). Regardless of its limitations, this study provided some evidences that areas most visited by whale sharks in the Gulf of Tadjoura were also those with higher concentrations of gelatinous zooplankton and chaetognaths.

At present, there is a lack of knowledge of long-scale movements outside the aggregation season and whale sharks might migrate to different locations either remain year-round in Djibouti. Analyses of carbon signature in animals is particularly valuable for estimating diet sources and identifying animal movement patterns (Post, 2002; Peterson and Fry, 2003). The use of SIA to understand animal movements is based on the retention of stable isotope signatures in an organism tissue that reflect the local food web where the animal lived for an extended period of time (Michener and Schell, 1994; Peterson and Fry, 2003). Skin tissues, which are less metabolically active compared to muscles and livers, are characterized by long retention time and will reflect longer and less recent period of dietary feeding (Macneil et al., 2005). Therefore, the high enrichment value of $\delta^{13}C$ measured in whale shark skin tissue collected in November-December possibly reflect the isotopic signature of a different feeding area, prior the Djibouti upwelling season. Indeed, the isotope values of lower trophic level organisms can vary temporally and spatially (MacAvoy et al., 1998; Syväranta and Rautio, 2010) and highly depleted $\delta^{13}C$ values are indicative of pelagic feeding ($\delta^{13}C$ =-22% to -17%) while enriched values reflect more inshore and/or benthic foraging ($\delta^{13}C$ > -17%) (West et al., 2006). The $\delta^{13}C$ measured in whale shark (-15.8‰) falls within the range of inshore/benthic values, suggesting that its diet is preferentially based on inshore sources. In addition, the $\delta^{13}C$ values were highly clustered with minimal variability, suggesting that whale sharks shared a similar feeding habitat. Only one individual showed highly depleted $\delta^{13}C$, which might indicate a wider home range or different movement patterns compared with other whale sharks. Alternatively, as showed for *Manta alfredi* (Couturier et al., 2013; Burgess et al., 2016), the high $\delta^{13}C$ enrichment compared with surface zooplankton might suggest that part of dietary intake was comprised also by a more carbon-enriched mesopelagic sources. Results from fatty acid analyses in whale sharks confirmed that similar food sources were likely to be important prey items (Rohner et al., 2013). The absence of sharks at the surface during visual surveys has led to the assumption that sharks disperse to places unknown during the long ‘off-seasons’ at most of aggregation locations (Cagua et al., 2005). However, apparent emigration from an aggregation area may actually result in a small-scale shift in habitat use, as showed in Mafia Island where sharks shifted their activity area away from the surface to deeper areas, presumably for a shift in prey availability (Cagua et al., 2005).

Similarly to $\delta^{13}C$, most whale sharks showed little intra-specific variation in $\delta^{15}N$ values, except for two specimens, whose $\delta^{15}N$ were 11.8‰ and 7.8‰ (Fig. 3.10). The shark with higher $\delta^{15}N$ was also the one that did not cluster with other sharks in term of $\delta^{13}C$. This strong variability in both elements probably reflected different movements pattern. Shifts in baseline can influence food-chain length, which affects the relative trophic position of a predator that forages in different oceanic regions (Vander Zanden and Fetzer, 2007; Jennings et al., 2008). The intra-specific variation observed in $\delta^{15}N$ might be also a consequence of ontogenetic shifts in prey selection (Wetherbee and Cortes, 2004). As the size of the shark increases, the contribution to the diet of small fish and/or of zooplankton of larger size and higher trophic level increases (Borrell et al., 2011). Due to a lack in whale shark length measurements, we cannot drive conclusions if intraspecific differences in $\delta^{15}N$ are linked to heterogeneities in locations where individuals forage, or to an ontogenetic shift in diet. Further analyses will be addresses on this direction with the support of satellite tags technology, since top predators can undergo large-scale movements and have extensive foraging ranges.

3.5. CONCLUSIONS

Results from this study indicate that the spatiotemporal distribution of whale shark is significantly correlated with the variation in zooplankton biomass and chlorophyll-a concentration, due to the monsoon winds and their seasonal reversal. Indeed, the variability in their distribution is related to the effects of summer upwelling that generates biological productivity, and whale sharks use the Gulf of Tadjoura as a feeding ground from October through February. This study highlighted discrete areas within the gulf that are most visited by animals. The core habitat for this species is located within a marine protected area (MPA), however half of the sharks encountered in the Gulf of Tadjoura were injured, indicating a strong anthropogenic pressure. Results from this study demonstrated the urgent need to establish conservation measures aimed to reduce boat collisions and fishery interactions. This study provided primary information useful for developing a new management strategies for protection of this endangered species, including the introduction of a code of conduct to avoid potential disturbance derived from unregulated tourism. With the global increasing interest in human encounters with whale sharks, there is a clear need for implementation of monitoring initiatives to reduce anthropogenic disturbance (Haskell et al., 2015), especially in Djibouti where effective conservation measurements are still minimal.

3.6. ACKNOWLEDGMENTS

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CHAPTER IV. Whale shark foraging on baitfish off Djibouti

Whale shark foraging on baitfish off Djibouti

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ABSTRACT

In Gulf of Tadjoura, Republic of Djibouti, whale sharks (*Rhincodon typus* Smith, 1828) are known to feed on a wide variety of zooplankton organisms. However, for the first time in this area, seven juvenile whale sharks were filmed feeding on a school of baitfish (anchovies). During the event, zooplankton samples were collected and biomass was calculated. Results support the hypothesis that, when dense patches of zooplankton are not available in the Gulf of Tadjoura, whale sharks remain in the area and feed on alternative energy rich prey, such as baitfish, to meet their energetic demands.

4.1. INTRODUCTION

The whale shark (*Rhincodon typus* Smith, 1828) is a suction filter-feeder that inhabits all oceans from tropical to temperate latitudes (Colman, 1997). Several studies on its preferences indicates that whale sharks congregate in coastal habitats to feed on dense patches of zooplankton organisms (Colman, 1997; Compagno, 2001; Stevens, 2007; Rowat and Brooks, 2012). Preys include copepods (Clark and Nelson, 1997), krill (Gunn et al., 1999; Wilson et al., 2001; Taylor, 2007), crab larvae (Meekan et al., 2009), sergestids (Rohner et al., 2015), fish spawn (Heyman et al., 2001; De la Parra Venegas et al., 2011), gelatinous zooplankton and chaetognaths (Rowat et al., 2007; Taylor, 2007). Recent studies indicates that whale sharks feed on a wider foraging range, including demersal and deep-water macrozooplankton, and fishes (Duffy, 2002; Borrell et al., 2011; Rohner et al., 2013; Marcus et al., 2016).

In Djibouti whale sharks aggregate on a regular basis from October to February (Rowat et al., 2007, 2011; Rezzolla and Storai, 2010), using this area as feeding ground during those months (Boldrocchi et al., 2018). The whale shark occurrence might be related to an increase in zooplankton biomass due to the south-western monsoon upwelling (Boldrocchi et al., 2018). In Djibouti, whale sharks have been observed targeting dense patches of copepods chaetognaths, salps and mantis shrimp larvae (Rowat et al., 2007; Rezzolla and Storai, 2010). In the present note, we provide the first observation of whale sharks feeding on a school of baitfish (anchovies) at the beginning of the aggregation period.

4.2. MATERIAL AND METHODS

On 16 October 2017 in the Gulf of Tadjoura (11°34.596' N 42°47.468' E) at 11:30 am, 7 whale sharks were filmed and photographed feeding on a school of anchovies (Fig. 4.1, complete video footage provided in Supplementary Material II). The baitfish recognition was made by Dr Moussa from the Centre d'Etude et de Recherche de Djibouti, who was witness at the event.

During the described feeding event, a zooplankton sample was collected in proximity (< 20 m) of the school of fish and whale sharks. Other samples were collected on the 17 and 18 October 2017 in the same location. Samples were collected vertically at a depth of 3 m from surface using a 200 µm mesh zooplankton net. Biomass was calculated for all of them by drying each sample at 60 °C and weighting it, using an analytical balance with a readability of 0.1 mg (Gibertini, Italy), until no weight variations were detected. The calculated biomass was expressed as mg m^{-3}.

4.3. RESULTS AND DISCUSSION

During the encounter, whale sharks were repeatedly circling the small school of baitfish from beneath and from the sides, charging to the center through the fish-ball (Fig. 4.1a-d), with their mouths wide open (Supplementary Material II), similarly to what reported by Andrewartha (1993) in Ningaloo Reef. Whale sharks generally closed their mouths as they exited the bait-ball. Just after the mouth closed in a gulping motion, the gill slits opened widely (Fig. 4.1e-f). The described event lasted for approximately one hour until the school of fish dispersed and whale sharks swam away. A similar observation was reported six month later, in April, by sport fishermen (personal communication). During the encounter, it was not possible to capture any anchovy specimen for species recognition however, based on the description of fish distributions in Djibouti (Myklevoll, 1981; Hata and Motomura, 2017) and on the morphological characteristics of anchovies, the specimens that the whale sharks were feeding on might belong to the genus *Encrasicholina* or *Stolephorus*.

Fig. 4.1 Feeding behavior exhibited by a juvenile whale shark (*Rhincodon typus*) when targeting baitfish. Time lapse from a video taken on 16 October 2017 showing the shark charging the bait-ball (a), feeding on fish (b-d), eject the water through the gills (e-f)

Although recent studies, based on fatty acid and stable isotope analyses, indicates that whale sharks feed on a wider range of prey, besides pelagic zooplankton (Borrell et al., 2011; Rohner et al., 2013; Marcus et al., 2016), in literature, direct observations of whale sharks targeting baitfish are still controversial. In Bahıa de Los Angeles, Mexico, whale sharks were observed feeding among large schools of baitfish (sardines, anchovies), but never directly preying upon these fish (Nelson and Eckert, 2007). Nelson and Eckert (2007) speculated that the association of baitfish schools with whale sharks represent a means by which whale sharks find concentrations of zooplankton. However, whale sharks can capture large, more active nektonic prey such as fishes and squid (Taylor et al., 1983; Rohner et al., 2013; Marcus et al., 2016). Indeed, in New Zealand whale sharks have been reported suction-feeding on schools of anchovy (*Engraulis australis*) (Duffy, 2002), similarly to what observed in Djibouti.

The observation reported here might indicate that a wider range of prey, besides zooplankton organisms, play important roles in attracting the whale sharks in the Gulf of Tadjoura. Whale sharks tend to aggregate in areas of high productivity (Rowat and Brooks, 2012). Whale sharks off Mafia Island feed in high-density prey patches (25.2 ± 22.8 mg m^{-3}), comparing to no-feeding areas (2.5 ± 6.1 mg m^{-3}) (Rohner et al., 2015). In Djibouti, analyses on zooplankton biomass during the whale shark aggregation season revealed an increasing trend up to December, when the zooplankton biomass reaches its maximum peak of 41.3 ± 36.4 mg m^{-3} (Boldrocchi et al., 2018). The zooplankton biomass measured during the bait-ball feeding event (15 mg m^{-3}) and the following days (21.5 ± 11.9 mg m^{-3}) were lower than what measured in November and December (Boldrocchi et al., 2018), when whale sharks were feeding on zooplankton patches. Zooplankton samples were not collected during the whale shark sighting in April, however we can assume the biomass was lower than the aggregative period, since the north-eastern winds generate a northern anticlockwise gyre preventing upwelling in the area (Rixen et al., 1996).

Whale sharks, as other large filter-feeders elasmobranchs including basking sharks and mobulid rays, face the challenge of meeting high energetic demands while consuming preys, that are many order of magnitude smaller their own body (Sims and Quayle, 1998; Marcus et al., 2016; Rohner et al., 2017, Stewart et al., 2017). To enhance cost-efficiency, they feed on dense aggregations of preys (Rohner et al., 2015; Marcus et al., 2016; Rohner et al., 2017, Stewart et al., 2017). However, plankton abundance strongly varies through time and space (Haury et al., 1978; Baars et al., 1990; Sugimoto and Tadokoro, 1997) and, therefore, most whale shark feeding aggregations are highly seasonal. Horizontal shark movements are generally poorly known outside these times, although large scale movement research have been conducted in areas relatively close to Djibouti, including the Red Sea and off Qatar (Berumen et al., 2014; Robinson et al., 2017). In the Gulf of Tadjoura, movements of whale shark in relation to feeding patches have not been understood yet, especially outside the aggregation period, when the frequency of sightings decrease (Dr Moussa personal communication).

The absence of sharks at the surface during visual surveys has led to the assumption that sharks disperse to other long-distance places during the ‘off-seasons’ at most locations, including Djibouti (Cagua et al., 2015). However, recent studies in Mafia Island (Tanzania) and in the Arabian Gulf showed that the majority of satellite-tagged sharks remained in the region year-round (Cagua et al., 2015; Robinson et al., 2017). Giving recent findings, whale sharks might remain close-by Djibouti area during the ‘off-seasons’, feeding on alternative energy rich preys, such as baitfish. Baitfish might allow whale sharks to meet their energetic demands, when no dense zooplankton patches are available.

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CHAPTER V: First concurrent assessment of elemental- and organic-contaminant loads in skin biopsies of whale sharks from Djibouti

First concurrent assessment of elemental- and organic-contaminant loads in skin biopsies of whale sharks from Djibouti

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ABSTRACT

The Gulf of Tadjoura (Djibouti) is an important site where the whale shark (*Rhincodon typus* Smith 1828) aggregates seasonally. Because of the proximity to the port of Djibouti that is located along one of the busiest shipping area in the world, whale sharks are potentially exposed to relatively high levels of trace elements and organochlorines. To assess their contamination status, concentrations of 15 trace elements, DDTs and PCBs were concurrently assessed for the first time in skin biopsies of 20 whale sharks. Additionally, 12 zooplankton samples were collected and analyzed for trace elements content. Concentration of As, Cu, Zn and Se were higher compared with previous studies on whale sharks. Whale shark samples exceeded the maximum allowable limits for Cr, Pb Se, Cd and Zn. In regards to PCBs, the predominant congener were Tetra-CB, accounting for 40.8% of total PCBs and Penta-CB for 22.9%, while for DDTs, the predominant metabolite was DDE contributing for 51.4% of total compounds. The maximum residue limit for DDTs in fish set by USEPA (2000) was exceeded in 50% of whale sharks, while the EU regulation for the ind-PCBs in 62.5% of samples. The p,p′ DDE/ΣDDT ratios were higher than the critical value, likely indicating possible recent inputs of technical DDTs in the area. This study suggests some concerns for the contamination status of whale sharks in Djibouti and, since major threats for this species include fisheries catches, highlights some level of risk from the exposure to elemental- and organic-contaminant via shark consumption.

5.1. INTRODUCTION

Contamination of marine environments has been linked to increasing lethal and sub-lethal effects to individuals, populations, and ecosystems (Todd et al., 2010; Ley-Quiñónez et al., 2013). Due to the high persistency, toxicity and the ability to bioaccumulate in the marine food web, elemental- and organic contaminants cause harmful effects on the organisms health (Storelli and Marcotrigiano, 2001a; Gelsleichter et al., 2006; Gelsleichter and Walker, 2010). Indeed, chronic or intermittent exposure to organochlorine compounds (OCs) and trace elements results in severe effects on aquatic organisms at different levels including physiological, cellular and behavioural (Thophon et al., 2003; Jepson et al., 2005; Ylitalo et al., 2005; Gelsleichter et al., 2006). Nevertheless, despite an increasing number of research, primary information on the contaminant loads in most elasmobranchs species is still unknown, as well as the potential physiological effects of pollutants on sharks (Mull et al., 2012; Lee et al., 2015).

The whale shark (*Rhincodon typus* Smith, 1828) is a filter feeder with a worldwide distribution found from tropical to temperate latitudes (Stevens, 2007). The spatiotemporal distribution and abundance of this species appear to be influenced by oceanographic processes such as upwelling, coastal currents, and fronts, which increase the biological productivity in localized areas and enhance high concentrations of food (Wilson et al., 2001; Duffy, 2002; Cárdenas-Palomo et al., 2010; Sleeman et al., 2010; Sequeira et al., 2012). Within the Gulf of Tadjoura (Djibouti), whale sharks aggregate seasonally from October to February (Rowat et al., 2007, 2011; Rezzolla and Storai, 2010; Boldrocchi and Bettinetti, 2019), using this area as a foraging ground during this time (Boldrocchi et al., 2018; Boldrocchi et al., unpublished results).

The whale shark is highly vulnerable to overfishing due to its K-selected life history (Stevens et al., 2000; Rowat and Brooks, 2012; Dulvy et al., 2014) and is listed as endangered by the IUCN Red list since 2016. Indeed, the slow growth rate, late maturation and extended longevity make the whale shark particularly slow to recover from exploitation or habitat disruption, and recent evidences indicate that whale sharks are declining in number (Stevens et al., 2000; Rowat and Brooks, 2012; Dulvy et al., 2014). Major threats include the growing market for shark products, targeted fisheries, bycatch as well as vessel strikes and inappropriate tourism (Hsu et al., 2012; Capietto et al., 2014; Dulvy et al., 2014; Pierce and Norman, 2016). Although Djibouti has adopted protection measures for whale shark conservation, so that capture and/or trading are forbidden (Rowat and Brooks, 2012), other concerns come from anthropogenic activity nearby the feeding ground of this species in the Gulf of Tadjoura (Boldrocchi et al., 2018). Indeed, the increasing human pressure in Djibouti has given rise to organic and inorganic contamination from industrial activities, wastewater discharge, vessels, agriculture run-off and organic as well as solid waste (Ahmed et al., 2017). Although pollution has not yet considered the main threat to shark survival, the negative effects on the organism health may worsen the already alarming situation (Camhi, 1998). Despite the growing scientific attention on the ecotoxicology of large filter feeding marine animals, considered as an early warning of the presence of a mixture of contaminants in the marine food web (Fossi et al., 2014, 2017), only few studies have been carried out since now. The presence of toxic trace elements has been investigated in whale shark skin biopsies collected from the Gulf of California (Pancaldi et al., 2019a), in two stranded individuals from the same area (Pancaldi et al., 2019b), in three specimens from South Africa (McKinney et al., 2016) and in bycatch specimens from the Chinese coast (Wang et al., 2014). In regards to persistent organic pollutants, only one study has been published on whale sharks from Mexico (Fossi et al., 2017).

With the global decline in shark numbers, evaluating the extent of contaminant loads and their impact on shark health is an urgent aspect, especially for species considered endangered (Camhi et al., 1998). To redress the knowledge gap on pollutant loads in the whale shark, especially in the Gulf of Aden, this study aims to investigate for the first time the presence of 1) legacy contaminants (PCBs and DDTs), 2) trace elements in skin tissues from Djibouti and 3) the biomagnification factor of trace elements in whale sharks. Furthermore, since whale sharks are routinely captured in several locations, including China and North-Western Indian Ocean (Riley et al., 2009; Li et al., 2012), this research aims to evaluate 4) the exposure health risks derived from the consumption of this species.

5.2. MATERIAL AND METHODS

Sharks examined for this study were collected from Djibouti (latitude 11°35'20.44"N, longitude 43°08'42.11"E), within the Gulf of Tadjoura (Fig. 5.1).

Skin biopsies were collected from 20 different whale sharks in November and December 2016-2018. Tissue samples were obtained by biopsy punch (using 30 mm sampling tips) on a pole spear (Ceta-Dart, Copenhagen, Denmark). Nine biopsies were used for PCBs and DDTs analyses however, due to the small amount of tissue, two biopsies were pooled for one analyses. For trace elements investigation, 12 biopsies were collected and analyzed individually, that included one sample from those evaluated for OCs compounds.

The collection of zooplankton samples (N = 12), at a depth of 3 m with a 200 μm mesh net, was performed when sharks were observed feeding. Sample were immediately stored on ice.

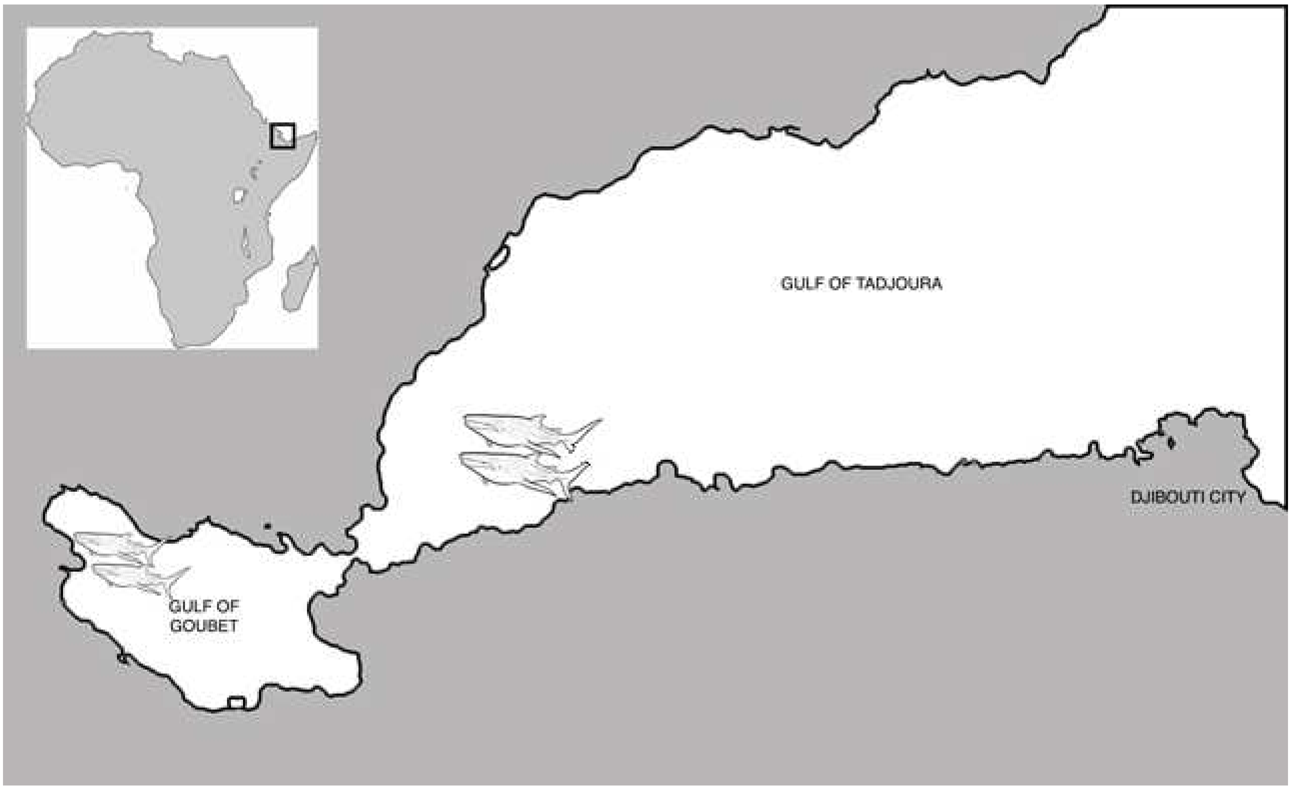

Fig. 5.1 The Gulf of Tadjoura within the Republic of Djibouti

5.2.1. PCBs and DDTs analyses

All frozen skin biopsies were freeze-dried and, subsequently, extracted following Bettinetti et al. (2016). The extracted samples were reduced to a final volume of 0.5 ml and, using a 1 µl on-column injection system, analyzed by gas chromatography (GC Carlo Erba, Top 8000) coupled with ^{63}Ni electron capture detector (Carlo Erba ECD 80). A capillary column CP-Sil-8 CB (50 m × 0.25 mm I.D., film thickness 0.25 µm, Varian, USA) was used for the separation, with helium as carrier gas (1 ml min^{-1}) and nitrogen as auxiliary gas (30 ml min^{-1}). The temperature program followed Bettinetti et al. (2016). External reference standards of p,p′DDT, p,p′DDE and p,p′DDD (Pestanal, Sigma-Aldrich, Germany) in isooctane (Carlo Erba, pesticide analysis grade) were used for sample quantification of DDTs, while Aroclor 1260 (Alltech, IL, USA) with the addition of PCB 28 and 118, for PCBs quantification. In this study, 13 different congeners were determined: PCB 18, PCB 28, PCB 31, PCB 44, PCB 101, PCB 118, PCB 138, PCB 149, PCB 153, PCB 170, PCB 180, PCB 194 and PCB 209. Depending on the organochlorine compound, detection and quantification limits varied from 0.1 to 0.5 and from 0.5 to 1 ng g^{-1} lw (lipid weight), respectively. Good laboratory practices were used when testing the standard reference materials BCR-598 and BCR-349 (Community Bureau of Reference, Brussels) for DDTs and PCBs residues, respectively. The percentages of recovery for the DDTs were 107.5 ± 4% (p,p′DDE), 106.2 ± 4% (p,p′DDD), and 106.2 ± 3% (p,p′DDT). The recoveries for the PCBs were between 91.3 ± 1.1% and 102.2 ± 1.6%.

5.2.2. Trace Element Analyses

In the present study, concentrations of 15 trace elements (As, Cd, Cu, Fe, Mn, Mo, Hg, Se, Sr, Zn, Cr, V, Co, Ni, Pb) were determined in skin biopsies of whale sharks and zooplankton samples. The analytical procedure followed Boldrocchi et al. (2019) and was validated through the analysis of certified reference materials BCR®-414, from the Institute for Reference Materials and Measurements in Belgium for zooplankton samples (Supplementary material III, Table S1). The procedure is briefly outlined in the following. Freeze dried biopsy (≈30 mg) and zooplankton (≈100 mg) samples were dissolved by microwave assisted digestion in an ETHOS One oven (Milestone) employing 2 ml of pure nitric acid produced by a sub-boiling Milestone DuoPur equipment (Monticelli et al., 2019). The microwave program was as follows: ramp to 50 °C: 10 min; isotherm 50 °C: 5 min; ramp to 100 °C: 5 min; isotherm 100 °C 20 min. The resulting solution was diluted to 30 ml in LDPE bottles. Ultrapure water produced by a Sartorius arium mini system was used throughout for sample dilution and standard preparation. All laboratory materials (sample bottles and digestion vessels) were decontaminated in a 3-stage process by soaking in a 0.4% w/w detergent solution (Nalgene L900, one week) rinsing with ultrapure water and followed by 2X soaking in a 2%, w/w HNO_3 solution (one week) and rinsing with ultrapure water. The quantification of trace elements was performed by Inductively Coupled Plasma–Mass Spectrometry (ICP-MS, mod. iCAPQ from Thermo Elemental, www.thermo.com), using a kinetic energy discrimination (KED) configuration and a standard addition method for calibration. Standard solutions were obtained by diluting a multistandard obtained from Merck (ICP multi-standard solution XXI for MS). Skin tissues were converted from dry weight (dw) to wet weight (ww) taking 70% of moisture (Bergés-Tiznado et al., 2015; Ooi et al., 2015) in order to compare the results from the current study with those from literature.

Biomagnification factor (BMF) was calculated using the equation (Gray, 2002): $BMF = C_D/C_P$, where C_D is the element concentration of the predator and C_P the one of the prey, and it is based on the assumption that the concentration in whale shark biopsies has reached steady state. The increase in concentration between trophic levels is determined when the calculated biomagnification factor is significantly > 1.0 (Bergés-Tiznado et al., 2019).

5.2.3. Statistical Analyses

All biomagnification factors were tested for significance using the T-test. To test any difference in the concentration of PCB congeners in whale sharks, the Kruskal-Wallis Anova test was used, since data was no normally distributed. The Post hoc Steel-Dwass method was performed to examine the mean differences among congener concentrations. Significance level used in study was set at $p < 0.05$.

5.3. RESULTS

5.3.1. Trace elements

Concentrations of selected trace elements in zooplankton samples and skin tissues of 12 whale sharks are showed in Table S2 (Supplementary material III). Fe and Sr had the highest concentrations in whale sharks (716 ± 481 and 516 ± 153 mg kg^{-1}, respectively), followed by Zn (126 ± 142 mg kg^{-1}), Ni (105 ± 60.5 mg kg^{-1}), Cr (61.4 ± 65.7 mg kg^{-1}), Mn (44.9 ± 33.7 mg kg^{-1}) and Cu (26.9 ± 36.6 mg kg^{-1}) (Fig. 5.2). A third group of elements, comprising Cd, Hg, V, Se, As, Pb, Mo and Co, was found to have lower concentrations and ranged from a maximum of 4.1 ± 3.7 mg kg^{-1} for Pb to a minimum of 0.2 ± 0.2 mg kg^{-1} for Hg.

In zooplankton samples, Fe (1136 ± 1135 mg kg^{-1}) and Sr (5110 ± 4858 mg kg^{-1}) were in highest concentrations, followed by Pb (231 ± 246 mg kg^{-1}) and Zn (242 ± 155 mg kg^{-1}) (Fig. 5.2). On the contrary, Mo (1.33 ± 0.70 mg kg^{-1}), Hg (1.14 ± 1.65 mg kg^{-1}) and Co (1.61 ± 0.93 mg kg^{-1}) were in lower concentrations (Fig. 5.2).

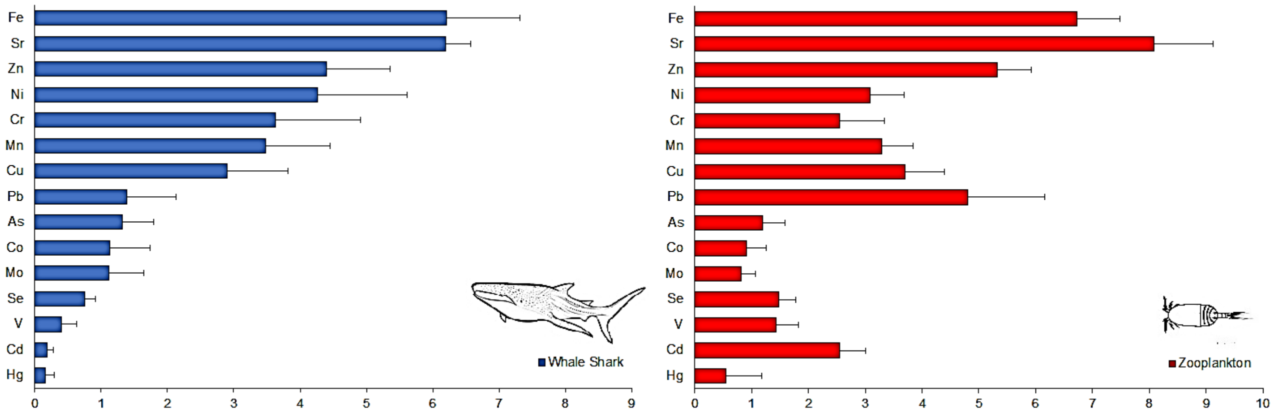

Fig. 5.2 Trace elements concentration (mg kg^{-1} dw) in whale shark skin tissues (left panel) and zooplankton samples (right panel) collected in Djibouti in 2016-2018

For whale sharks, BMFs through zooplankton samples were higher than 1 for Cr, Mn, Co, Ni, As, Mo (Table 5.1). However, only for Cr, Ni and Mo they were statistically higher than 1. Cr BMF in these sharks ranged from 0.03 to 10.6; Ni BMF from 0.02 to 8.01 and Mo 0.2 to 6.0 (Table 5.1).

Table 5.1 Biomagnification factor (BMF) calculated for whale sharks

	BMF		**P-VALUE**
	Mean	Std	
Cr	4.5	2.9	p = 0.0014
Mn	1.9	1.6	
Co	1.7	1.3	
Ni	4.2	2.3	p = 0.0006
As	1.4	0.8	
Mo	2.4	1.8	p = 0.0197

The level of trace elements found in whale sharks inhabiting the Gulf of Aden area were compared with those previously published in literature (Table 5.2). Samples from Djibouti showed higher mean concentration for As, Cu, Zn and Se. Furthermore, all whale shark samples exceeded the maximum allowable limit (MAL) for Cr, while 83.3% for Pb and Se, 58.3% for Cd and 33.3% for Zn (Table 5.2).

Table 5.2 Mean concentrations of trace elements (mg kg^{-1} ww) in tissues of whale sharks sampled in Djibouti and from literature, as well as the maximum allowable limits (MAL) for each metal

	N	Hg	Cd	As	Pb	Cu	Zn	Se	Cr
MAL		**1^a**	**0.05^a**	**3^b**	**0.2^a**	**30^c**	**30^c**	**0.3^d**	**0.1^e**
Present study	12	0.05 ± 0.06	0.06 ± 0.03	0.96 ± 0.7	1.24 ± 1.12	8.1 ± 11	37.8 ± 42.6	0.35 ± 0.09	18.4 ± 19.7
Pancaldi et al (2019a)	38 32	0.009 ± 0.007 0.004 ± 0.002						0.23 ± 0.14 0.28 ± 0.2	
Pancaldi et al (2019b)	2	0.09 0.03	0.3 0.5	0.03 0.07	6.8 0.7	5.8 1.6	0.01 0.21	0.1 0.1	
Wang et al (2014)		0.0002 ± 0.00001 0.001 ± 0.00001	0.2 ± 0.0008 0.2 ± 0.0008	0.3 ± 0.0003 0.3 ± 0.0004	0.4 ± 0.01 0.4 ± 0.01	2.8 ± 0.2 2.8 ± 0.1	29 ± 0.1 22 ± 0.1		
McKinney et al (2016)	3	0.08							

Notes: ^{a} FAO, 2003; ^{b} DOH, 2004; ^{c} FAO, 1983; ^{d} Bosch *et al.*, 2016; ^{e} Tarley et al. (2001)

5.3.2. PCBs and DDTs

In skin tissues of whale sharks, the mean PCBs concentration was 624.9 ± 570 ng g^{-1} dw. All congeners have been found, however PCB 149 was detected only in one individual, as for PCB 118. The predominant congener was Tetra-CB, accounting for 40.8% of total PCBs (Fig. 5.3), in particular PCB 52 (188.7 ± 255 ng g^{-1} dw) that comprised 35% of all congeners. Penta-CB was also in higher concentrations, accounting for 22.9% of total PCBs. Within Penta-CB, PCB 101 (180.8 ± 159.6 ng g^{-1} dw) contributed for 22% of all congeners (Fig. 5.3). Hepta-CB, Octa-CB and Deca-CB were in lower concentrations, accounting for 2%, 0.8% and 0.6%, respectively (Fig. 5.3).

The Wilcoxon Kruskal-Wallis test showed a statistical difference in the mean concentration of congeners (X^2 = 24.2, df = 6, p = 0.0005). The Post Hoc Test Steel-Dwass All Pairs showed a significant difference in the mean concentration of Tri-CB with Octa-CB (p = 0.0384), and with Deca-CB (p = 0.0247).

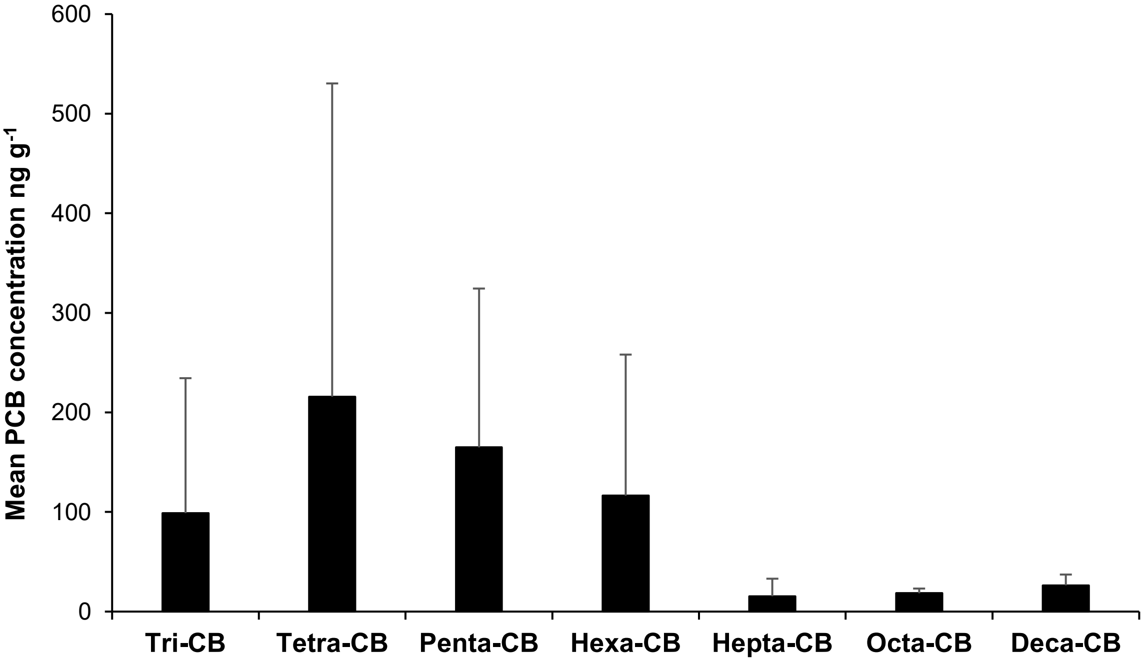

Fig. 5.3 Mean (±s.d.) concentration of PCBs in whale shark skin biopsies from Djibouti, grouped according to the chlorine content of each congener of all samples

The predominant DDT metabolite in whale shark skin tissues was DDE contributing for 51.4% of total compounds and in particular o,p′DDE (164.7 ± 150 ng g^{-1} dw) was the metabolite found in higher concentrations. Other metabolites, DDT and DDD, accounted for 31.4% and 17.2%, respectively (Fig. 5.4). Within DDT compounds, p,p'DDT was the parent compound found in higher concentration (86.9 ± 83.3 ng g^{-1} dw). The p,p′ DDE/ΣDDT ratio, measured in those whale sharks where both p,p'DDE and DDT metabolites were detected (N = 5), had a mean value of 0.9 ± 0.7.

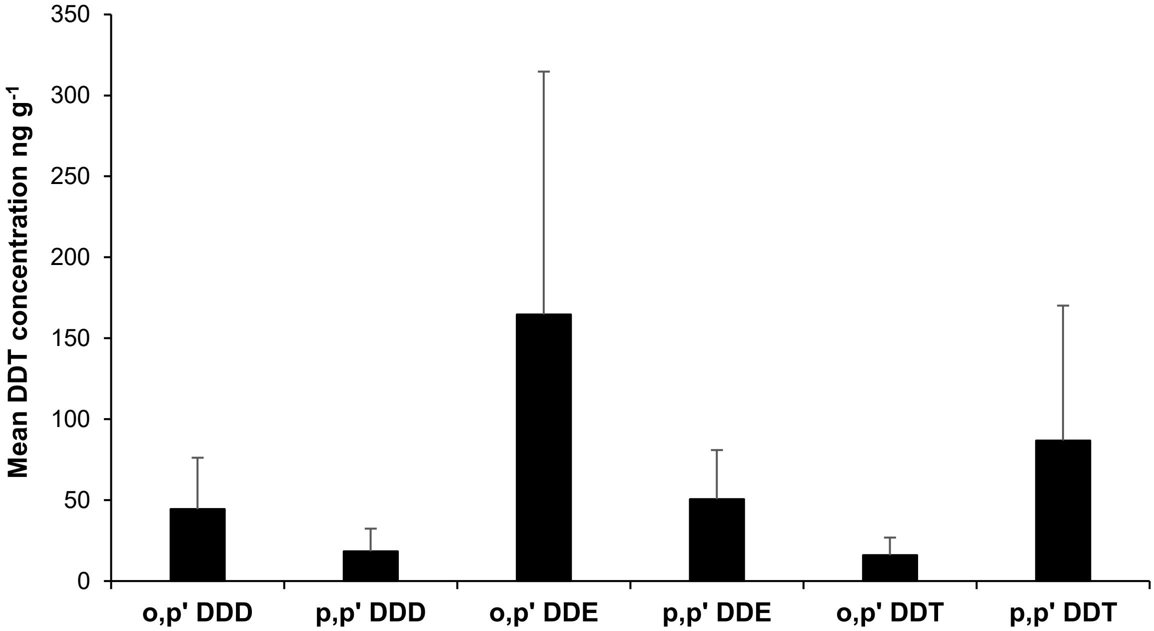

Fig. 5.4 Mean (±s.d.) concentration of DDTs metabolites in whale shark skin biopsies from Djibouti

In order to evaluate the status of contamination by OCs in whale sharks in the Gulf of Aden area, results from this study were compared with the MAL for each compound (Table 5.3). Although the European Union does not provide a maximum residue limit for DDTs in fish muscles, USEPA (2000) has established a DDTs limit of 14.4 ng g^{-1} ww. This limit was exceeded for 50% of whale shark samples. In regards to PCBs, the EU concentration threshold of ∑ ind-PCBs (PCB 28, PCB52, PCB101, PCB138, PCB153 and PCB180) in fish is 75 ng g^{-1} ww (European Commission, 2006) and comparison with skin tissues showed that 62.5% of whale sharks exceeded this limit as well (Table 5.3).

No data on PCBs and DDTs were available for zooplankton samples.

Table 5.3 Mean concentrations of PCBs and DDTs (ng g^{-1} dw) in skin tissues of whale sharks (Ws) sampled in Djibouti and the maximum allowable limits (MAL) for each compound

OCs		**Ws1**	**Ws2**	**Ws3**	**Ws4**	**Ws5**	**Ws6**	**Ws7**	**Ws8**	**MAL**
PCB										
	28+31		2.9	15.9			28.3	255.1	25.2	
	52	37.1	70.5	252.6	7.4	175.5	732.2	45.4		
	101	69.3		102.9	2.7	47.9	374.8	348.6	319.5	
	153	38.9		55.4	<LOD	39.0	57.0		23.0	
	138	14.1		289.8		4.8	52.9	24.9	54.1	
	180	2.1		14.4	<LOD		31.7		19.1	
	Total	161.5	73.4	731.0	10.0	267.1	1277	674.0	440.9	
	Total ww	48.5	22.0	219.3	3.0	80.1	383.1	202.2	132.3	75[a]
DDT	p,p'DDT	2.4		196.8	<LOD	24.1	178.7	87.7	31.4	
	Total	2.4		703.6	7.4	151.3	641.9	409.9	300.2	
	Total ww	0.7		211.1	2.2	45.4	192.6	123	90.1	14.4[b]

Notes: [a] European Commission, 2006; [b] USEPA, 2000

5.4. DISCUSSION

This study is the first concurrent assessment of elemental- and organic-contaminant loads in the skin biopsies of whale sharks from Djibouti. Whale sharks showed higher concentrations of Hg, As, Cu, Zn and Se compared with results from literature (Wang et al., 2014; McKinney et al., 2016; Pancaldi et al., 2019a,b). Indeed, Hg concentration in whale shark samples from the Gulf of Tadjoura had a mean value of 0.05 mg kg^{-1} ww, higher than what reported from Mexico (0.009 and 0.004 mg kg^{-1} ww, Pancaldi et al., 2019a), and from China (0.0002 and 0.001 mg kg^{-1} ww, (Wang et al., 2014). McKinney et al. (2016) found an Hg concentration of 0.08 mg kg^{-1} ww in 3 stranded whale sharks from South Africa, which is comparable with results from this study. Trophic position is a major factor in Hg accumulation and, as a filter feeder, whale sharks exhibit lower Hg concentration respect to other predator shark species (Pancaldi et al., 2019a). However, Hg levels clearly exceeded the European EQS of 0.02 mg kg^{-1} ww for total Hg (European Directive 2013/39/EC). The biota EQS is designed for the protection of aquatic life against secondary poisoning and consequently protects both marine ecosystems and human health (Vignati et al., 2013). Moreover, the biota EQS is also attributable to application for the protection of prey fish (Vignati et al., 2013). Therefore, Hg levels in whale sharks, above the biota EQS, might indicate possible harmful effects to their health.

Since, over 90% of total Hg in fish tissue is in the methylmercury form (MeHg), and it reaches up to 98% in sharks (Storelli et al., 2001b), levels of Hg in whale sharks were compared with those established by the Canadian Council of Ministers of the Environment for protection of aquatic life by MeHg. Hg levels in whale sharks were higher than the threshold value of 0.033 mg kg^{-1}, highlighting some concerns for their health and that of top predators. Indeed, fish, marine mammals, and birds accumulate most of their Hg body load through their diet (Rodgers, 1994) consequently, top predators in the Djibouti area are assumed to be at risk of secondary poisoning. MeHg is a strong neurotoxicant, affecting also the organism reproduction. Indeed, effects on fish reproduction have been reported above a threshold concentration of 0.04 mg kg^{-1} (Depew et al., 2012), that was exceeded by Hg levels in whale sharks. Overall, the Hg concentrations measured in this study indicate that whale sharks could suffer physiological impact and may pose at risk the food web. The established limits for fish and fishery products intended for human consumption are less rigid and allow a maximum of 1 mg kg^{-1} (FAO, 2003; EC, 2006). This limit was not exceeded in any of the samples analyzed here. However, it is worth to point out that both the biota EQS and human consumption limits are established for muscle tissue (or for whole fish), where Hg levels can be one order of magnitude higher than in skin tissue; i.e. the matrix analyzed in the present study (Pethybridget et al., 2010). Arsenic concentrations from Djibouti were much higher compared with the range of 0.03-0.3 mg kg^{-1} ww found in literature (Wang et al., 2014; Pancaldi et al., 2019b) as well as for Pb, except for a specimen stranded in the Gulf of California (Pancaldi et al., 2019b). Overall, the Pb concentration from this study ranged from 0.2 to 2.9 mg kg^{-1} ww, greatly exceeding the maximum allowable limit of 0.2 mg kg^{-1} ww (FAO, 2003). Cu and Zn concentrations were both higher than what reported from the Gulf of California, South Africa and China and the mean value of Zn exceeded the MAL of 30 mg kg^{-1} ww (FAO, 1983). Se concentrations were also higher in this study, however more in line with results from literature (Pancaldi et al., 2019a,b). In regards to Cr, no information is currently available from literature and therefore no comparison could be carried out. Although few shark species have been investigated for Cr bioaccumulation (Gelsleichter and Walker, 2010), whale sharks from Djibouti appeared to bioaccumulate high Cr concentrations. Indeed, values measured in skin biopsies from this study were much higher than those in predatory shark species from the same area (Boldrocchi et al., 2019), and exceeded the maximum allowable limit of 0.1 (Tarley et al., 2001). Such high levels of Cr could indicate high availability in the areas visited by whale sharks outside the Djibouti aggregation season, and/or the ability of this species to bioaccumulate Cr in specific tissues rather than others (Pancaldi et al., 2019b). Overall, the trace elements concentration found in the Djibouti whale shark appeared to be elevated. The finding is consistent with ecotoxicology results reported for the hammerhead and milk sharks in Djibouti (Boldrocchi et al., 2019). Indeed both species appeared to accumulate elevated concentrations compared with studies reported from other locations (Boldrocchi et al., 2019). Industrial ports are hotspots of anthropogenic contamination due to their specific land based source of pollution (Bu-Olayan and Thomas, 2014). Djibouti city is one of the major port within the Gulf of Aden and it is located in one the busiest shipping areas in the world. Djibouti is facing a rapid increase in coastal activities due to an increasing urbanization, sea traffic, and industrial development. However, untreated and improperly treated wastewater are released regularly into marine environment (Ahmed et al., 2017), resulting probably in such high concentrations of trace elements in the Djiboutian shark species. Indeed, assuming that the analyzed surface zooplankton is representative of the main food source of whale sharks in Djibouti, contaminants discharged into coastal waters are first assimilated by planktonic organisms and, then, biomagnify in higher trophic level organisms, including whale sharks. Results from this study suggested that Cr, Ni and Mo biomagnified in this species, but not Hg and Cd, as their concentration in whale sharks was lower than in zooplankton. The whale shark is highly migratory and is believed to spend only few months in the Gulf of Tadjoura (Rowat et al., 2007, 2011), consequently Hg and Cd levels may reflect the lower concentrations of other foraging areas visited by whale sharks outside the aggregation season. Alternatively, the detoxification mechanism of sharks, considered an important factor influencing the Hg accumulation (Escobar-Sánchez et al., 2011), might reduce the Hg levels in whale sharks. Indeed, Se and Hg appear to behave antagonistically so that high Se concentrations protect against Hg toxicity through Hg sequestration (Wang et al., 2001; Ralston et al., 2007; Branco et al., 2014; Bergés-Tiznado et al., 2015). In this study, Se concentrations greatly exceded Hg levels, suggesting that juvenile whale sharks likely metabolize and excrete Hg more effectively due to the excess of Se accumulated (Bergés-Tiznado et al., 2015). Alternatively, skin biopsies might not represent the appropriate indicator tissues for biomagnification processes, as well as for internal loads of Cd and Hg. Indeed, Hg bioaccumulates preferentially in shark muscles, due to its high affinity for sulfhydryl groups (Gelsleichter and Walker, 2010), and Cd in pancreas and liver (Corsolini et al., 2014), while skin has been reported as the tissue with lower Hg and Cd-organotropism (Corsolini et al., 2014).

Boldrocchi et al. (2018) reported a strong contamination by PCBs and DDTs in zooplankton organisms, hammerhead sharks and milk sharks from Djibouti (Boldrocchi et al., 2019), which is in line with the values found in whale sharks, highlighting a strong anthropogenic pollution in this area. An indicator of new DDTs inputs in the environment is the p,p′DDE/ΣDDT ratio, that exceded the critical value of 0.6, indicating that recent inputs of technical DDTs might be released in the Gulf of Tadjoura (Storelli et al., 2005; Boldrocchi et al., 2018). Indeed, although the use of OCs has been banned in the developed countries since 1970s, they can be still found in the sub-Saharan Africa, including Djibouti (Bon et al., 2015; Boldrocchi et al., 2018, 2019). Therefore, it is not surprising that whale sharks exhibited elevated concentrations of both DDTs and PCBs, also compared with those measured in whale sharks from La Paz (Mexico) (Fossi et al., 2017). Indeed, concentrations in this study were in line with levels reported for predatory shark, such as the blue shark from the Mediterranean Sea (Storelli et al., 2005) and the white shark from South Africa (Marsili et al., 2016). The PCBs content was mostly dominated by congeners with medium-low chlorine content such as PCB 52 and 101 with contributions >20%, which is not what usually described in biotic matrices, where PCB 153, 138 and 180 constitute the major bulk of PCBs load. However, Fossi et al. (2017) reported a similar accumulation profile in skin biopsies of whale sharks from La Paz (Mexico), as well as Boldrocchi et al. (2019) in fin tissues of hammerhead and milk sharks from the Gulf of Tadjoura. Indeed the higher-chlorinated and more hydrophobic congeners (such as PCB 138, 153 and 180) tend to bioaccumulate in tissues more enriched in lipid content such as liver rather than muscles or skin tissues (Mull et al., 2012; Storelli et al., 2003).

Whale sharks showed high intraspecific variability in the contamination load by OCs that might indicate different movement patterns. Indeed, the apparent absence of whale sharks in the Gulf of Tadjoura, outside the aggregation season, has led to the hypothesis that sharks migrate to other locations, which might explain the different accumulation profile found in those specimens.

Although several countries have established protection measures for the whale shark (Rowat and Brooks, 2012), in China, fins are requested at high price and large individuals are targeted by fishermen (Li et al., 2012). Uncontrolled catches in China, poaching activities and illegal trade in the North-Western Indian Ocean (Riley et al., 2009) not only are likely to make efforts of other countries for the whale shark protection vain (Li et al., 2012), but also rise some concerns for the human health due to shark consumption. Seafood is considered an important pathway for pollutant accumulation in human body, resulting in various adverse effects for its health (Bosch et al., 2016). And, this study highlights several risks from whale shark consumption, not only for the overall load of pollutants, but also because some elemental- and organic-contaminants exceeded the MAL.

5.5. CONCLUSIONS

This study provides for the first time information on the contamination status of the whale shark in Djibouti. Levels of elemental and organic contaminants were consistent with the contamination profile previously described for this species. However, the Djiboutian whale sharks appeared to bioaccumulate higher concentration of As, Cu, Zn, and Se compared with other locations. Hg levels in whale sharks likely indicate that they may suffer physiological impact and may pose at risk top predators consuming this species. Furthermore, contamination by OCs in this species was comparable with those of predatory sharks, typically top predators, which highlights the high level of pollution in this area. The rapid increase in urbanization and coastal utilization in Djibouti result in chronic discharge of pollutants into the marine environment. Considering the role of whale sharks as early warning bioindicators of regional pollution and that pollution plays an important role in the understanding of the decline of sharks, further research should be carried out to monitor contaminant status in the Gulf of Aden. Furthermore, due to poaching activities and illegal trade in North-Western Indian Ocean of this species, this study suggests particular attention in the consumption of whale sharks from this region. Indeed, the analyzed samples exceeded the MAL for Cr, Pb, Se, Cd and Zn. Further investigations are needed to assess the horizontal movements of this species in the Gulf of

Aden and Indian Ocean to evaluate if whale sharks from Djibouti migrate in areas where illegal fishing is still undergoing, posing a risk for human health.

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CHAPTER VI. Trace elements and POPs in two commercial shark species from Djibouti: implications for human exposure

Trace elements and POPs in two commercial shark species from Djibouti: implications for human exposure

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ABSTRACT

Within Djibouti (Gulf of Aden), the scalloped hammerhead shark (*Sphyrna lewini*) and milk shark (*Rhizoprionodon acutus*) are important components of the artisanal fishery and they are caught to be exported or sold for local consumption. However, little scientific information exists on the contamination load of these species in this area of the world. With global populations of elasmobranchs in decline, understanding the extent of contaminant exposure is critical to future conservation as well as to assess the health risks for consumers of these species. The contaminants analyzed in this study comprised PCB, DDT and trace elements in livers, muscles and fins of both hammerhead sharks and milk sharks. The overall organochlorine compounds (OCs) and trace elements concentrations were similar among the two sharks' species and the pattern of PCB and DDT tissue distribution showed the highest burdens in livers compared with muscles and fins. However, the different accumulation profiles of OCs among shark species suggest species-specific accumulation of these contaminants. The p,p'DDE/∑DDT ratios were equal or slightly higher than the critic value of 0.6, suggesting possible recent inputs of technical DDT in the area. Concentration of trace elements from this study were generally comparable to those found in sharks from other areas of the world and, highlight the wide variation in metal concentrations between species, individuals and tissues. As far as Hg is concerned, scalloped hammerhead sharks showed higher accumulation in muscles compared with milk sharks. Both species showed elevated concentration of Se, which might

be related to high Hg levels since Se inhibits Hg toxicity. The potential cancer risk for PCB, Cd, Ni, Cr and As fell within the range of 10^{-6}-10^{-4}, suggesting some concerns for the overall contamination levels in both species. Indeed, consuming of fish involves a mixture of all analyzed elements, and therefore, some potential risk might arise from regularly consuming these species.

6.1. INTRODUCTION

Ocean pollution has been linked to emerging diseases in individuals, populations, and ecosystems (Aguirre and Lutz, 2004; Ley-Quinonez et al., 2013). Legacy contaminants such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT) compounds, as well as trace elements are persistent in the environment, and because of their bioaccumulative nature and toxicity have negative effects on the organisms health, particularly to higher trophic level species (Storelli and Marcotrigiano, 2001; Gelsleichter and Walker, 2010). Although several studies have focused on the accumulation of trace metals and organochlorine contaminants (OCs) in marine vertebrates and birds (Storelli and Marcotrigiano, 2000; Braune et al., 2002; Tanabe, 2002; Storelli et al., 2003; Jepson et al., 2005; Gardner et al., 2006; Kennedy, 2011), information about predatory fish is scarce and mainly comes from specific parts of the world or tends to examine only a limited number of species (Tarassoli et al., 2012; Lee et al., 2015). High tissue levels of contaminants have been shown to have deleterious effects on aquatic vertebrates including pathological changes of varying severity in kidneys, liver and gills (Battaglini et al., 1993; Thophon et al., 2004), impairment of reproduction and growth (Cross, 1988; Hose et al., 1989), and possible immune suppression (Lahvis et al., 1995; Jepson et al., 2005; Ylitalo et al., 2005; Gelsleichter et al., 2006). However, little is known on the baseline contaminant loads in elasmobranchs, as well as the potential physiological effects of pollutants on sharks (Mull et al., 2012; Lee et al., 2015).

Sharks accumulate high levels of bioavailable contaminants via the food web (Johnson-Restrepo et al., 2005; Gelsleichter and Walker, 2010). Characteristics associated with high longevity, low metabolic rate, high trophic position and large lipid-rich livers make elasmobranchs particularly susceptible to contaminant uptake and accumulation in tissues throughout their lifetime (Johnson-Restrepo et al., 2005; Mull et al., 2012). Trace metal and OCs concentrations in sharks vary within and among species (Storelli and Marcotrigiano, 2002; McMeans et al., 2007; Lee et al., 2015) and differences are likely due to variations in prey type (Storelli and Marcotrigiano, 2002; Domi et al., 2005; McMeans et al., 2007; Lee et al., 2015). Trace metal and OCs concentrations also vary among tissue types (Storelli and Marcotrigiano, 2001; Gelsleichter et al., 2005; Cornish et al., 2007; Pethybridge et al., 2010) and typically, the highest concentrations of OCs are found in shark livers (Storelli and Marcotrigiano, 2001; Gelsleichter et al., 2005; Mull et al., 2012), while trace metals are preferentially accumulated in protein-rich tissues (Corsolini et al., 2014).

Fish and shellfish consumption has been reported as an important route of human exposure to a variety of chemical contaminants (Llobet et al., 2003; Storelli et al. 2005), especially within coastal communities. Since the human demand for shark products, including fins, has increased dramatically since the 1950s (Dulvy et al., 2014; Dent and Clarke, 2015), it seems crucial to examine the level of trace metals as well as OCs in sharks tissues to better understand the associated human health risk. Within the Gulf of Aden (Africa), elasmobranchs are an important component of the artisanal fishery and they are caught to be exported or sold for local consumption (Bonfil, 2003; Henderson et al., 2007; Spaet and Berumen, 2015; Jabado and Spaet, 2017). A great proportion of shark catch is from small-scale fisheries from Djibouti, Saudi Arabia, Yemen, Somalia, and Eritrea, and do not appear in any fishery statistics or record (Gladstone et al., 2003; Alabsi and Komatsu, 2014; Jabado and Spaet, 2017). Although several species have been recorded in this area, few of them form the bulk of these landings, which include the milk shark *Rhizoprionodon acutus* (Ruppell, 1837) and the scalloped hammerhead shark *Sphyrna lewini* (Griffith & Smith, 1834) (Bonfil, 2003; Henderson et al., 2007;

Spaet and Berumen, 2015). Both species are regularly harvested in the coastal water of Oman, (Henderson et al., 2009), Persian Gulf (Adel et al., 2017), in the Saudi Arabian Red Sea (Spaet and Berumen, 2015) and in the Gulf of Aden (Bonfil, 2003). The scalloped hammerhead shark is a semi-oceanic species globally distributed throughout tropical and temperate oceans (Compagno, 1984; Baum et al., 2009). Currently, *Sphyrna lewini* is listed as endangered on the International Union for Conservation of Nature and Natural Resources (IUCN) Red List (Baum et al., 2009) due to its high mortality in longline fisheries (Morgan and Burgess, 2007) and low population rebound potential (Smith et al., 1998; Hayes et al., 2009). The milk shark occurs primarily throughout the coastal regions of the Indian Ocean, Indo-Pacific and the western coast of Africa (Compagno, 1984). In contrast to the scalloped hammerhead shark, *R. acutus* has a generation time much shorter with rapid sexual maturity and a maximum lifespan of 8 years that provides resilience to exploitation (Simpfendorfer, 2003). *Rhizoprionodon acutus* is listed as Least Concern by the IUCN. However, due to the coastal distribution and accessibility in shallow bays and continental shelves of both species, the milk shark and the scalloped hammerhead shark comprise a large proportion of elasmobranch catches from commercial fishing activities and are regularly found in fish markets worldwide (Chen et al., 1988; Castro, 1993; Simpfendorfer, 2003). Even if both species are commonly harvested for local consumption in the Gulf of Aden, little scientific information exists on the contamination load of these species in this part of the world.

Even if banned since the 1970s, large quantities of POP have been released into the environment in the past and due to their propensity for long-range transport, high environmental persistence, bioaccumulation and biomagnification potential, inherent toxicity, nowadays they represent a global problem. In sub-Saharan Africa pesticide choice, frequency of application and use are hardly controlled. Quite often, not licensed pesticides end up in the food chain (De Bon et al., 2014). Similarly to other pollutants, trace element levels have been recently increasing compared to their natural background conditions, exceeding international limits (Yabe et al., 2010). The Red Sea and the surrounding areas, for instance, have suffered from trace metals contamination due to the growing anthropogenic pressure and the consequent increase in sewage effluents, industrial, shipping and coastal activities (Kress et al., 1999; Ahmed et al., 2017).

To redress the lack of scientific information on pollutant loads in elasmobranchs, especially in Africa, this study focuses on two commonly exploited species: the milk shark and the scalloped hammerhead shark. With global populations of elasmobranchs in decline, understanding the extent of contaminant exposure is a critical aspect, since chemical pollution may exasperate the current situation (Camhi, 1998), especially for regularly targeted species. Therefore, the aim of this study is to: 1) evaluate for the first time the presence of legacy contaminants (PCB and DDT) and trace elements in two commercial shark species in Djibouti, 2) investigate the species-specific accumulation of these contaminants, 3) examine the distribution of pollutants in different tissue types (fin, muscle and liver), and 4) estimate the exposure health risks derived from the consumption of milk shark and scalloped hammerhead shark by the target hazard quotient (THQ) and the carcinogenic risk (CR).

6.2. MATERIALS AND METHODS

Sharks examined for this study were collected in the Gulf of Tadjoura (Djibouti, latitude 11 40°N, longitude 43 00°E), located in the Gulf of Aden at the southern entrance to the Red Sea. The Gulf of Tadjoura is an inlet of the Indian Ocean created by the fault line of the northerly end of the East African Rift Valley that transects Djibouti, Ethiopia and Kenya (Boldrocchi et al., 2018). The seabed shelves steeply from the coast of the Gulf dropping to 100 m depth around 2 km offshore and to around 450 m depth in the center of the Gulf of Tadjoura (Dauteuil et al., 2001). The coastline is composed of a mixture of eluvial and colluvial deposits, taluses, sheet floods, dunes and beach deposits with a tidal range of 0–3 m relative to Chart Datum (Schlüter, 2008).

Eleven individuals of scalloped hammerhead shark and 5 specimens of milk shark were caught by artisanal fishery from November 2016 to February 2017 and from October 2017 to January 2018 within the coastal waters of Djibouti (Table S.1, Supplementary material IV). According to fishermen, all captures were within 15 km from Djibouti city. Samples of hepatic, muscle and fin tissues were collected from different individuals of scalloped hammerhead shark and milk shark (Table S.1, Supplementary material IV) for a total of 6+5 hepatic tissue samples, 2+3 muscle samples and 7+2 fins samples. However, due to the small amount of tissues collected for scalloped hammerhead sharks, 3 fins from specimens of same size were pooled together for analyses, as well as for other two individuals. Within the sampled milk sharks, the liver of a pregnant female and of its fully term embryo were collected. Biological information such as body length (total length, TL) and sex were recorded for each individual (Table S.1, Supplementary material IV). After dissection of the sharks, the collected tissues were kept frozen at -20 °C in Djibouti, during transportation and at the University of Insubria, until analysis.

6.2.1. PCB and DDT analyses

All frozen samples were first lyophilized and then analyzed following Bettinetti et al. (2016). Briefly, for each sample, a subsample of approximately 0.5 g dw (dry weight) was extracted with 50 ml of an acetone/n-hexane 1:1 v/v mixture. All reagents were purchased from Sigma Aldrich (Sigma Aldrich, Milwaukee, WI, USA) with purity always higher than 98%. The gravimetric determination of lipids was carried out by weighting each sample, until no weight variation was detected. Acetone/n-hexane extracts of samples were evaporated under nitrogen stream. Lipids were then suspended in 2 ml of n-hexane and digested with 5 ml of H_2SO_4 (98%, Carlo Erba, Italy). The organochlorine compounds were then recovered by several washings with n-hexane. The n-hexane extracts were concentrated and subsequently cleaned on a Florisil column. The resulting samples were reduced in volume to 0.5 ml and analyzed by gas chromatography (GC Carlo Erba, Top 8000) coupled with ^{63}Ni electron capture detector (Carlo Erba ECD 80) using a 1 μl on-column injection system. The column was a WCOT fused silica CP-Sil-8 CB (50 m × 0.25 mm I.D., film thickness 0.25 μm, Varian, USA), and the temperature program used was from 60 °C to 190 °C at 20 °C min^{-1}, followed by a run from 190 °C to 250 °C at 1.5 °C min^{-1} and a further run from 250 °C to 270 °C at 3 °C min^{-1} and a final isothermal step at 270 °C for 17 min, with helium as carrier gas (1 ml min^{-1}) and nitrogen as auxiliary gas (30 ml min^{-1}). Sample quantification was performed using external reference standards of p,p'DDT, p,p'DDE and p,p'DDD (Pestanal, Sigma-Aldrich, Germany) in isooctane (Carlo Erba, pesticide analysis grade). Arochlor 1260 (Alltech, IL, USA) with the addition of PCB 28 and 118, was used for PCB quantification. The analyzed congeners were PCB 18, PCB 28, PCB 31, PCB 44, PCB 101, PCB 118, PCB 138, PCB 149, PCB 153, PCB 170, PCB 180, PCB 194 and PCB 209. The method detection limit and the method quantification limit varied from 0.1 to 0.5 and from 0.5 to 1 ng g^{-1} lw (lipid weight), respectively, depending on the organochlorine compound. Good laboratory practices were used when testing the standard reference materials BCR-598 and BCR-349 (Community Bureau of Reference, Brussels) for DDT and PCB residues, respectively. The percentages of recovery for the DDTs were 107.5±4% (p,p'DDE), 106.2±4% (p,p′DDD), and 106.2±3% (p,p′DDT). The recoveries for the PCBs were between 91.3±1.1% and 102.2±1.6%.

6.2.2. Trace element analysis

In the present study, concentrations of 14 trace elements (As, Cd, Cu, Fe, Mn, Hg, Se, Zn, Cr, V, Co, Ni, Ag, Pb) were determined in muscle samples and 15 elements in liver tissues (As, Cd, Cu, Mn, Mo, Hg, Se, Sr, Zn, Cr, V, Co, Ni, Ag, Pb) of both scalloped hammerhead shark and milk shark. Ultrapure water produced by a Millipore MilliQ A10 system was used throughout for sample dilution and standard preparation. Ultrapure grade hydrochloric acid was purchased from Fluka (37% HCl Fluka TraceSelect), whereas pure, concentrated nitric acid was produced in the laboratory by a sub-boiling Milestone DuoPur equipment (Monticelli et al., 2019). All laboratory materials (sample bottles and digestion vessels) were decontaminated in a 3-stage process before use. First, all materials were washed with ultrapure water and immersed in a 0.4% w/w detergent solution (Nalgene L900) for one week. Bottles and vessels were then rinsed with ultrapure water and left for one week in a 0.03% w/w HCl solution. Finally, they were decontaminated with a HNO_3 solution (2%, w/w) for one week and thoroughly rinsed with ultrapure water before use. Sample digestion was conducted as follows. Lyophilized aliquots of liver and muscle samples, approximately 0.200 g of dry tissue, were digested in perfluoroalkoxy (PFA) vessels (5 ml with cap from Savillex) with 2 ml of concentrated HNO_3 and slowly heated to 100 °C on a hot plate until complete digestion. Samples were diluted with ultrapure water to a final volume of 60 ml. Liver samples were additionally diluted 1:10. The quantification of trace metals was performed by ICP-MS (mod. iCAPQ from Thermo Elemental, www.thermo.com), using a kinetic energy discrimination (KED) configuration and a standard addition method for quantification. The analytical procedure was validated through the analysis of certified reference materials ERM®-BB422, from the Institute for Reference Materials and Measurements in Belgium for muscle samples, and DOLT-5 from the National Research Council in

Canada for liver samples (Table S.2, Supplementary material IV). To ensure accuracy, for every lot of shark samples digested, blank reagents, standard reference materials and replicate samples were analyzed at the same time as well.

6.2.3. Exposure risk assessment

Human health risk of DDT and PCB from consumption of contaminated fish (muscle and fin tissues) was calculated for both species. Risks associated with cancer and non-cancer health effects were considered separately. For the non carcinogenic risk, the Hazard Ratio (HR) was calculated as the ratio between the estimated daily intake (EDI) and the recommended USEPA reference dose (RfD):

$$\mathrm{EDI} = \frac{\mathrm{C \times DR}}{\mathrm{BW}} \qquad \text{and} \qquad \mathrm{HR} = \frac{\mathrm{EDI}}{\mathrm{RfD}}$$

where C is the concentration of OC (ng g^{-1} ww), DR is the estimated daily consumption rate of fish (10.2 g $person^{-1}$ day^{-1}, from FAOSTAT database) and BW is the estimated average adult body weight (70 kg). RfD is equal to 500 ng kg^{-1} day^{-1} for DDT (it refers to the RfD proposed for p,p'DDT; USEPA, 1999a) and 20 ng kg^{-1} day^{-1} for PCB (it refers to the RfD proposed for Aroclor 1254; USEPA, 1999b).

In order to assess the potential carcinogenic risks posed by OCs through contaminated fish consumption, both cancer risk estimate and hazard ratio were calculated according to USEPA guidelines. The cancer risks (CR) associated with OCs were estimated using the following equation:

$$\mathrm{CR} = \mathrm{EDI \times CSF}$$

where CSF is the cancer oral slope factor, i.e. 3.4×10^{-7} and 2×10^{-6} (ng kg^{-1} day^{-1}) for DDT and PCB, respectively (USEPA, 1999a, 1999b). Results are interpreted as follows: $<10^{-6}$ is considered acceptable, between 10^{-6} and 10^{-4} is considered an area of concern, and carcinogenic risks $>10^{-4}$ are considered unacceptable (USEPA, 2005). The hazard ratio for carcinogenic risk was calculated following Jiang et al. (2005):

$$\text{HR} = \frac{\text{EDI}}{\text{BMC}} \quad \text{and} \quad \text{BMC} = \frac{\text{Risk x BW}}{\text{Fish consumption x CSF}}$$

where the BMC refers to the benchmark calculation for cancer effect. The risk is set as one in one million due to the lifetime exposure, fish consumption is the amount of fish consumed per kg body weight of the individual per day (g kg^{-1} day^{-1}). Hazard ratio greater than one indicates that there is a potential risk to human health (Dougherty et al., 2000).

Human health risk for trace elements from consumption of contaminated fish (muscle tissue) was calculated for both shark species. The target hazard quotient (THQ), which is used to express the risk of non-carcinogenic effects, was calculated according to the following equations (Chien et al., 2002):

$$\text{THQ} = \frac{E_f E_d \text{ FIR C}}{\text{Wab TA RfD}} \times 10^{-3}$$

where Ef is exposure frequency (365 days $year^{-1}$); Ed is the exposure duration (70 years) equivalent to the average lifetime; FIR is the food ingestion rate (10.2 g $person^{-1}$ day^{-1}, from FAOSTAT database, http://www.fao.org/fishery/statistics/global-consumption/en); C is the metal concentration in the food (mg kg^{-1} ww); RfD is the oral reference dose; Wab is the average body weight (70 kg for adult) and TA is averaging exposure time for non-carcinogens (365 days $year^{-1}$ x number of exposure years).

For carcinogens, risks are estimated as the incremental probability of an individual to develop cancer, over a lifetime as a result of exposure to a potential carcinogen (USEPA, 1989). Level $<10^{-6}$ is considered acceptable, between 10^{-6} and 10^{-4} is considered an area of concern, and carcinogenic risks $>10^{-4}$ are considered unacceptable (USEPA, 2005). The carcinogenic risk (CR) was calculated by multiplying the oral carcinogenic potency slope factor (CSF), set by USEPA (0.5 mg kg^{-1} for Cr; 1.7 mg kg^{-1} for Ni; 1.5 mg kg^{-1} for As; 6.3 mg kg^{-1} for Cd and 0.0085 mg kg^{-1} for Pb), by the EDI. The equation used for calculating the risk of cancer is as follows:

CR = CSF x EDI

Because muscle samples were lyophilized, data were converted from dw to wet weight (ww) taking 70% of muscle moisture in order to calculate HR, CR and THQ values (Bergés-Tiznado et al., 2015; Ooi et al., 2015).

6.2.4. Statistical analyses

Shapiro test was used as a normality test. To test any difference in the concentration of PCB congeners in the liver of milk shark and scalloped hammerhead shark, the One-way Anova test was performed and the Post hoc Tukey's method was carried out to examine the mean differences among congeners concentrations. The Kruskal-Wallis Anova test was used to evaluate differences in PCB and DDT concentrations across tissues. The Post hoc Steel-Dwass method was carried out to examine the mean differences among tissues. To identify species-specific differences in the contaminants accumulation of hepatic tissue, the Wilcoxon test has been applied for DDT since data was non-normally distributed, while the Independent-samples t-test for PCB. Simple linear regression model was performed to correlate the size of sharks with the DDT and PCB concentration in hepatic tissues.

Trace elements concentrations with parametric distribution (statistical test: ANOVA) and with non-parametric distribution (statistical test: Kruskal-Wallis) with Tukey's method were carried out to examine the mean differences among element concentrations in each tissue. Kruskal-Wallis non-parametric method has been applied to test element distribution among tissues type. A Wilcoxon test was used to identify significant differences in the trace element concentration in the hepatic tissue between the two species. Simple linear regression model was performed to correlate the size of sharks with the different trace elements concentrations in hepatic tissues. When data were not normally distributed, a log transformation has been performed. The p values of <0.05 were used to determine the significant differences. Analyses were conducted on a dry weight basis. All statistical results were analyzed using the SAS statistical software program JMP, version 14.

6.3. RESULTS

6.3.1. PCB and DDT

The pattern of PCB tissue distribution (ΣPCB) in both species showed the highest burdens in livers compared with muscles and fins that accumulate in similar concentrations (Table 6.1).

Table 6.1 Summary of PCB and DDT concentrations expressed as ng g^{-1} dry weight (dw) and lipid weight (lw) in the muscle, liver and fin samples of the scalloped hammerhead shark and milk shark collected in Djibouti

Species	**Tissue**	**N**	**Lipid %**	**ΣPCB**			**ΣDDT**		
			Mean ± SD	Range dw	Mean ± SD dw	Mean ± SD lw	Range dw	Mean ± SD dw	Mean ± SD lw
R. acutus	Liver	5	62.4 ± 9.9	24.6 - 201	135 ± 72.4	230 ± 132	7.1 - 75.7	33.9 ± 26.2	53.1 ± 39.1
	Muscle	2	4.0 ± 0.3	21.1 - 57.6	39.4 ± 25.8	1005 ± 710	3.0 - 32.9	18.0 ± 21.1	466 ± 558
	Fin	2	1.0 ± 0.2	22.7 - 47.3	35.0 ± 17.4	3705 ± 2428	0.7 - 38.2	19.4 ± 26.5	2217 ± 3054
S. lewini									
	Liver	6	64.3 ±	39.3 - 471	258 ± 160	415 ± 272	17.5 - 740	229 ± 306	406 ± 545
	Muscle	2	12.4	47.3 - 51.0	49.1 ± 2.6	3045 ± 700	12.2 - 16.2	14.1 ± 2.8	857 ± 18.5
	Fin	4	1.6 ± 0.3	10.1 - 13.7	62.0 ± 63.3	7904 ± 7559	<LOD - 17.4	12.9 ± 23.1	1523 ± 2709
			0.8 ± 0.1						

In the hepatic tissues of hammerhead sharks, all congeners have been found, except for PCB 209 that could not be detected in any samples. The predominant congener was PCB 153 (80.9 ± 48.6 ng g^{-1} dw) accounting for 31.3% of total PCB, followed by PCB 180 and PCB 138 that contributed for 22% and 14% respectively (Fig. 6.1). The One-way Anova Test showed a statistical difference in the mean concentration of congeners ($F(5.30) = 9.216$, $p<0.001$). Post hoc Tukey's HSD test showed a significant difference in the mean concentration of Hexa-CB with Tri-CB ($p = 0.002$), Tetra-CB ($p = 0.0083$), Penta-CB ($p = 0.0123$) and Octa-CB ($p < 0.001$). Post hoc Tukey's HSD test also revealed a difference between Hepta-CB with Octa-CB ($p = 0.0039$) and Tri-CB ($p = 0.0150$).

In fins, the predominant congeners were PCB 52 (20.1 ± 33.6 ng g^{-1} dw) that accounted for 32% of total PCB, followed by PCB 18 and PCB 101, contributing for 17.4% and 17.0% respectively (Fig. 6.1). On the contrary, PCB 149 was never detected. In muscles, the predominant congeners were PCB

44 (11.6 ± 9.1 ng g^{-1} dw), PCB 101 (8.4 ± 11.9 ng g^{-1} dw) and PCB 28+31 (8.2 ± 11.5 ng g^{-1} dw), accounting for for 23.5%, 17.1% and 16.7% of the total PCB. PCB 52, 149, 118 and 170 were never detected (Fig. 6.1). No statistical analyses were performed for PCB congeners in fins and muscles due to the limited sample size.

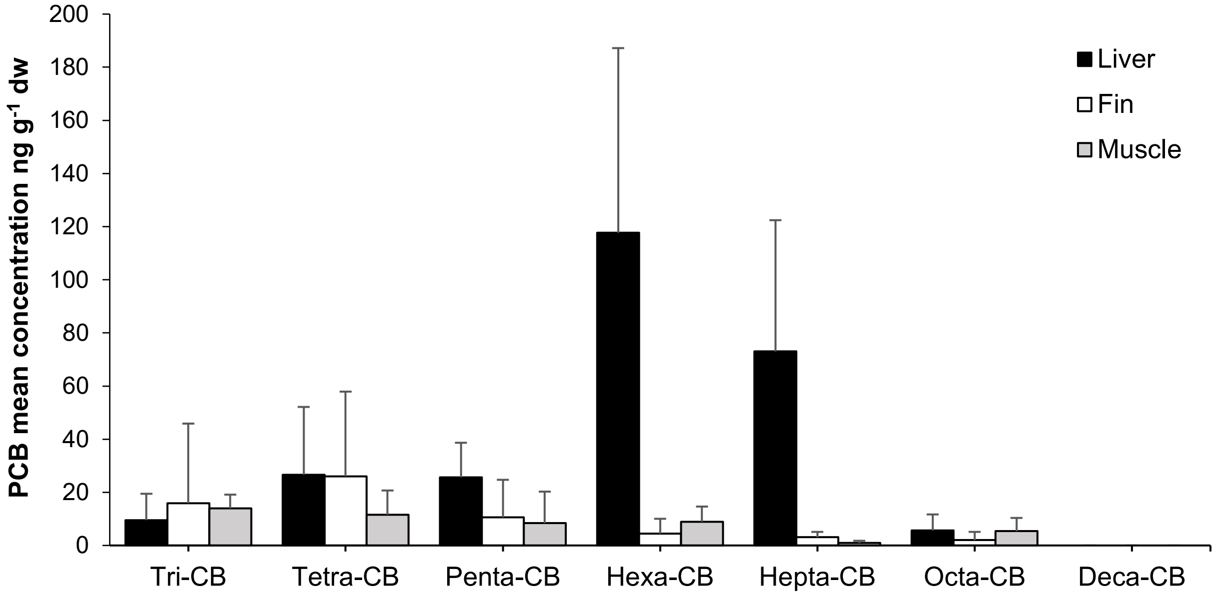

Fig. 6.1 Accumulation profiles of PCB in the scalloped hammerhead shark, in livers (N = 6), fins (N = 4) and muscles (N = 2). Error bars represent ±1 standard deviation

In liver of milk sharks, all congeners have been found and the predominant was PCB 153 (28.2 ± 18.2 ng g^{-1} dw), accounting for 20.9% of total PCB, followed by PCB 180, PCB 18 that contributed for 17.9% and 12.8%, respectively (Fig. 6.2). The One-way Anova Test showed a statistical difference in the PCB concentration between congeners (F(5.24) = 3.3815, p = 0.0188). Post hoc Tukey's HSD test showed a significant difference in the mean concentration of Hexa-CB with Octa-CB (p = 0.0104).

In fins, the predominant congeners was PCB 101 (19.5 ± 7.6 ng g^{-1} dw) representing by itself 55.6% of total PCB, followed by PCB 44 and PCB 18 that contributed for 12.7% and 11.8% (Fig. 6.2). PCB 149, 118, 153, 170 and 209 were never detected. In muscles, the predominant congeners were PCB

44 (8.8 ± 4.6 ng g^{-1} dw) thatcontributed for 22.3% of total PCB, followed by PCB 153 and PCB 28+31 with a percent of 21.4% and 14.4%, respectively (Fig. 6.2). In muscles PCB 149, 118, 170 and 209 were never detected.

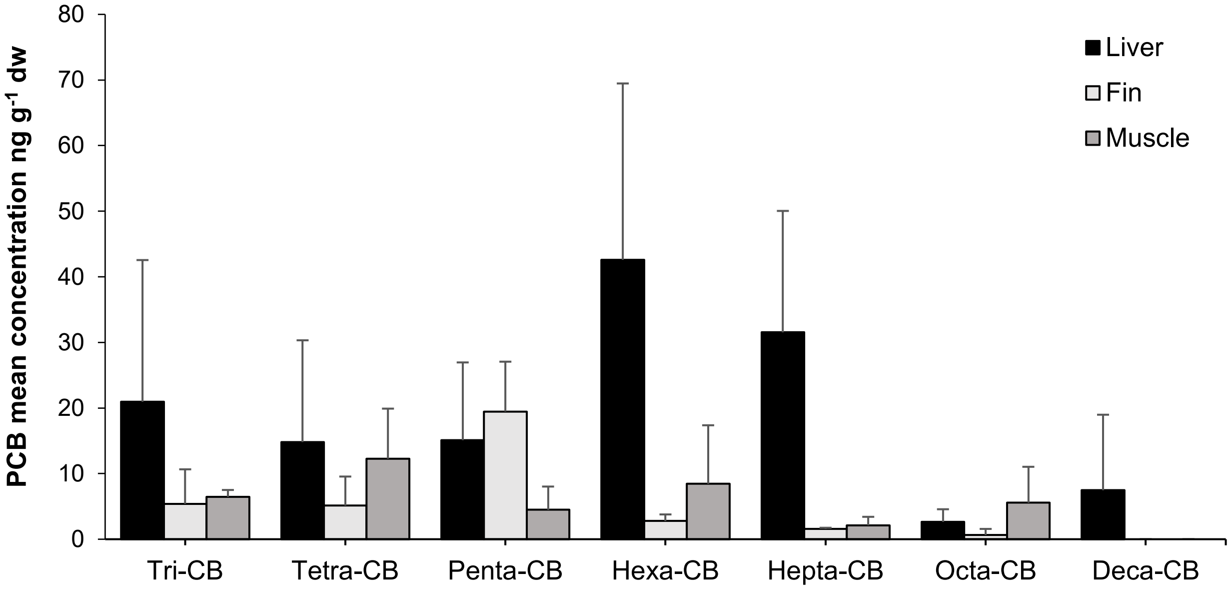

Fig. 6.2 Accumulation profiles of PCB in the milk shark, in livers (N = 5), fins (N = 2) and muscles (N = 2). Error bars represent ±1 standard deviation

The milk shark mother and its embryo showed the same congeners, except for the PCB 52, detected only in the embryo, and PCB 44, quantified only in the mother. The adult specimen showed a total PCB concentration of 201 ng g^{-1} dw (378 ng g^{-1} lw), while the embryo of 159 ng g^{-1} dw (284.3 ng g^{-1} lw) (Fig. S.1, Supplementary material IV).

An Independent-samples t-test was conducted to compare the total PCB concentration between the two species. Results did not show any statistical difference in the PCB contamination between species (t(7.2) = 1.686, p = 0.1344) (Fig. 6.3). However, when comparing the PCB concentration across tissues, the Kruskal-Wallis Test showed a significant difference ($X^2 = 8.357$, df = 2, p = 0.0153). The Post Hoc Test Steel-Dwass All Pairs showed that livers accumulate more PCB than fins (p = 0.0368) (Fig. 6.3).

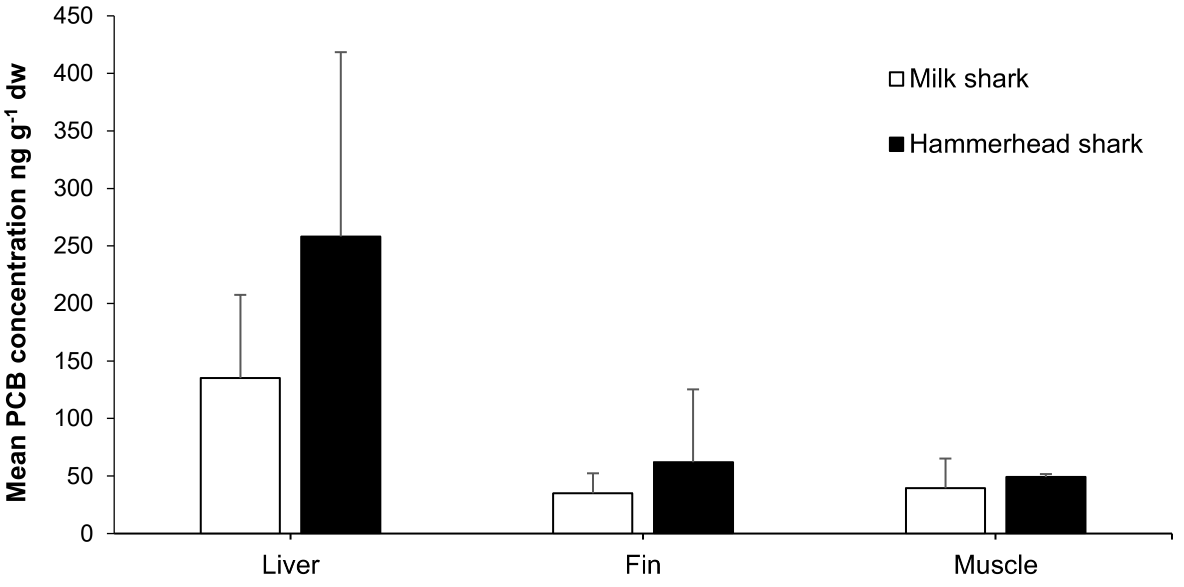

Fig. 6.3 Accumulation profiles of PCB in fins (N = 6), muscles (N = 4) and livers (N = 11) of milk and scalloped hammerhead sharks. Error bars represent ±1 standard deviations

DDT concentration in hammerhead shark was higher in livers, followed by muscles and fins (Table 6.1, Fig. S.2, Supplementary material IV). The predominant metabolite in liver was DDE 83.4% while DDT and DDD accounted for the 10.9% and 5.7% of total DDT, respectively (Table 6.2). In muscles, DDE was the predominant metabolite, 59.1%, followed by DDT, 34%, and DDD, 5.9%. In fins, DDE accounted for 47.3%, while DDD for 29% and DDT for 23.6%. In all tissue analyzed, p,p'DDE was the major metabolite reaching the highest level in livers, then in muscles and in fin (Table 6.2). Considering only livers, the p,p'DDE/∑DDT ratio, had a mean of 0.7 ± 0.2.

DDT concentration in milk sharks was higher in livers, followed by fins and muscles (Table 6.1, Fig. S.2, Supplementary material IV). The predominant metabolite in liver was DDE contributing for 52.9% of total DDT and in particular p,p'DDE that was the major compound. Other metabolites, DDT and DDD, accounted for the 27.6% and 19.4%, respectively (Table 6.2). In muscles, DDT was the predominant metabolite, in particular p,p'DDT that contributed for 60.8% of total DDT, followed by DDD, 24.8%, and DDE, 13.5%. In fins, DDE accounted for 98.4%, in particular o,p'DDE, while

DDD for 1.6% and DDT were <LOD (Table 6.2). The p,p’DDE/∑DDT ratio in livers had a mean of 0.6 ± 0.2.

The milk shark mother and its embryo showed a similar trend where p,p’DDE was the main metabolite with a mean concentration of 5 and 10.4 ng g^{-1} dw, respectively. In both specimens DDD was not detected and p,p’DDT had a value of 2.1 ng g^{-1} dw in the mother and 0.8 ng g^{-1} dw in the embryo. The latter had a relative high concentration of o,p’DDE, which reached 8.9 ng g^{-1} dw, contributing to 44% of total DDT, contrarily to the mother in which o,p’DDE was not detected.

Table 6.2 Concentrations of DDT (dw ng g^{-1}) and related compounds in liver, muscle, or fin of *Sphyrna lewini* and *Rhizoprionodon acutus* collected in Djiboutian waters

DDT metabolites	***Sphyrna lewini***			***Rhizoprionodon acutus***		
	Liver	Fin	Muscle	Liver	Fin	Muscle
DDE	191 ± 275	6.09 ± 11.3	8.36 ± 1.1	18 ± 13.5	19.1 ± 26.1	2.44 ± 2.77
o,p' DDE	0.52 ± 1.27	< LOD	0.60 ± 0.85	5.45 ± 4.15	18.4 ± 26.1	<LOD
p,p' DDE	190 ± 275	6.08 ± 11.3	7.85 ± 2.07	12.5 ± 10.2	0.68 ± 0.03	2.43 ± 2.77
DDD	13.1 ± 11.8	3.73 ± 7.47	0.84 ± 1.2	6.60 ± 8.99	0.32 ± 0.45	4.46 ± 5.06
o,p' DDD	7.24 ± 9.48	3.33 ± 6.67	0.84 ± 1.19	5.67 ± 5.80	<LOD	4.22 ± 5.39
p,p' DDD	5.82 ± 12.2	0.40 ± 0.80	< LOD	0.93 ± 2.08	0.32 ± 0.45	0.24 ± 0.33
DDT	24.9 ± 27.1	3.04 ± 4.45	4.85 ± 2.8	9.36 ± 6.92	<LOD	10.9 ± 13.1
o,p' DDT	0.45 ± 0.45	0.33 ± 0.66	< LOD	< LOD	<LOD	0.16 ± 0.22
p,p' DDT	24.4 ± 27.1	2.71 ± 3.82	4.85 ± 2.8	9.36 ± 8.99	<LOD	10.9 ± 13.1

Notes: Values presented are means ± SD.

Due to the low sample number of muscles and fins, only DDT concentration in livers was taken into consideration for statistical analyses. The Wilcoxon test did not show any statistical differences between species (X^2 = 1.6333 df = 1, p = 0.2012). When considering all tissue samples, despite the species, the Wilcoxon/Kruskal-Wallis (Rank Sums) Test showed a difference in the total DDT concentration (X^2 = 7.217, df = 2, p = 0.0271). The Wilcoxon Post Hoc test showed a statistical difference in the mean concentration between liver and muscle (p = 0.0306), as well as with fins (p = 0.0430). The mean concentration in liver was statistically higher than the other two tissues.

A simple linear regression was calculated to predict both PCB and DDT concentration (ng g^{-1} dw) based on length. However no significant relationship was found neither for PCB (F(1.9) = 0.120, p = 0.737) with an R^2 of 0.013 or DDT (F(1.9) = 3.595, p = 0.091) with an R^2 of 0.285. The ratio between DDT and PCB (ΣDDT/ΣPCB) was used to characterize the magnitude of the contributions from agricultural and industrial sources to shark contamination. For the majority (73%) of investigated sharks the ∑DDT/∑PCB values were between 0.04-0.4 indicating a high PCB preponderance.

6.3.2. Trace Elements

The concentrations of selected trace elements in muscles and livers of both species are presented in Table 6.3.

Table 6.3 Descriptive statistics of trace elements concentrations (ppm dw) based on species and tissue

Metals	**Milk Shark**				**Hammerhead Shark**			
	Hepatic tissue n = 5		**Muscle Tissue** n = 3		**Hepatic Tissue** n = 6		**Muscle Tissue** n = 2	
	Mean	Dev. Std.	Mean	Dev. Std.	Mean	Dev. Std.	Mean	Dev. Std.
V	0.07	0.08	0.09	0.10	0.02	0.02	0.07	0.05
Mn	1.52	1.52	0.53	0.17	2.29	1.16	0.63	0.31
Co	0.06	0.05	0.03	0.02	0.03	0.02	0.01	0.01
Cu	6.41	2.97	1.77	0.58	14.6	18.6	1.20	0.37
Ni	<LOD		0.1	0.06	<LOD		0.13	0.04
Zn	27.4	20.96	14.1	8.6	18.8	8.9	15.6	3.29
As	25.3	14.1	19.7	5.7	18.7	17.0	12.2	1.08
Se	16.3	14.9	4.8	0.84	12.2	8.0	3.65	0.91
Cd	14.5	22.6	0.22	0.13	0.48	0.82	0.004	0.0002
Pb	0.16	0.22	0.14	0.2	0.08	0.05	0.05	0.02
Cr	0.3	0.4	0.34	0.3	0.13	0.07	0.60	0.38
Hg	0.68	1.32	0.29	0.14	0.35	0.48	2.51	0.20
Ag	0.27	0.27	0.02	0.01	0.25	0.32	0.002	0.003
Sr	1.9	0.62			3.27	2.47		
Mo	0.2	0.11			0.25	0.09		
Fe			37.7	9.03			16.4	7.83

Notes: Missing values were not measured in this study

Concentration of Zn, Se and As were statisticallly higher than those of all other elements in liver samples of milk shark and scalloped hammerhead shark (Table S.3, Supplementary material IV).

Similarly, Fe, As and Zn were the elements in higher concentration in muscles of both species (Table 6.3; Table S.3, Supplementary material IV). However, due to the low sample size of hammerhead shark muscles, no statistical analyses could be performed. No differences were found between the two species in the trace element concentrations of liver samples. Analyses aimed at evaluating differences in the muscle concentration of trace elements between the two species were not performed due to the low sample size (Fig. S.3, Supplementary material IV).

When comparing trace elements concentration in muscle and liver samples of both species, Mn, Cu, Zn, Ag and Se were in higher concentrations in livers than in muscle samples (Table 6.3; Table S.3, Supplementary material IV). Liver tissues of the scalloped hammerhead shark showed a significant negative relationship between Zn, Se, and Ag concentration and shark size, while Cu only showed a similar behavior in milk shark.

However, when combining both species and analyzing the relationship of trace elements with shark size on the base of sex, strong significant decreasing relationships were found for Zn, Se, Hg, Mo and Cu, but only for male specimens (Table S.3, Supplementary material IV). When combining muscle samples of both species, a positive trend was found between Fe, As and Cd concentrations and shark size. Contrarily, Cr, Mn, Ni, Zn and Pb concentrations showed a negative relationship with increase of shark length. However, due to the low sample size, no statistical analyses were performed.

6.3.3. Exposure risk Assessment

The HR values were <1 for both non carcinogenic and carcinogenic risks for all analyzed tissues (Table 6.4). In regards to CR, PCB values were between 10^{-4} and between 10^{-6} for both species and both tissues analyzed (Table 6.4). CR for DDT was smaller than 10^{-6} and below the threshold value.

The THQ value for each trace element analyzed in muscle samples of both species are presented in Table 6.5. The THQ value were less <1 for all trace elements and therefore the exposed population should not experience any non-carcinogenic adverse health hazard. The CR was calculated for five trace elements in muscles of both species and values are presented in Table 6.5. THQ and CR for As were calculated for the inorganic form, which generally accounts between 3% and 10% of total As (Rahman et al., 2012; Copat et al., 2013). For this study, both 3% and 10% of total As were calculated for the exposure risk assessment.

Table 6.4 The Hazard Ratio (HR) for non carcinogenic risk, for carcinogenic risk and the carcinogenic risk (CR) for milk sharks and hammerhead sharks consumed in Djibouti

OCs	Species	Tissue	HR	HR carcinogenic risk	CR
PCB	Hammerhead shark	Fin	0.14	0.011	$5.4\text{x}10^{-6}$
		Muscle	0.11	0.009	$4.3\text{x}10^{-6}$
	Milk shark	Fin	0.08	0.006	$3.1\text{x}10^{-6}$
		Muscle	0.09	0.007	$3.4\text{x}10^{-6}$
DDT	Hammerhead shark	Fin	0.001	0.0004	$1.9\text{x}10^{-7}$
		Muscle	0.001	0.0004	$2.1\text{x}10^{-7}$
	Milk shark	Fin	0.002	0.0006	$2.9\text{x}10^{-7}$
		Muscle	0.002	0.0006	$2.7\text{x}10^{-7}$

Table 6.5 The target hazard quotient (THQ) and the carcinogenic risk (CR) values for Djiboutian population calculated for each trace element (ppm ww) analyzed in muscle samples of hammerhead and milk sharks

Trace element	**Rfd ug g^{-1} d^{-1}**	**CSF mg kg^{-1} d^{-1}**	**Concentration**		**THQ**		**CR**	
			R. acutus	***S. lewini***	***R. acutus***	***S. lewini***	***R. acutus***	***S. lewini***
V	0.005		0.03	0.02	0.001	0.001		
Cr	0.003	0.5	0.1	0.18	0.005	0.009	7.5x10^{-6}	1.3x10^{-5}
Mn	0.14		0.16	0.19	0.0002	0.0002		
Fe	0.7		11.3	4.93	0.002	0.001		
Co	0.0003		0.01	0.003	0.005	0.001		
Ni	0.02	1.7	0.03	0.04	0.0002	0.0003	7.7x10^{-6}	9x10^{-6}
Cu	0.04		0.53	0.36	0.002	0.001		
Zn	0.3		4.22	4.67	0.002	0.002		
As	0.0003	1.5	0.18* (0.59)	0.11* (0.37)	0.09* (0.39)	0.05* (0.18)	3.9x10^{-5}* (1.3x10^{-4})	2.4x10^{-5}* (8x10^{-5})
Se	0.005		1.43	1.09	0.04	0.032		
Ag	0.005		0.006	0.001	0.0002	0.00002		
Cd	0.001	6.3	0.07	0.001	0.01	0.0002	6.1x10^{-5}	1.1x10^{-6}
Pb	0.004	0.0085	0.04	0.01	0.002	0.001	5.2x10^{-8}	1.8x10^{-8}
Hg	0.0003		0.09	0.75	0.04	0.366		

Notes: The value represents the As inorganic form. Numbers* were calculated taking into account 3%, while those in brackets 10%.

6.4. DISCUSSION

6.4.1. PCB and DDT

The overall OCs concentrations were similar among the two sharks' species. Both *R. acutus* and *S. lewini* are widely distributed throughout the world and are some of the most commonly landed coastal sharks in many fisheries (Capape et al., 2006; Henderson et al., 2008; Moore et al., 2012). Research on milk shark and scalloped hammerhead shark suggests both species usually feed on a variety of small teleost fish, crustacean and cephalopod preys (Klimley, 1983; White et al., 2004; Torres-Rojas et al., 2006). Bioaccumulation and biomagnification are likely related to the feeding habits of sharks and preys are the major exposure pathway of sharks to OCs (Lee et al., 2015). Based on dietary compositions, it has been estimated that the scalloped hammerhead shark and the milk shark occupy the same trophic level (Cortes, 1999), which would explain the similar OCs concentration found among species in the present study.

Although total OCs concentrations were similar in the scalloped hammerhead shark and the milk shark, the accumulation profiles of PCB and DDT differed among species, suggesting species-specific accumulation of these contaminants. In this study, the hammerhead shark livers showed a preferential accumulation of Hexa-CB and Hepta-CB that together accounted for the greatest proportion of total PCB, up to 70% (Fig. 6.1). On the contrary, the milk shark livers showed a less predominant contribution of Hexa-CB and Hepta-CB with respect to other congeners, such as Tri-CB, which accumulated in greater proportion (Fig. 6.2). In addition, milk sharks showed the capability to accumulate Deca-CB that was never detected in the scalloped hammerhead shark. The different accumulation profiles of PCB implying species-specific differences in metabolic capacities for PCB congeners (Lee et al., 2015). Although shark species had different accumulation features of PCB, the higher-chlorinated PCB 153, PCB 138 and PCB 180 dominated in liver tissue. Accumulation profiles were similar to those reported for shark livers from different part of the world, such as the Mediterranean Sea (Storelli et al., 2005; Storelli and Marcotrigiano, 2001), Canary Islands in the North African Atlantic Ocean (Serrano et al., 1997, 2000) Indian, Pacific Oceans, coastal water of Korea (Lee et al., 2015), and southeastern coast of Brazil (Cascaes et al., 2014).

Compared with livers, fins and muscles of both species accumulated less higher-chlorinated congeners. Differences in PCB accumulation pattern between livers and muscles/fins might be explained by the fact that higher-chlorinated, more hydrophobic congeners (such as PCB 138, 153 and 180) are less easily metabolized (Arnold and Feeley, 2003) and bioaccumulate in lipid-rich tissues such as the liver (Storelli et al., 2003; Mull et al., 2012). Furthermore, the lipid composition including fatty acids in the different organs of fish vary significantly (Chakraborty et al., 2005). Regardless of the species, PCB concentration in fin and muscle tissues generally did not correlate with those in liver that most likely accumulates OCs in elasmobranchs because of its high lipid content (Gelsleichter et al., 2005). In fact, many OC contaminants found in livers of scalloped hammerhead shark and milk shark were not detected in the fins and muscles from the same individuals. However, if not recommended as indicators of internal contaminant load, assessments of muscle and fin OC concentrations in sharks have merit for characterizing the risks that aquatic contaminants pose to human populations that consume meat from these fish (Gelsleichter et al., 2005).

In regards to DDT, predominant DDT compound in most sharks was p,p’DDE, especially for hammerhead sharks. DDT are abiotically and biotically degraded to metabolites such as DDD or DDE. The p,p’DDE/∑DDT ratio, an indicator of new DDT inputs in the environment, was 0.7 in hammerhead sharks and 0.6 in milk sharks. These ratios were equal or slightly higher than the critic value of 0.6, which suggest that recent inputs of technical DDT might be experienced in the area (Storelli et al., 2005; Boldrocchi et al., 2018). The use of chemical pesticides has been developing in plenty countries through sub-Saharan Africa for more than 40 years (Bon et al., 2014). Although the use of POPs has been banned or restricted, developing countries still use them for agricultural and health purposes, such as the use of DDT in combating malaria, and they can be still found in aquatic ecosystems (Boldrocchi et al., 2018). The ratio between DDT and PCB (ΣDDT/ΣPCB) was used to characterize the magnitude of the contributions from agricultural and industrial sources to shark contamination. Generally, the ratio is higher in water masses closer to agricultural areas and lower in waters closer to industrialized areas, independently of the congener number determined. For the majority (73%) of investigated sharks the $\sum$DDT/$\sum$PCB values were between 0.04-0.4 indicating a high PCB preponderance. The latter confirms the predominance of an industrial contamination profile over an agriculture source both at a local or/and at a wider scale. OCs concentrations in both species in our study were not significantly related to length, consistent to previous research (Gelsleichter et al., 2005; Lee et al., 2015), although the sample size in this study was too small to drive any definitive conclusion.

Comparison of organochlorine concentrations with other studies may be limitedly significant: to our knowledge, only two studies examined organochlorines in scalloped hammerhead sharks, one from Japan (Nomiyama et al., 2011) in which only PCB metabolites in the blood were measured, and one from U.S. Atlantic waters (Lyons and Adams, 2015), that measured DDT and PCB in the hepatic tissues. Therefore, comparison was possible only with the latter one. Our study showed a DDT concentration in hammerhead shark hepatic tissues that ranged from 23 to 1210 ng g^{-1} lw, while Lyons and Adams (2015) reported values much higher in specimens collected from U.S. Atlantic waters. Indeed, livers of adult females had a DDT concentration of approximately 3000 ± 790 ng g^{-1} lw, while the only sampled male of 1400 ng g^{-1} lw. In regards to PCB, concentration levels measured in this study (approximately 415 ng g^{-1} lw in hepatic tissues) were much lower than the 11100 ng g^{-1} lw reported for the adult females in the U.S. Atlantic (Lyons and Adams, 2015). Scalloped hammerhead sharks from this study showed OCs concentrations more similar to those reported for *Sphyrna tiburo* in Florida (Gelsleichter et al., 2005), whose levels of total DDT rarely exceeded 100 ng g^{-1} lw, while the mean PCB never exceed 400 ng g^{-1} lw (Gelsleichter et al., 2005). Contamination in hammerhead sharks from this study was much higher than other species of the same genus, such as *Sphyrna zygaena* from the Indian and Pacific Oceans (Lee et al., 2015).

As concern the milk shark, results from this study showed higher accumulation levels for both PCB and DDT compared with muscle tissues of specimens collected from coastal waters of Korea, that showed a mean PCB and DDT concentrations of 24.2 ± 23.0 ng g^{-1} lw and 1.90 ± 0.2 ng g^{-1} lw, respectively (Lee et al., 2015). This suggests a stronger OCs pollution level in the Gulf of Tadjoura compared with Korean waters. Comparing with species from the same genus, milk sharks showed a PCB accumulation in muscle tissues lower than what reported from the coastal waters of Florida for the Atlantic sharpnose shark, *Rhizoprionodon terraenovae* (Johnson-Restrepo et al., 2005). Contamination of hepatic tissues of milk sharks was also lower than what reported for *Rhizoprionodon lalandii* from the southeastern coast of Brazil (Cascaes et al., 2014).

Accumulation studies on the scalloped hammerhead shark and milk shark are still scarce worldwide and therefore it is difficult to drive any conclusion on the OCs contamination in the Gulf of Tadjoura. Unfortunately, comparing our results with other bioaccumulation studies on other marine organisms in the study area is equally complex. Almost nothing is known about the existence of organochlorine contaminants in the Red Sea and Gulf of Aden, even if they have been used for more than four decades (Al-Shwafi et al., 2009). To the best of our knowledge, only one research evaluated the presence of PCB and DDT in commercial fish tissues from the Gulf of Aden and reported an average concentration of 2.9 ng g^{-1} dw for DDT and 0.4 ng g^{-1} dw for PCB (Al-Shwafi et al., 2009), much lower than levels presented in our study. However, the study by Al-Shwafi et al. (2009) focused on teleost fishes and shellfish species, which likely occupy trophic levels lower than sharks and, therefore, are expected to accumulate less pollutants.

Sharks have a number of biological traits that make them particularly prone to accumulate high levels of these pollutants. In addition, OCs bioaccumulation represents a potential concern for those species where maternal energy investment in embryo development occurs in both egg- and live-bearing species via yolk and/or placental nourishment and the potential exists for the transfer of the maternal PCB burden (Olin et al., 2014). Female sharks mobilize a significant proportion of their liver lipids to developing embryos during gestation (Hussey et al., 2010). In lines with other findings (Gelsleichter et al., 2007; Lyons and Lowe, 2013; Olin et al., 2014), in this study, the presence of high concentrations of organic contaminants in the milk shark embryo, prior to any exogenous exposure, provides clear evidence of maternal offloading (Fig. S.1, Supplementary material IV). Indeed, the fully term embryo had an OCs concentration similar to older individuals, indicating that the extent of such maternal offloading is enough to generate OCs concentrations in neonates that are of consistent magnitude with those associated with increased toxicological risk (Olin et al., 2014). Exposure of marine animals to these persistent organic pollutants is, in fact, of serious concern due to their negative effects including birth defects, endocrine disruption, immune system dysfunction and reproductive problems (Tanabe, 2002; Ylitalo et al., 2005; Jepson et al., 2005). A study on geographic variations in OC contaminants in *S. tiburo* from Florida (Gelsleichter et al., 2005), revealed that populations with greater concentrations of OCs exhibited markedly higher rates of infertility (Parsons, 1993; Gelsleichter et al., 2005). The evidence for a relationship between OC exposure and reproductive impairment in *S. tiburo* was circumstantial, however if demonstrated, it could indicate that specimens and closest species with comparable OCs loads, as scalloped hammerhead sharks from Djibouti, might experience similar adverse effects.

6.4.2. Trace elements

In regards to trace metals, within the same shark species, metals accumulate differently and even within the same specimen, one metal accumulate at different levels in different tissues (Turoczy et al., 2000; Canli and Atli, 2003). Similarly to what reported for OCs, trophic level and associated diet, as well as species ecology, appear to be the most important factors determining metal concentration differences between species (Turoczy et al., 2000; Pethybridge et al., 2010). In this study, milk shark and hammerhead shark showed a similar load of trace elements, which is consistent with the similar feeding ecology and habitat use of these species. Both accumulated Zn, Fe and As in elevated concentrations in muscles and livers (Table 6.3). Milk sharks showed a Zn concentration in muscle similar to what reported for specimens of the same species collected in Malaysian waters, but lower than those captured in the Persian Gulf (Ong and Gan, 2017; Adel et al., 2017). Hammerhead sharks from Djibouti accumulated higher Zn concentration in muscles if compared with the same species from Papua New Guinea (Powell et al., 1981) and three time higher in the hepatic tissue if compared with hammerhead sharks from Malayasia (Ong and Gan, 2017). Overall, Zn accumulate more in liver and its concentration decreased with increased body length similarly to what reported in other shark species (Endo et al., 2008). Zn was the most abundant element in all analyzed tissues. This finding is consistent with the fact that Zn is an essential metal needed to regulate metabolic activities (Ong and Gan, 2017). In line with other studies (Vas and Gordon, 1993; Endo et al., 2008; Moore et al., 2015), Cu and Mn distributed preferentially in liver rather than in muscles and their concentrations decrease with the increase in length of sharks. A possible explanation is an increased clearance of trace elements in larger and more metabolically active sharks (Moore et al., 2015). In this study, Cu concentration in muscles of both milk sharks and hammerhead sharks were similar to what reported for the same species from Malaysia and Papua New Guinea (Powell et al., 1981; Ong and Gan, 2017). On the contrary, hepatic tissues of milk sharks from Djibouti appeared to accumulate more Cu compared with those reported from Malaysia (Ong and Gan, 2017). Cu is another essential nutrient, together with Zn, and is normally found to be more concentrated than other metals because it is needed by the organisms to undergo metabolism (Al-Weher, 2008).

The average Hg concentration in muscles of milk sharks was slightly lower than in liver samples (Table 6.3). Overall, Hg accumulation in muscles was comparable with levels measured in the same species from Malaysia (Ong and Gan, 2017). On the contrary, Hg accumulation in liver was extremely high in this study. In regards to hammerhead sharks, Hg concentration in muscles was much higher than in livers, similarly to other studies (Hurtado-Banda et al., 2012; Bergés-Tiznado et al., 2015). The detoxification processes and the higher metabolic activity of liver, where the protein synthesis and degradation can be more rapid than in muscle tissue, might be the explanation for the lower levels of Hg found in liver (Bergés-Tiznado et al., 2015). The available data in literature fails to reach a consensus as some authors indicate lower concentrations of Hg in livers than muscles (Branco et al., 2007; Endo et al., 2008), others suggest similar levels (Marcovecchi et al., 1991; Endo et al., 2008) and still others reported similar or higher concentrations of Hg in the liver (Hornug et al., 1993). Hammerhead sharks showed a higher Hg concentration in muscles compared with milk sharks, similarly to what reported by other authors (Adel at al., 2017). Adel at al. (2017) speculated that it might be related to a more benthic feeding habits of hammerhead sharks. It was observed that Hg concentrations in marine organisms, including sharks, are positively correlated with size, and that accumulation occurs preferentially in certain tissues, mainly in muscle and liver (Ravera, 2001; Gomez et al., 2004). Contrary to expectations, no body length-dependent accumulation of Hg was observed in liver and muscle samples of both sharks. A possible explanation for the absence of correlation is that the sample population and range of body lengths were too limited in this study.

Se concentration was higher in livers than muscles in both species and, overall, in elevated concentrations (Table 6.3). This finding is congruent with what reported for livers and muscles of hammerhead sharks from the Gulf of California (Bergés-Tiznado et al., 2015). In general, the elevated concentrations of Se in sharks might be related to high Hg levels, since Se inhibits Hg toxicity in several marine species, including sharks (Moore et al., 2015).

Cd concentrations in milk shark tissues were higher than what reported for the same species by other authors (Ong and Gan, 2017; Adel et al., 2017). In both species, Cd tended to accumulate preferentially in the liver of sharks (Marcovecchi et al., 1991). When comparing both species, Cd concentration in milk sharks was higher than in hammerhead sharks, which levels were also lower than the same species from Trinidad and Tobago (Mohammed and Mohammed, 2017). High concentrations of As and Cd have been attributed to a diet richer in crustaceans and cephalopods than in fish (Bustamante et al., 1998; Turoczy et al., 2000). This could contribute to the significantly higher

As found in both milk and hammerhead sharks, and Cd concentrations in milk shark (Table 6.3). It should be noted that mean Cd levels in hepatic tissues were extremely high due to one female specimen, pregnant at the time of capture. Moore et al. (2015) speculated that high levels of As found in the smoothtooth blacktip shark livers could be linked to a reduced liver condition that can be found in female sharks in the latter stages of pregnancy. Elevated concentration of Cd in milk sharks from this study might be attributed to the same condition as well. No body length-dependent accumulation of Cd was observed in the liver samples from both species, however a positive trend was found in muscles, with Cd concentration increasing with length, as reported for smoothtooth blacktip sharks (Moore et al., 2015).

Hammerhead and milk sharks from this study appear to accumulate elevated concentrations of As, also compared with other studies (Powell et al., 1981; Mohammed and Mohammed, 2017). Background As levels in marine environment do not seem a likely explanation for the elevated concentration found in these sharks. Indeed, anthropogenic pollution from urbanized coastlines has been associated with elevated concentrations of metals and metalloids in shark tissues (Mull et al., 2012) and this may partly explain the high concentrations of As. Both sharks occur in coastal and continental shelf waters, therefore, it is likely that these animals accumulated most of their contaminant loads in Djiboutian waters. However, despite the elevated concentrations found in this area, only inorganic As is of toxicological concern and generally accounts between 3% and 10% of total As (Rahman et al., 2012; Copat et al., 2013).

Other metals appeared to be within the ranges reported from studies elsewhere in the world. As for smoothtooth blacktip shark (Moore et al., 2015), Ni was found in muscles, but was below detection limits in livers. Few shark species have been investigated for Cr presence (Gelsleichter and Walker, 2010), and values in livers were similar to those in muscles, as in this study (Table 6.3). Pb concentration in milk shark had similar concentration to what reported for livers and muscles of the same species in Malaysia and in the Persian Gulf (Ong and Gan, 2017; Adel et al., 2017). Contrarily, hammerhead sharks in Djibouti accumulated less Pb than at other locations (Mohammed and Mohammed, 2017).

Overall, results from this study are generally comparable to those found on trace elements in sharks from other areas of the world and, highlight the wide variation in metal concentrations between species, individuals and tissues.

6.4.3. Exposure risk assessment

Coastal sharks may face unique risks for inhabiting polluted regions on a more regular basis for their dependence on these degraded areas as sites for breeding and juvenile refuge (Castro, 1993; Gelsleichter et al., 2007). Nowadays, pollution is not yet the main threats to shark species, however the negative effects of contaminants may worsen their already dramatic situation (Camhi, 1998). Therefore, it is important to further evaluate the contamination levels in sharks, and especially in those species, such as the scalloped hammerhead shark and the milk shark, that are commonly landed in many coastal fisheries, transfer a substantial amount of contaminants to their offspring and possibly experience greater contamination risks by visiting highly polluted areas on a regular basis. Although the use of POPs has been banned or restricted, in sub-Saharan Africa pesticide choice, quality, and frequency of application and use are not hardly controlled. Quite often, pesticides not licensed for use on food crops end up in the food chain (Bon et al., 2014). In addition, many African countries are burdened by obsolete pesticide stocks (Haylamicheal and Dalvie, 2009). Ethiopia, for instance, that borders Djibouti, has accumulated obsolete pesticide stocks since pesticides were first imported in the 1960s. However, banned pesticides are still being sold in open markets (EPA and SEG, 2003). The activities concentrated around Djibouti City, such as industries, sea traffic, slaughterhouses and the domestic wastewater, result in chronic release of pollutants, mostly untreated (Boldrocchi et al., 2018). Indeed, trace elements contamination in the Djiboutian marine sediments has been attributed to surface run-off and input organic wastes from municipal sewage and solid wastes (Ahmed et al., 2017). This underlines the importance of wastewater treatment before discharging in the sea for reducing the risk of anthropogenic pollution in Djiboutian marine ecosystems (Awaleh et al., 2015).

Within the Gulf of Aden, including Djibouti, sharks are a component of the artisanal fishery and are consumed by locals (Bonfil, 2003; Henderson et al., 2007; Spaet and Berumen, 2015; Jabado and Spaet, 2017). Subsequently, pollutants accumulated in sharks might migrate to the human body through diet and results in various adverse health effects (Bosch et al., 2016). Exposure risk assessment from this study suggest some concerns for the overall contamination levels of both species. The potential cancer risk for PCB was higher than 10^{-6}, highlighting some level of concern from the daily exposure to PCB via fish consumption (Table 6.4). In regards to trace metals, the THQ values for all trace elements were below the threshold however, the CR values calculated for Cd, Ni and Cr in both species were within 10^{-6} and 10^{-4}, suggesting some level of concerns as well (Table 6.5). The mean concentration of total As in muscles of hammerhead shark and milk sharks was 3.66 mg kg^{-1} ww and 5.91 mg kg^{-1}ww, respectively. These values were both higher than the limit of 3 mg kg^{-1}set by the African Department of Health. The CR for inorganic As was higher than 10^{-5} in the best case (3% of total As), while exceeded 10^{-4} in the worst (10% of total As), which is considered unacceptable (Table 6.5) (USEPA, 2005). However, it should be noted that these values were calculated as the worst scenario, taking into account an exposure frequency of 365 days year^{-1} and considering the overall fish ingestion rate of 10.2 g (FAOSTAT database), as it was referred exclusively to sharks consumption, since no specific data on elasmobranchs were available. Never the less, this study clearly highlighted some level of concern from the daily exposure to PCB, Cd, Ni, Cr and As via shark consumption, encouraging further studies in this direction. Indeed, even if the average consumption per person per day is only 10.2 g, a portion of shark fin/muscle during a meal will likely be higher. This means that the consumer will be temporally exposed to levels far exceeding safe limits after a meal. Moreover, consuming of fish involves a mixture of all analyzed elements, and therefore, some potential risk might arise from regularly consuming these species.

6.5. CONCLUSIONS

Both hammerhead shark and milk shark comprise a large proportion of elasmobranch catches, regularly found in Djibouti fish markets. Both species exhibited comparable overall OCs and trace elements loads, however, they also showed different accumulation profiles of pollutants, implying species-specific differences in metabolic capacities. In addition, within the same shark species, specimens accumulate at different levels in different tissues. To evaluate the status of both sharks in Djibouti, the accumulation load of OCs and trace elements of hammerhead shark and milk shark was compared with other research worldwide focused on the same species. However, there is a general lack on ecotoxicology studies related to elasmobranchs. Information mainly comes from specific parts of the world or tends to examine only a limited number of species. Considering the general lack of research, this study provided important baseline information on the pollution status in Djibouti and contaminant loads in the scalloped hammerhead shark and milk shark. Pollution is an important aspect in the understanding of the population decline of sharks, therefore further investigations should be carried out to understand the extent of contaminant exposure in different marine environments, including the Gulf of Aden. Furthermore, elasmobranchs are a component of the worldwide artisanal fishery and seafood consumption is as an important route of human exposure to a variety of chemical contaminants. Hotspots of contamination have been identified associated with specific land based anthropogenic source of pollution, such as industrial ports (Alshemmari et al., 2010; Bu-Olayan and Thomas, 2014). The port of Djibouti is at the crossroads of one of the busiest shipping routes in the world. This study has estimated for the first time the exposure health risks derived from the consumption of the milk shark and scalloped hammerhead shark. The results highlighted some level of concern from the daily exposure to PCB, Cd, Ni, Cr and As, as well as the need to further investigate the level of contaminants within the marine food web of Djiboutian waters.

6.6. ACKNOWLEDGMENTS

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CHAPTER VII. Conclusions and Conservation Implications

7.1. THE WHALE SHARK ECOLOGY

In Djibouti the whale shark aggregation is dominated by juvenile males, however the frequency of females was not constant over the sampling years, indicating that the number of females may increase significantly from one season to another. Results from photo-identification technique showed that some specimens have a prolonged residency and site fidelity, while others appear to be transient. Further photo-identification data should be collected in the upcoming years to fully understand the residency patterns of the whale sharks occurring in Djibouti.

Results of the fieldwork carried out for this thesis demonstrated that spatiotemporal distribution of whale sharks in Djibouti is mainly driven by the upwelling phenomena generated by the southwest monsoon. Indeed, from July to September, the southwest monsoon winds induce an eastward movement of surface water, generating biological productivity in the Gulf of Tadjoura, as supported by the monthly variation in chlorophyll-*a* concentration, which induces an increase in zooplankton biomass. On the contrary, in winter months, the northeast monsoon winds induce a surface water flow toward the Gulf of Tadjoura, which prevents the upwelling phenomenon and induces a decrease in zooplankton biomass. This study showed that the spatiotemporal distribution of whale shark is significantly correlated with the variation in zooplankton biomass so that they most likely occur in Djibouti at the end of summer and leave the area during winter months, when the abundance of food source decreases.

During the aggregation season, whale sharks occur preferably in the extreme west and southeast part of the Gulf of Tadjoura. The affinity to these main hotspots is strongly linked to the greater food availability in those areas. Indeed, sites with greater abundance of whale sharks coincided with those where zooplankton biomass was higher. Here, whale sharks were feeding on zooplankton patches with a mean of 42.2 ± 31.9 mg m^{-3}, almost 1.5 higher than those measured in control stations.

Zooplankton composition might also play an important role in driving the presence of whale sharks in the hotspot bays within the Gulf of Tadjoura. Indeed, this study provided for the first time a characterization of the zooplankton community and identified the main target preys of whale sharks in Djibouti. In the Gulf of Tadjoura, the majority of organisms comprise copepods, reaching 82% of the whole zooplankton mean density, in particular Calanoida and Poicilostomatatoida. At genus level, the most abundant organisms are *Acartia*, *Paracalanus*, *Canthocalanus*, *Nannocalanus*, *Macrosetella*, *Oncaea* and *Corycaeus*. Besides copepods, Appendicularia and Sagittoidea are also important taxa, as well as gelatinous plankton (Ctenophora). Anecdotal observations reported that in the Gulf of Tadjoura, whale sharks fed on dense concentrations chaetognaths. Results from this study confirmed a potential preference of whale sharks for these organisms. Indeed, in feeding samples the mean density of Chaetognatha was statistically higher than those of control areas. Chaetognatha represents a significant food source for a wide variety of large organisms, as they are probably energetically richer than other zooplankton prey (Øresland, 1990, 1995; Postel et al., 2000); therefore, whale sharks might target these organisms so they can meet their energy requirements over a shorter time. Besides Chaetognatha, Ctenophora can be also considered important driver for the whale shark's presence in hotspot bays. Indeed, although the contributions of gelatinous zooplankton to the calculated zooplankton biomass could not be quantified, this study provided some evidences that areas most visited by whale sharks are also those with higher concentrations of gelatinous zooplankton.

At present, a detailed picture of whale shark movements and behaviour outside the aggregation season in Djibouti has not investigated yet. Whale sharks might migrate to different areas either remain year-round in the Gulf of Tadjoiura. However, results from SIA, possibly reflect the isotopic signature of a different feeding area, since whale sharks showed a high enrichment in δ^{13}C compared with that of Djibouti zooplankton samples. Alternatively, this discrepancy with δ^{13}C of surface zooplankton may suggest that part of the whale shark diet comprises mesopelagic sources, which are more carbon-enriched. Indeed, the absence of sharks at the surface outside the aggregation phase may actually indicate a shift in habitat use, from the surface to deeper areas.

Whale sharks showed little intra-specific variation in the isotopic signature, suggesting they may share similar movement patterns and feeding habitats outside the aggregation season. Only one individual did not cluster with other sharks in term of both $\delta^{13}C$ and $\delta^{15}N$ values, probably reflecting different movements. However, the intra-specific variation might be also a consequence of an ontogenetic shift in prey selection (Wetherbee and Cortes, 2004). As the length of the shark increases, the contribution to the diet of small fish and/or of zooplankton of larger size and higher trophic level increases (Borrell et al., 2011). Unfortunately, due to a lack in whale shark measurements and information on their long scale movements, at present it is difficult to drive conclusions related to the isotopic signatures measured in whale sharks. Further analyses will be addresses on this direction with the support of satellite tags technology, which hopefully, will shed some lights on vertical and horizontal movements of whale sharks during the ‘off-season’.

Overall, this study underlines the dependence of whale sharks for the Gulf of Tadjoura, which is used as a foraging site throughout October-February, and the urgent need of improvement of the current protective measurements. Indeed, half of the whale sharks encountered during the fieldwork were injured, probably due to the fact they spend a significant amount of time feeding on surface and, consequently, are exposed to boat collisions and floating fishing gears. Surprisingly, the core habitat for this species identified in this study is located within a marine protected area, however, no actual conservation measurements were ever put in place during the three years of fieldwork. Results from this study demonstrated the necessary of establishing conservation measures aimed to reduce the potential for negative impacts on the sharks. These include limiting the number of people per boat and the total number of boats in the interaction area; reducing in speed when approaching the whale shark hotspot and maintening a reasonable distance from sharks to vessels (such as 20 m) (Pierce et al., 2010; Schleimer et al., 2015). In addition, regulating the number of people per shark and keeping a distance from the shark body (such as 3-4 m) result in a reduction of avoidance by sharks, the risk of accidental touching and also the perceptions of crowding (Pierce et al., 2010; Schleimer et al., 2015).

With this study, I hope to have provided an overall picture of the ecology of the whale shark and baseline information useful for developing new management strategies, indeed the endangered whale shark requires enhanced protection and a dedicated management strategy in Djibouti.

7.2. THE ECOTOXICOLOGY OF THE DJIBOUTI FOOD-WEB

This study has characterized for the first time part of the Djibouti marine food-web from an ecotoxicology point of view. Results presented in this thesis highlight the urgent need of wastewater treatments in Djibouti. Indeed, even the lower trophic level organisms (zooplankton) showed a massive bioaccumulation of both DDT and PCBs. Similarly, whale sharks exhibited elevated concentrations of the same compounds, comparable with high trophic level shark species, such as the blue shark (Storelli et al., 2005) and the white shark (Marsili et al., 2016). The contamination load by PCBs and DDT in whale sharks was also in line with the values measured in both hammerhead sharks and milk sharks from Djibouti, highlighting a strong contamination in this area. The p,p′DDE/ΣDDT ratio measured in zooplankton samples, whale sharks, hammerhead and milk sharks exceeded the critical values suggesting that recent inputs of technical DDT might be experienced in the area.

The trace elements concentration found in whale sharks appeared to be elevated. Whale sharks exceeded the maximum allowable limit (MAL) for Cr, Pb, Se, Cd and Zn; furthermore, Hg levels were above the biota EQS, designed for the protection of aquatic life, indicating that those concentrations may possible cause harmful effects to their health. Whale sharks bioaccumulate also high concentrations of certain trace elements compared with other locations. This finding is consistent with the ecotoxicology results showed for the hammerhead and milk shark. Indeed, all the three shark species accumulated elevated concentrations of elemental and organic pollutants compared with studies on the same species from other areas of the world.

Sharks have a number of biological traits that make them particularly prone to accumulate high levels of pollutants. In addition, coastal species, such as those analyzed in this study, face greater threats of contamination since they inhabit polluted areas on a more regular basis due to their dependence on these areas as sites for feeding, breeding and juvenile refuge (Castro, 1993; Gelsleichter et al., 2007). Djibouti is rapidly expanding in coastal utilization due to an increase in urbanization, fisheries and industrial development that result in chronic discharge of pollutants into the marine environment, affecting the quality of water (Awaleh et al., 2015). Marine environment is extremely important to Djibouti as a renewable food and desalinated water source, as well as for recreational activities. Therefore, the need of a proper coastal environmental management is highly pressing for the protection of the marine environment, and people health (Awaleh et al., 2015). Elasmobranchs are a component of the artisanal fishery in Djibouti and seafood consumption is as an important route of human exposure to a variety of chemical contaminants. Results from this study suggest some concerns for Djiboutians from the daily exposure to elemental and organic pollutants via milk and hammerhead shark consumption. In regards to whale sharks, although they are protected in Djibouti, they might temporarily migrate in areas where illegal fishing is still undergoing and still pose a risk for human health.

Overall, I hope that results presented here on the contamination of elasmobranchs from the area of Djibouti and the associated risk for humans from their consumptions might be an incentive for reducing their fisheries and discouraging their consumption, contributing overall to the shark protection.

APPENDIX

Supplementary Material I

The Ecology of the Whale Shark in Djibouti

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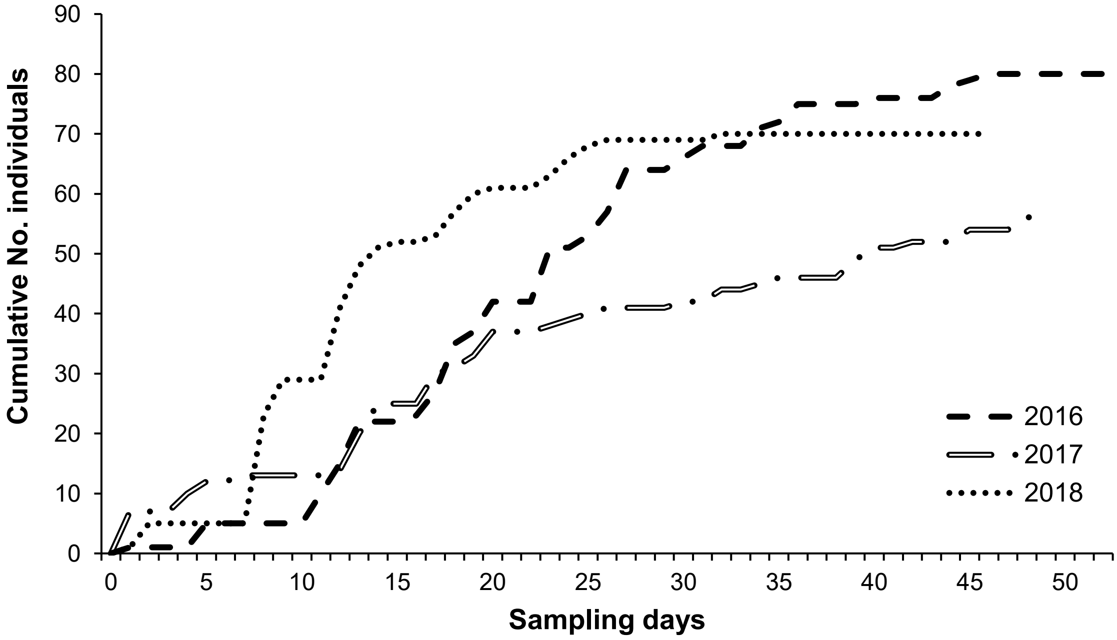

Fig. S.1 Discovery curve for newly identified whale shark in Djibouti (2016-2018). Cumulative total individuals identified is plotted against sampling days

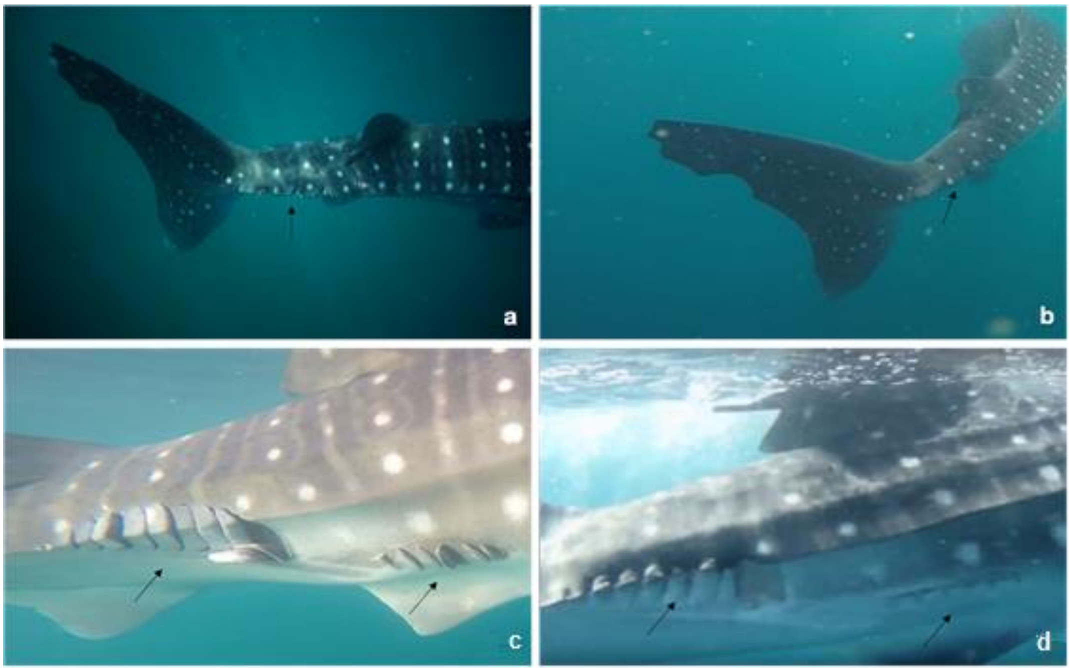

Fig. S.2 Healing process of two individuals injured by a boat propeller. For individual 1 (a-b), pictures were take in a year distance, for individual 2 (c-d), in a month distance

Table S.1 Percentages of taxonomic counts from whale shark feeding plankton samples. Only taxa with >1% are shown here

Taxon			%		
Copepods			**85.0**		
	Copepods nauplii			1.2	
	Calanoids			53.9	
		Acartiidae			5.9
		Calanidae			10.8
		Paracalanidae			25.9
		Centropagidae			2.2
		Pontellidae			1.9
	Poecilostomatoids			26.9	
		Oncaeidae			15.5
		Corycaeidae			11.3
	Harpacticoids			2.4	
		Miraciidae			2.4
Non-copepods			**15.0**		
	Malacostraca			2.9	
		Lucifer spp.			2.2
	Oiklopleurids			2.4	
	Chaetognaths			4.9	

Footnote: Taxa <1% included calanoids copepods (Candaciidae, Eucalanidae, Clausocalanidae, Temoreida, Euchaetidae, Tortanidae, Arietellidae), poecilostomatoids (Sapphirinidae, Peltidiidae), cyclopoids (Oithonidae); Amphipodae, decapod larvae, polychaete larvae, cirripeds larvae, ostracods, *Doliolum spp*, echinoderms larvae, undefined eggs.

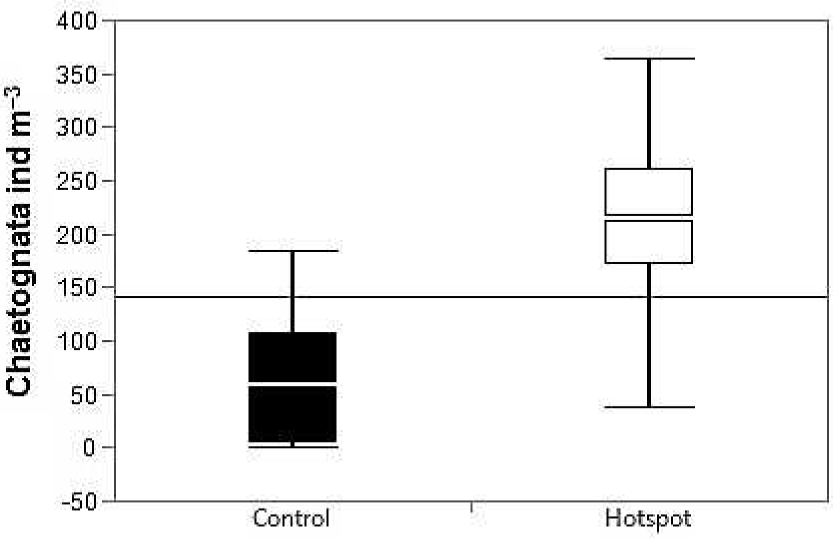

Fig. S.3 Boxplot showed the mean density of Chaetognatha expressed as number of individual per m^3 at control and hotspot stations in Djibouti

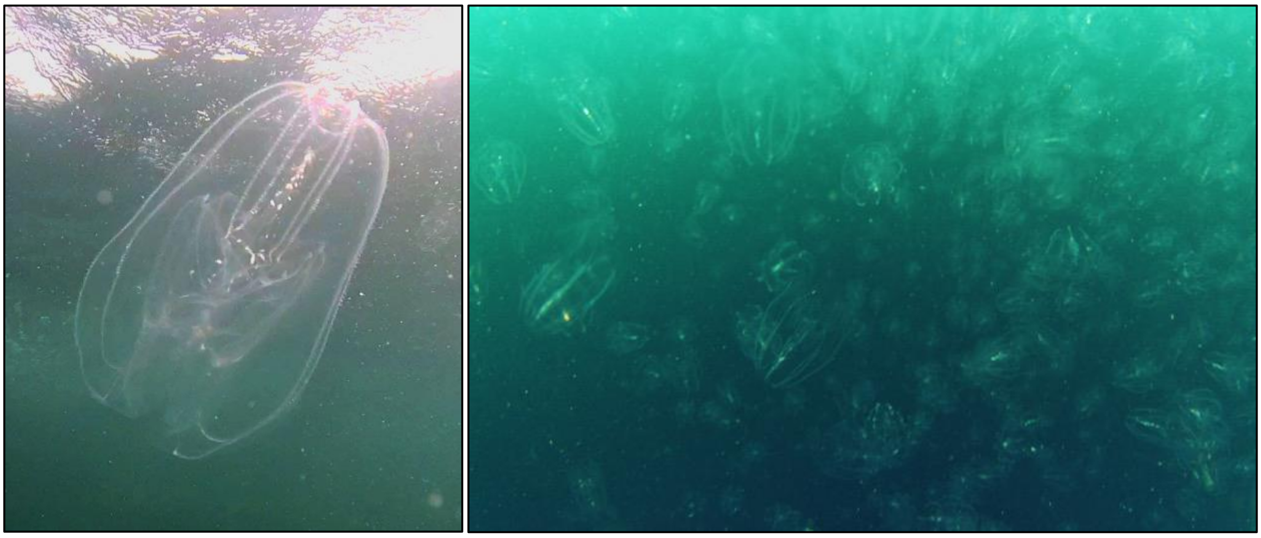

Fig. S.4 Pictures showing Ctenophora organisms, possibly *Bolinopsis spp.*, in the Gulf of Tadjoura during the whale shark aggregation season

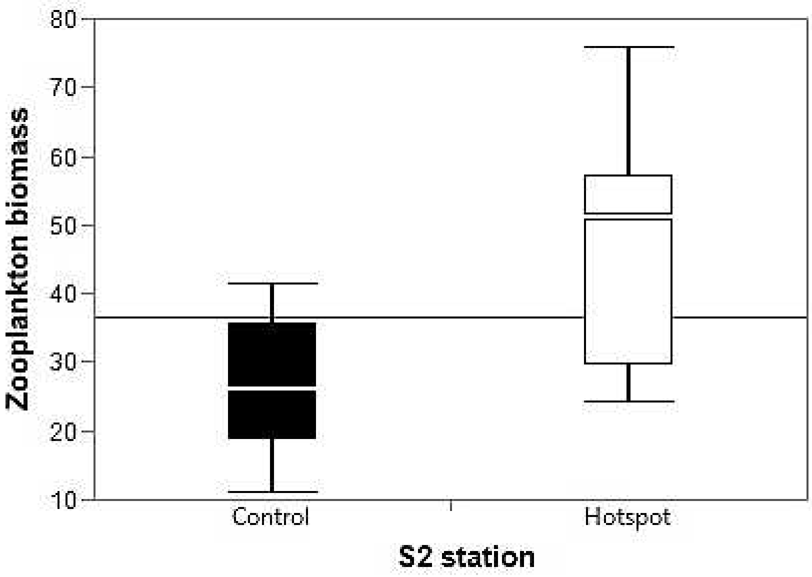

Fig. S.5 Box plots showed the mean zooplankton biomass (mg m^{-3}) collected in S2 at control (black boxplot) and hotspot (white boxplot) stations, in 2016-2018

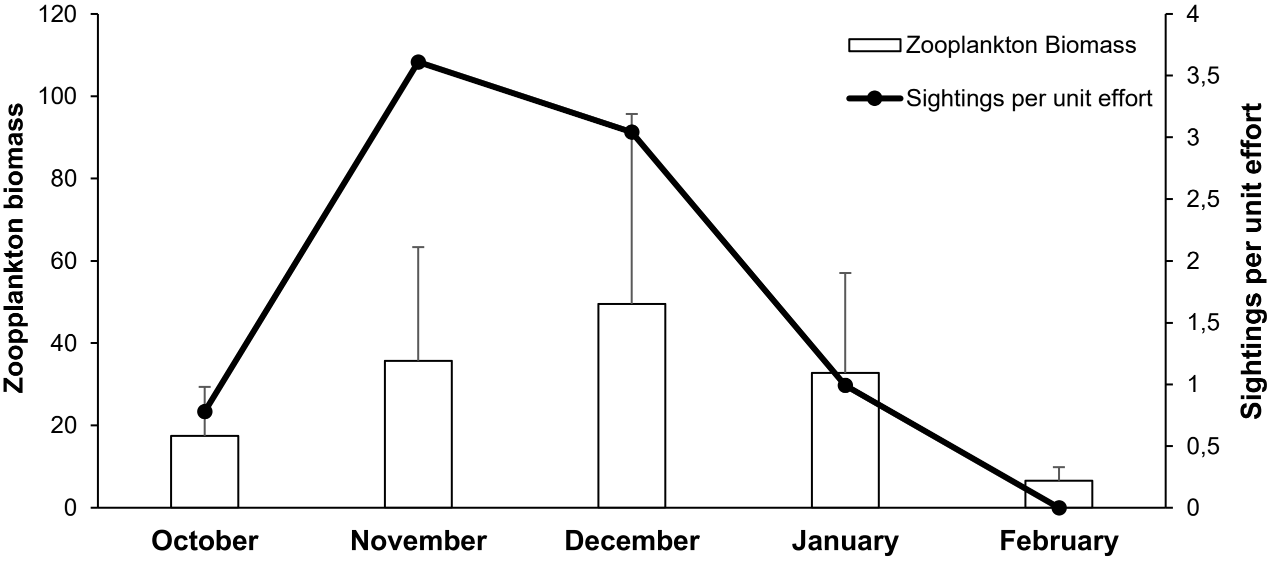

Fig. S.6 Temporal trend of whale shark sightings per unit effort (black line) and that of zooplankton biomass from October to February in 2016-2018 in Djibouti

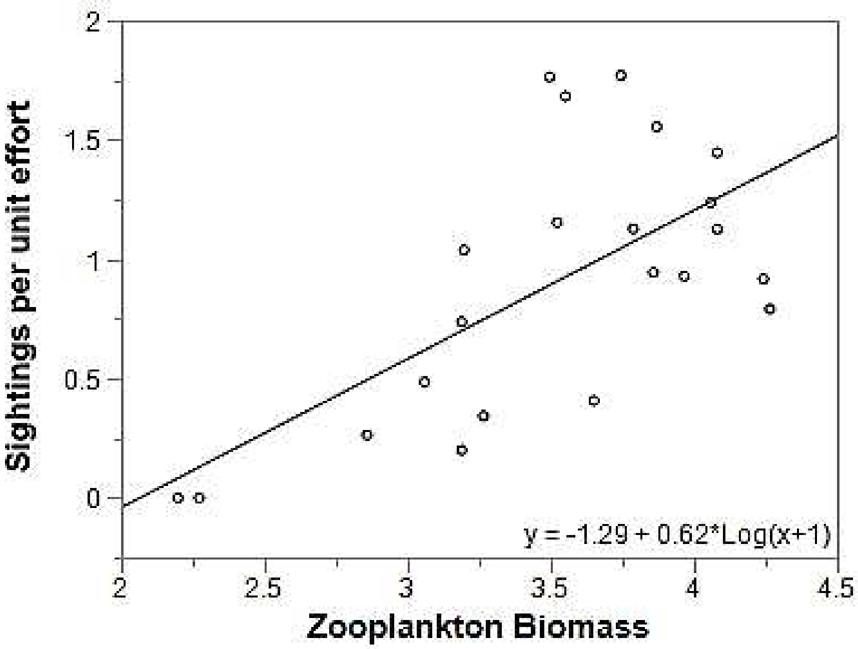

Fig. S.7 The relationship between zooplankton biomass and total sightings per unit effort [log10 (x + 1)] recorded off Djibouti area in November-January ($R^2 = 0.4$, N = 22, P = 0.0012)

Supplementary Material II

Whale shark foraging on baitfish off Djibouti

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Video available at: https://doi.org/10.1007/s12526-018-00934-8

Supplementary Material III

First concurrent assessment of elemental- and organic-contaminant loads in skin biopsies of whale sharks from Djibouti

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Table S.1 Certified and experimental trace metal concentrations in zooplankton samples (mg kg^{-1} dw) (mean ± standard deviation)

Element	**Zooplankton samples (ppm dw)**		
	Certified value	Uncertainty	Experimental value (n = 5)
As	6.82	0.28	7.07 ± 0.35
Cd	0.383	0.014	0.369 ± 0.024
Cr	23.8	1.2	23.1 ± 0.8
Cu	29.5	1.3	28.8 ± 0.8
Hg	0.276	0.018	0.32 ± 0.08
Mn	299	13	281 ± 7.1
Pb	3.97	0.19	3.44 ± 0.03
Se	1.75	0.10	1.86 ± 0.19
Zn	111.6	2.5	89.4 ± 5.4
Ni	18.8	0.8	18.1 ± 0.5
V	8.10	0.18	8.19 ± 0.27

Table S.2 Descriptive statistics of trace elements concentrations (mg kg^{-1} dw) measured in skin tissues of whale sharks from Djibouti in 2016-2018

Trace element	**Whale shark samples** N = 12		**Zooplankton samples** N = 12	
	Mean ± sd	Range	Mean ± sd	Range
Hg	0.18 ± 0.19	0.04 - 0.75	1.14 ± 1.65	0.09 - 4.22
Cd	0.20 ± 0.11	0.05 - 0.38	12.8 ± 4.6	3.53 - 19.0
V	0.54 ± 0.36	0.14 - 1.34	3.48 ± 1.60	0.95 - 6.40
Se	1.15 ± 0.30	0.48 - 1.49	3.58 ± 1.35	1.75 - 6.20
Mo	2.46 ± 1.77	0.17 - 5.62	1.33 ± 0.70	0.64 - 3.15
Co	2.67 ± 2.29	0.09 - 7.17	1.61 ± 0.93	0.61 - 3.28
As	3.18 ± 2.33	1.05 - 9.46	2.56 ± 1.72	0.56 - 7.61
Pb	4.15 ± 3.73	0.61 - 9.74	231 ± 246	9.95 - 848
Cu	26.9 ± 36.6	1.70 - 140	53.0 ± 59.5	16.0 - 236
Mn	44.9 ± 33.7	4.13 - 97.2	29.4 ± 14.0	7.71 - 62.7
Cr	61.4 ± 65.7	0.25 - 251	18.0 ± 28.1	5.31 - 106
Ni	105 ± 60.5	0.47 - 200	25.1 ± 18.6	7.9 - 76.7
Zn	126 ± 142	15.1 - 510	242 ± 155	79.9 - 650
Sr	516 ± 153	197 - 700	5110 ± 4858	721 - 15565
Fe	716 ± 481	30.3 - 1382	1136 ± 1135	375 - 4377

Supplementary Material IV

Trace elements and POPs in two commercial shark species from Djibouti: implications for human exposure

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Table S.1 Biological information on *Rhizoprionodon acutus* and *Sphyrna lewini* specimens collected in Djibouti

Species	Total length (cm)	Sex	Tissue collected
Rhizoprionodon acutus	91	F	Liver
	87	F	Liver
	85	M	Liver Muscle Fin
	69	M	Liver Muscle Fin
	49	F	Liver Muscle
Sphyrna lewini	119	M	Liver
	94	M	Liver
	94	M	Liver
	105-94*	F-F	Fin
	75	F	Liver
	56	F	Liver Muscle Fin
	54	M	Liver Muscle Fin
	50-51-52*	F-F-M	Fin

Notes: specimens with * were pooled together for analyses

Table S.2 Certified and experimental trace metal concentrations in muscles and hepatic tissues (mg/kg dw) (mean ± standard deviation)

Element	**Muscle samples (ppm dw)**			**Liver samples (ppm dw)**		
	Certified value	Uncertainty	Experimental value (n = 7)	Certified value	Uncertainty	Experimental value (n = 4)
As	12.7	0.7	11.2 ± 0.6	36.6	2.4	31.2 ± 0.67
Cd	0.0075	0.0018	0.006 ± 0.001	14.5	0.6	13.8 ± 0.23
Cu	1.67	0.16	1.54 ± 0.06	35	2.4	35.8 ± 0.8
Fe	9.4	1.4	8.4 ± 0.5	1070	80	Not measured
Hg	0.601	0.030	0.604 ± 0.058	0.44	0.18	0.68 ± 0.01
Mn	0.368	0.028	0.33 ± 0.01	8.91	0.7	8.77 ± 0.13
Se	1.33	0.13	1.34 ± 0.14	8.3	1.8	7.0 ± 0.09
Zn	16.0	1.1	15.1 ± 1.8	105.3	5.4	104.8 ± 2.03
Mo				1.41	0.22	1.25 ± 0.02
Sr				3.73	0.26	3.85 ± 0.19
Ni				1.71	0.56	0.75 ± 0.07
V				0.51	0.06	0.52 ± 0.01
Co				0.267	0.026	0.26 ± 0.001
Ag				2.05	0.08	2.20 ± 0.03
Cr				2.35	0.58	1.45 ± 0.12

Table S.3 Statistical results on trace metal analyses performed on *Sphyrna lewini* and *Rhizoprionodon acutus* tissues

Species	Tissue	Trace element	Concentration ppm dw	Statistical results
R. acutus	Liver	Zn	27.35 ± 21.0	F(13, 20.997) = 5.4990, p = 0.0003)
		As	25.3 ± 14.1	
	Muscle	Fe	37.7 ± 9.0	X^2 = 36.2, df = 13, p = 0.0006
		Zn	14.1 ± 8.6	
		As	19.7 ± 5.7	
	Liver vs TL	Cu		R^2 = 0.80, $p < 0.05$
S. lewini	Liver	Zn	18.8 ± 8.9	F(13, 26.2) = 8.629, p = >0.0001
		As	18.7 ± 17	
	Muscle	Fe	16.4 ± 7.8	
		Zn	15.6 ± 3.3	
		As	12.2 ± 1.08	
	Liver vs TL	Zn		R^2 = 0.95, $p < 0.05$
		Se		R^2 = 0.81, $p < 0.05$
		Ag		R^2 = 0.78, $p < 0.05$
R. acutus + *S. lewini*	Muscle vs Liver*	Mn	0.57 ± 0.21 2.6 ± 1.68*	X^2 = 6.8182, df = 1, p = 0.009
		Cu	1.54 ± 0.55 20.2 ± 17.2*	X^2 = 6.8182, df = 1, p = 0.009
		Zn	14.7 ± 6.4 24.3 ± 6.9*	X^2 = 3.9382, df = 1, p = 0.0472
		Se	4.3 ± 0.96 12.4 ± 7.04*	X^2 = 6.8182, df = 1, p = 0.009
		Ag	0.01 ± 0.01 0.33 ± 0.35*	X^2 = 3.9382, df = 1, p = 0.0472
	Liver vs TL by M	Zn		R^2 = 0.89, $p < 0.05$
		Se		R^2 = 0.81, $p < 0.05$
		Hg		R^2 = 0.67, $p < 0.05$
		Cu		R^2 = 0.83, $p < 0.05$
		Mo		R^2 = 0.66, $p < 0.05$

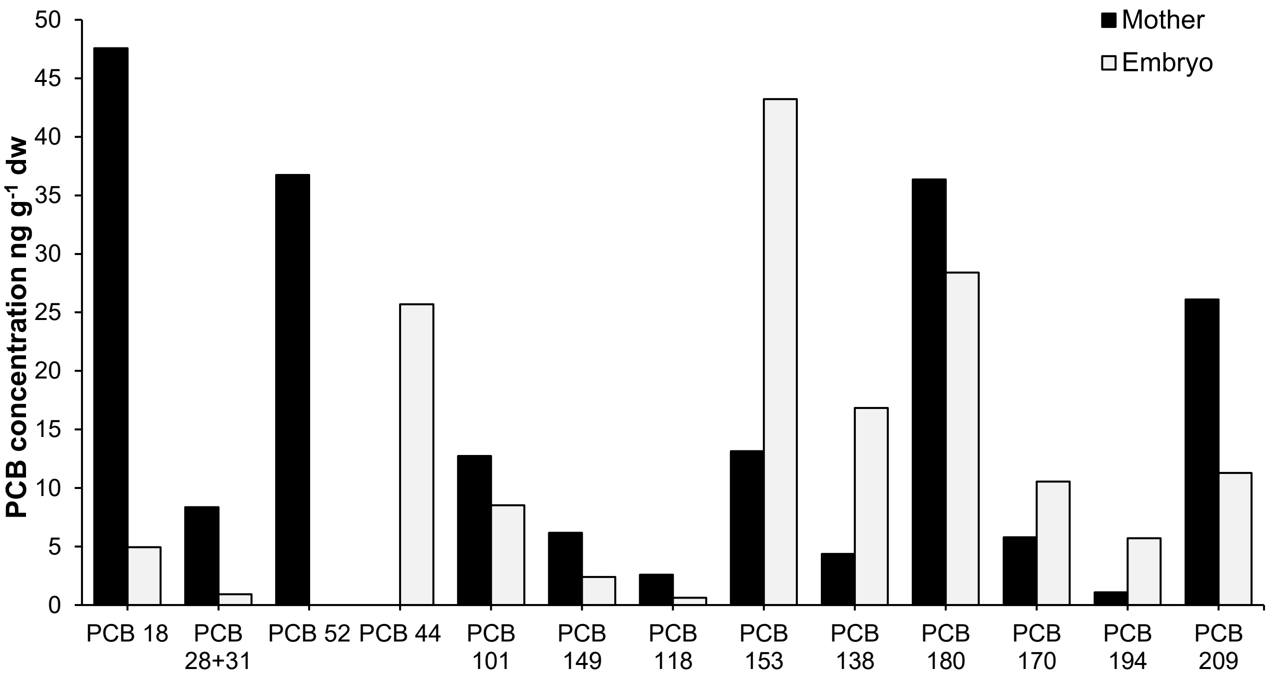

Fig. S.1 Accumulation profiles of PCB in the milk shark pregnant female and its embryo. Black bars represent the hepatic samples collected from the female, while the white correspond to the embryo

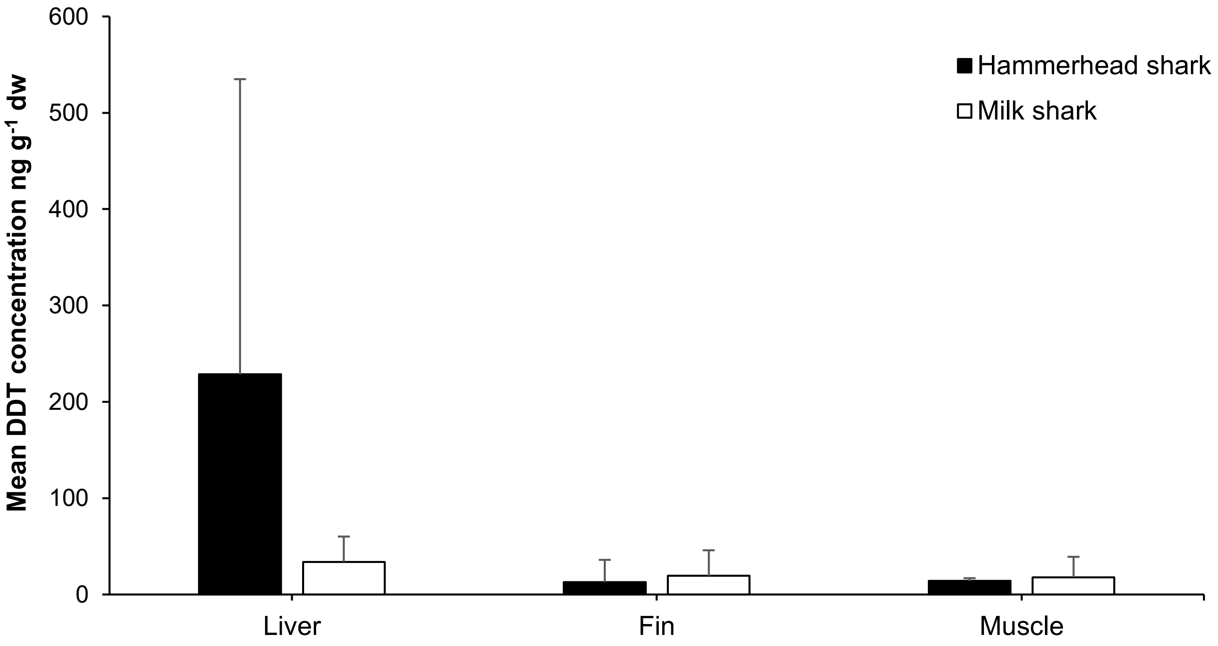

Fig. S.2 Accumulation profiles of DDT in fins (N = 6), muscles (N = 4) and livers (N = 11) of milk and scalloped hammerhead sharks. Error bars represent ±1 standard deviations

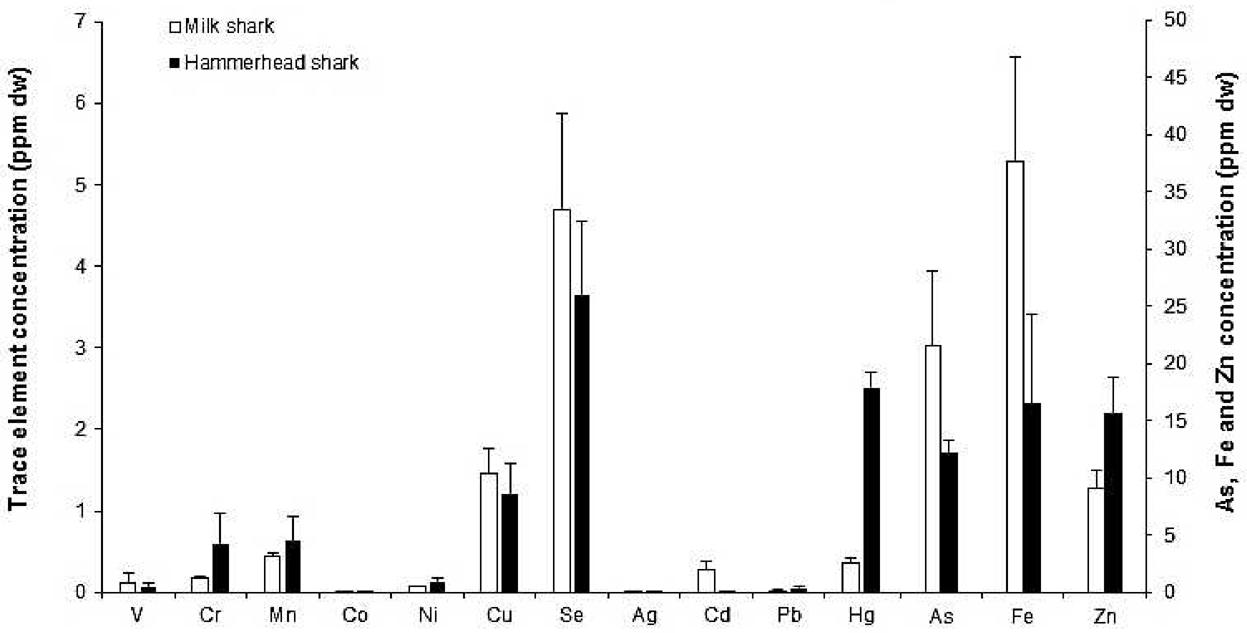

Fig. S.3 Concentration (ppm dw) of trace elements in muscle samples of hammerhead sharks (N = 2) and milk sharks (N = 3). Error bars represent ±1 standard deviation

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