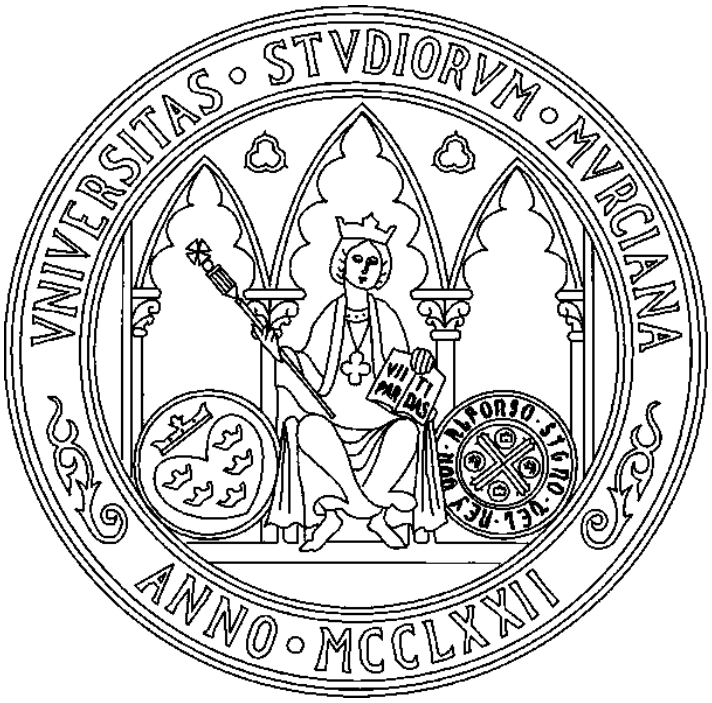


UNIVERSIDAD DE MURCIA

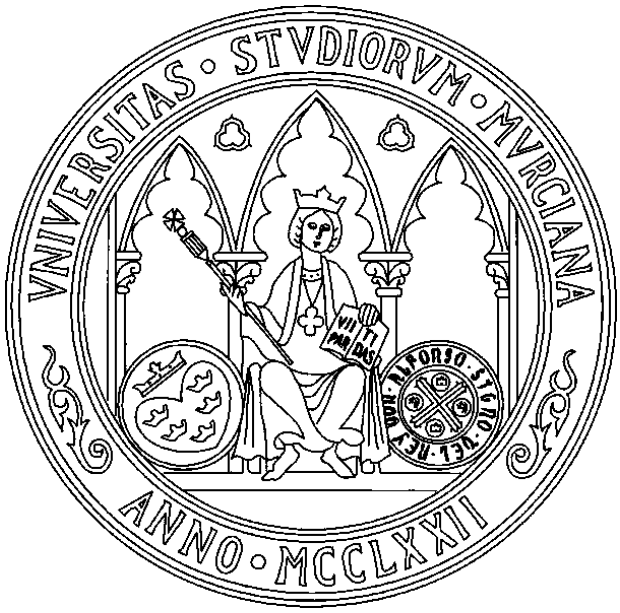
ESCUELA INTERNACIONAL DE DOCTORADO

Inorganic elements in marine turtles (*Lepidochelys olivacea*) from “La Escobilla” beach (Mexico) and biomarkers related to heavy metal pollutants

Elementos inorgánicos en Tortugas marinas (*Lepidochelys olivacea*) de la playa de “La Escobilla” (México) y biomarcadores asociados a contaminación por metales pesados

Dª Adriana Azucena Cortés Gómez

2017



UNIVERSIDAD DE MURCIA
ESCUELA INTERNACIONAL DE DOCTORADO

Inorganic elements in marine turtles (*Lepidochelys olivacea*) from "La Escobilla" beach (Mexico) and biomarkers related to heavy metal pollutants

Elementos inorgánicos en Tortugas marinas (*Lepidochelys olivacea*) de la playa de "La Escobilla" (México) y biomarcadores asociados a contaminación por metales pesados

Memoria presentada para optar al grado de doctor por

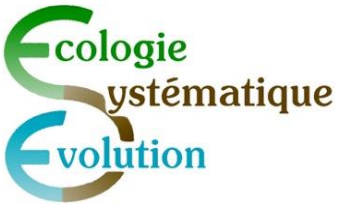
ADRIANA AZUCENA CORTÉS GÓMEZ

DIRECTORES:
Diego Romero García (Universidad de Murcia)
Marc Girondot (Universidad Paris-Sud)
Gisela Fuentes Mascorro (Universidad Autónoma "Benito Juárez" de Oaxaca)

2017

The present work has been possible thanks to the grant received from the *Consejo Nacional de Ciencia y Tecnología* from Mexico (CONACYT)

Also for the support of the University of Murcia, the University Paris-Sud, the Centre National de la Recherche Cientifique (CNRS), the AgroParisTech, University Paris Saclay and the University of Oaxaca



Agradecimientos

Al Consejo Nacional de Ciencia y Tecnología por la beca y oportunidad otorgadas, sin este apoyo nunca hubiera sido posible esta experiencia increíble y que me ha cambiado completamente la vida.

A mis directores, a Diego, por haberme recibido de esa manera tan incondicional y por el apoyo recibido durante estos 5 años. Marc, merci pour m'avoir reçu quand j'en avais le plus besoin, pour être une vraie inspiration et pour m'apprendre à toujours poser des questions et aller toujours plus loin, vraiment, merci ! Dra. Mascorro, por haberme inspirado y animado a iniciar esta aventura, sin esa inspiración quizá nunca lo hubiera hecho. Thanks to you all for your patient, knowledge and support!!

Muchas gracias a los miembros del tribunal que aceptaron participar en mi tesis, los admiro mucho y estoy muy agradecida y contenta de poder contar con ustedes para esta fecha tan importante.

A Julien, por haber estado siempre ahí, darme tu amor y apoyo incondicional, celebrar, reír, por tu paciencia en los momentos difíciles. Sin ti esto nunca hubiera sido posible ♥.

A mis padres, por ser mi base y mi apoyo incondicional desde siempre y hasta siempre. Gracias por ser quienes son y haberme hecho lo que soy.

A mi hermano y toda mi familia ¡es tan grande que mejor no digo nombres para no olvidar a nadie!, por su apoyo, ánimos, platicas, comidas y cariño, siempre.

A mis abuelitas, que se adelantaron en el camino este año, la base de mis familias, mujeres fuertes y guerreras que siempre me inspiraron. Las extrañaré siempre.

A mis queridísimas Tere y Erikoy, sin ustedes mi trabajo de campo nunca hubiera podido ser posible. Y más allá de eso por su amistad, las risas, las comidas, todo, todo ¡las quiero inmensamente!

A todos los que, sin pedir nada a cambio, incluso sin conocerme muchas veces, me ayudaron tanto en mi recolección de muestras, gracias a ustedes también estoy hoy aquí: Alba, Arturo, Ilse, Luz, May, Dioselin, Joel y a todos los que estuvieron un momento o unas horas apoyándome, todo fue invaluable. Gracias infinitas.

A todos mis nuevos amigos que he hecho durante estos 5 años de aventura y que llegaron para quedarse:

Mi introducción increíble a la vida española dada por mi increíble grupo del máster de gestión en fauna silvestre (2012-2013), ¡son los mejores!

Mis queridísimas amigas de veterinaria: Pilar, Mari Paz, Laura… Las quiero mucho y las extraño siempre ☺.

Mis amigos del grupo de Biología Celular ¡nunca trabajar tanto fue tan increíble! Con tan buen ambiente y tanto apoyo, de verdad ¡son fenomenales!: Patri, Yule, Fran, Cristóbal, Héctor… Gracias por recibirme como parte de su grupo. Alberto, por su guía y apoyo inestimable, por permitirme ser parte de su grupo, mil gracias. Teresa, por esa buena energía y esas fiestas en tu casa ☺! A todos los que nos conocimos y compartimos momentos en ese laboratorio (siempre buenos), todos y cada uno de verdad, son geniales.

A la gente del laboratorio de Patología Animal, mi estancia fue corta, pero siempre bien recibida y sintiéndome en casa, mil gracias a José Cerón por la oportunidad de la colaboración. A Asta y Susana por su invaluable ayuda y conocimientos.

Después vino Francia…
A la nueva familia española que formé en Francia: Raquel, Laura, Magda, Irene. Mil gracias por ser tan increíbles amigas y apoyo, por aguantar quejas, llorar y reír conmigo (que les toco aguantar la peor parte jaja) ¡las extrañaré como no imaginan!, llegaron para quedarse.

À la famille Danos, pour la patience, le support, les repas, les sourires, pour être toujours là pour moi, pour rendre ma vie plus facile, pour avoir pris soin de notre Josephine pendant ces 5 années, merci du fond de mon cœur !

À tous dans le labo ESE, pour m'avoir fait sentir comme faisant partie du laboratoire a part entière ! À Jonathan pour ton support et pour être un super collègue et surtout un ami ! Merci ! À Michelle, pour toujours avoir un sourire et un mot gentil pour tout le monde et rendre le quotidien plus agréable. À tous et a toutes pour votre aide et votre patience !

À Nathalie et Maxime, merci de m'avoir reçu chez-vous comme partie de votre famille, vraiment, ma vie en France a été génial, et en très grande partie c'était grâce à vous, je ne pourrai jamais assez vous remercier ! Vous êtes les meilleurs !

INDEX

SUMMARY

Long-lived marine species such as marine turtles are becoming an important tool in ecotoxicology because of their sensitivity to marine environmental change, especially regarding pollution. They usually occupy diverse trophic levels in the marine food web (depending on the age and species), and can therefore accumulate different pollutants over their lifetimes in different ecological niches (Aguirre and Lutz, 2004; Camacho et al., 2013b). Turtle blood is thought to be a good tool for the simultaneous monitoring of environmental contaminants and clinical parameters (Camacho et al., 2013b). However, only the elements of a recent exposition can be found in blood (acute exposure). In order to better elucidate the chronic exposure and accumulation of these elements, it is still necessary to use their accumulation target organs. Inorganic elements, on the other hand, such as Pb, Cd, Cu As, Se and Ni, have been proven to provoke toxicological effects in many aquatic animal species, but marine turtles have been little investigated in this regard. Thus, the aim of this Doctoral Thesis was to evaluate the concentration of inorganic elements in blood and tissues (liver kidney, bone, muscle, brain and fat) from a large number of *Lepidochelys olivacea* turtles (241 nesting marine turtles and tissues from 58 dead turtles) from La Escobilla beach (Oaxaca, Mexico). In this manner, the actual situation of this population in this area could be analyzed, alongside different possible molecular, biochemical and anatomical biomarkers to assess their possible utility regarding turtle health and their relation with these inorganic elements over 3 years (2012-2014).

The first part of the Introduction (section 1.1) describes the principal characteristics of the Olive Ridley turtles and La Escobilla beach. This section touches upon the ecological importance of marine turtles, the morphological characteristics of the studied species, their feeding habits, reproductive behaviour, distribution, habitats and their principal threats.

The second part of the introduction (Chapter I) is a state of the art review and meta-analysis of the most studied inorganic elements worldwide (Pb, Cd, Hg, Al, As, Cr, Cu, Fe, Mn, Ni, Se and Zn) for the 7 marine turtle species. We show that all these elements are above the detection limit, at least in some individuals of all the species and populations

studied. This meta-analysis also showed some features of contamination, and the distribution of these pollutants regarding sea basins, species and tissues.

In the Experimental Chapters, the first part (Biomonitoring) contains 2 sections (Chapters II and III). Here, the concentrations of 14 inorganic elements in the blood of 241 live turtles and different tissues (liver, kidney, muscle, brain, bone, fat and blood) of 58 dead turtles are described. These samples were taken over three different years during 8 different arribadas. The results of this biomonitoring program allow us to better understand the distribution of these elements; this was especially informative for some elements not commonly analyzed (Sr, Ti, Tl). An alarming level of Cd was also found in this population. A decreasing tendency in many of these inorganic elements through those three years was also observed. Since blood is commonly used in biomonitoring programs, the relationship between blood and tissues of dead individuals was also tested to check if there were significant relationships that might indicate that blood could be used to predict the accumulation of these elements in tissues.

The second part of the Experimental Chapters (Biomarkers) runs from chapters IV to VII. These sections (3.1 to 3.5) contain studies evaluating commonly used biomarkers in many species to determine the effects of inorganic elements on the health of the animals, although they have been little studied in marine turtles and none of them have been previously described in *Lepidochelys olivacea*. Additionally, a possible new biomarker using the asymmetry of the carapace of these turtles was developed. Firstly, molecular biomarkers were studied (section 3.3): the presence of metallothionein (MT), superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) transcription and/or enzymatic activities related to some inorganic elements (Al, As, Cd, Cr, Cu, Fe, Li, Ni, Pb, Sb, Se, Sr, Ti, Tl and Zn) was determined.

The most common biochemical analytes related to pollutants were also determined (sections 3.4.1 and 3.4.2) through biochemical analysis in serum samples from two different years. ALT, AST, ALP, albumin, creatinine, glucose, urea, cholesterol, cortisol and esterase activity (EA) were determined in samples from two years (2013 and 2014).

These biochemical parameters were also related to some inorganic elements (As, Cd, Cr, Mn, Ni, Pb, Sr, Ti, Zn and Se).

In the final chapter of this Thesis (VIII), a new tool for measuring the asymmetry of the carapace was developed (DIx) and related to the concentrations of 15 inorganic elements (Bi, Cd, Li, Pb, Sb, Sr, Ti, Tl, Al, As, Co, Cr, Cu, Ni, and Se) from 17 dead turtles (blood, liver, kidney, muscle, fat, bone, brain and egg parts). *Lepidochelys olivacea* is characterized by remarkable morphological variability in the number and shape of scutes, the origin of which is thought to be based on a permissive genetic background. The influence of pollutants on developmental instability and one of its consequences, the asymmetry of individuals, has been demonstrated in several species. However, the use of this asymmetry as a biomarker of contamination in adult individuals has never been explored. Thus, we developed an index to quantify developmental instability (DIx) based on the number and relative size of costal carapace scutes. The link between DIx inorganic element concentrations was then explored in various tissues and egg components of stranded dead Olive Ridley females from the Southern Pacific coast of Mexico (3 arribadas from 2014).

1. INTRODUCTION

1.1 The Olive Ridley (*Lepidochelys olivacea*) and the study area (*La Escobilla* beach)

1.1.1 Introduction

Despite consisting of only seven species, marine turtles are distributed worldwide, inhabit all oceans and occupy various ecological niches, with intra-specific variations in population size, trends, reproduction and morphology (Fowler, 2005; Wallace et al., 2011). Currently, the 7 marine turtle species of the world are listed as: Vulnerable (Olive Ridley, *Lepidochelys olivacea*), Endangered (Loggerhead, *Caretta caretta*; Green Turtle, *Chelonia mydas*), Critically Endangered (Kemp's Ridley, *Lepidochelys kempii*; Hawksbill, *Eretmochelys imbricata*; Leatherback, *Dermochelys coriacea*), and Data Deficient (Flatback, *Natator depressus*) on the Red List (IUCN, 2012). Threats to marine turtles vary across regions, but general categories include fisheries bycatch (i.e. incidental capture by marine fisheries operations targeting other species), take (e.g. utilization of eggs, meat or other turtle products), coastal development, pollution and pathogens, and climate change (Finlayson et al., 2016; Rees et al., 2016; Wallace et al., 2011).

The status of sea turtles and the need for conservation to aid population recovery has captured the interest of government agencies, non-governmental organizations (NGOs) and the general public worldwide. This has facilitated increased research focusing on a wide variety of topics relating to sea turtle biology and conservation. Management actions are often hindered, however, by the lack of basic data on turtles themselves, data such as turtle population status, threats, health parameters, and human interactions (Rees et al., 2016). Characteristics of populations and relative impacts of thee threats to populations are vital components in the assessment of extinction risk (Wallace et al., 2011).

Oceans have long been considered dumping grounds where domestic and industrial wastes could be discarded. It is not surprising, then, to now measure significant concentrations of such contaminants in the water, sediments and inhabitants of the marine environment. Over the past few decades, marine environments have been of increasing concern with regards to environmental pollution. Inorganic elements are one of the most studied groups of pollutants due to their potential threat to the health of most animal species as well as their global distribution. They are usually divided into

essential (Zn, Cu, Cr, Se, Ni, Al, As) and non-essential elements (Hg, Cd, Pb, Tl, Sb, Sr). Many of the elements in the latter group are potentially toxic to many species, even at low concentrations (Vos et al., 2003). Metal contamination sources can be both anthropogenic and natural, and distinguishing between the two is often very difficult. The natural background component of the input of inorganic elements in marine ecosystems may be just as important as the anthropogenic one, and in some areas appears to be the primary source. This is important because it emphasizes that marine animals have often been exposed to these elements long before the development of human activities. This is the case, for example, in the Mediterranean Sea and the Arctic, which are known for their high natural metal levels: mercury (Hg) in the Mediterranean Sea and cadmium (Cd) in the Arctic (Bilandzic et al., 2012; Caurant et al., 1994; Lamborg et al., 2014; Vos et al., 2003).

Since the distribution of these inorganic elements in the marine environment is not homogeneous and a considerable variation of the concentrations may occur regionally and temporally (Gelcich et al., 2014), the use of some species as bioindicators offers a useful alternative in pollution monitoring studies. Marine turtles appear to be valuable indicators of the level of heavy metals accumulated in the marine environment. According to their various positions in the trophic network (from herbivorous to carnivorous), their long-life spans and the long biological half-time of their elimination of pollutants, these animals accumulate high levels of chemicals such as heavy metals (Barbieri, 2009; Frias-Espericueta et al., 2006; García-Fernández et al., 2009; Gardner et al., 2006; Jerez et al., 2010b; van de Merwe et al., 2010a). Furthermore, they are far easier to monitor than other long-life marine species, such as marine mammals, especially nesting females. On the other hand, it is very important to understand more about the impact of these pollutants on the health of the turtles. The high resistance of turtles to xenobiotics compared with mammals and other reptiles has been described previously (Dyc et al., 2016; Jakimska et al., 2011; Venancio et al., 2013), but both the toxic limits and the chronic effects of these elements are unknown. Due to the longevity of turtles, the health risk to these animals from the exposure to various xenobiotics may be very high due to the bioaccumulation of these compounds.

Although many on these metals are properly characterized as toxicants in laboratory rodents or in humans (ATSDR, 2015), the demonstration of their toxic effects in marine fauna represents a significant challenge. This is especially true in turtles because of the relatively limited normal physiological activity database currently in existence. the evaluation of physiological impairments or alterations is almost impossible. Controlled experiments are unavailable to establish any definite causal relationship between these pollutant concentrations and any physiological problem. Moreover, many studies had been undertaken with a limited number of individuals, leaving doubts about the general applicability of collected values at which an effect at individual or population level might be expected (Finlayson et al., 2016). Most of these published works have been conducted only during a short sampling period. Their results are interesting in an ecotoxicology context (as bioindicators), but it is hard to interpret whether the results found were a reflection of the common metal amounts in the population (risk assessment) or a reflection of a one-time exposure (especially when a high concentration of some element was present).

Few studies exist regarding the effects of biomarkers of inorganic elements that bioaccumulate in marine turtles. For instance, Aguirre et al. (1994b) and da Silva et al. (2016a) measured the relationship of some metals with fibropapillomatosis (FP) in Green Turtles. In their work, da Silva et al. (2016a) mention the possible effect of oxidative stress as an immune-suppressor caused by metals which may favor FP etiology. In this regard, there is only one work attempting to relate antioxidant defenses to metal (and other pollutant) concentrations in Green turtles. In this work, Labrada-Martagon et al. (2011a) detected significant correlations between organic and inorganic contaminants and several oxidative stress enzymes, including GST, catalase (CAT), super-oxide dismutase (SOD), as well as an indicator of lipid peroxidation measured using thiobarbituric acid reactive substances (TBARS). Finally, Andreani et al. (2008b) measured metallothioneins (MT), a biomarker of exposure to metals. MT is a defense mechanism involved in detoxifying metals by binding them, thus making metals biologically unavailable. Andreani et al. (2008b) found significant positive correlations

between MT and Cu and Cd concentrations in the liver and kidney of Loggerhead and Green turtles, suggesting their use as biomarkers of exposure in more than one species.

In this regard, the establishment of long term biomonitoring programs of inorganic elements and other xenobiotics, and their biological effects in key populations should be included in marine turtle conservation and ecotoxicological programs. It will also be valuable to marine rescue centers. No less importantly, all this information may also be used as a part of more general ocean health assessments of ocean pollutants dispersion using marine turtles as a flag species.

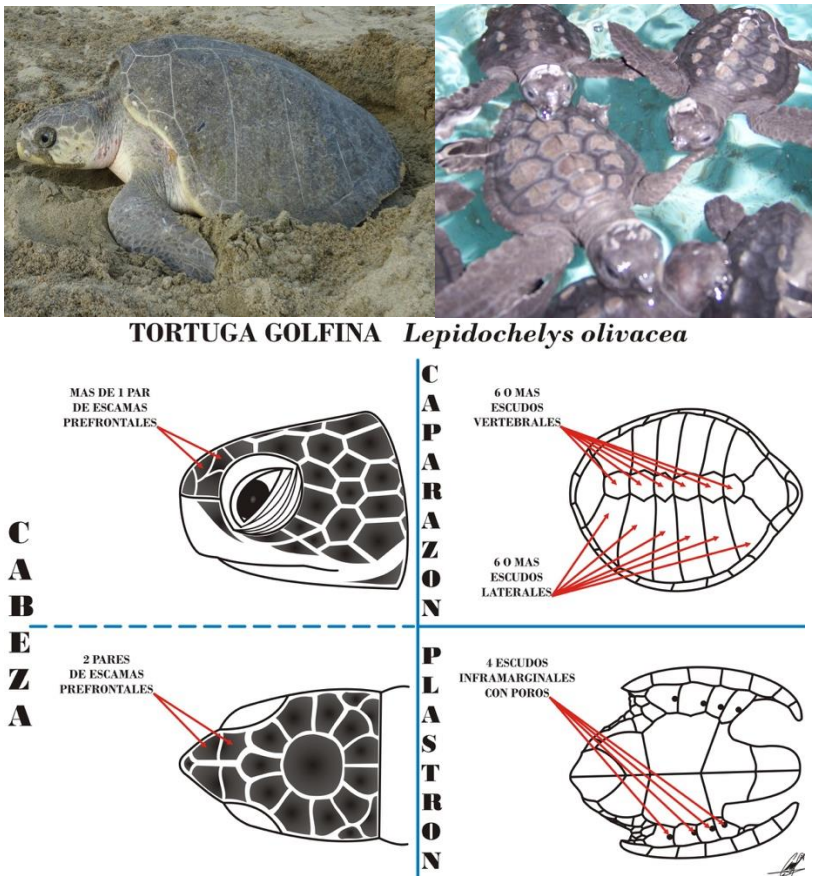
1.1.2 The Olive Ridley Turtle *(Lepidochelys olivacea*)

Olive Ridley (*Lepidochelys olivacea*) is the smallest of the seven existing species of sea turtles worldwide. The curved carapace length (CCL) of adults ranges from 62 to 78 cm; the weight from 35 to 50 kg, and the maximum age recorded is 45 years. Hatchlings vary are dark gray to completely black and are born with an average length of 5 cm. It is also the most recovered and most abundant of all marine turtle species worldwide (Pritchard and Mortimer, 2000; Wyneken, 2001). Their identification characteristics are presented in Figure 1.

1.1.2.1 Feeding habits

During their first week of life, sea turtles feed exclusively on their reserve located in the yolk sac, which provides them with the energy needed to swim to their feeding areas without stopping and there begin to feed themselves independently (Musick and Limpus, 1997; Musick and Lutz, 1997). The turtle, while demonstrating a very varied diet depending on its stage of growth and location, is an omnivorous species. In adulthood, its diet consists of pelagic organisms in oceanic waters (lobsters, fish eggs, etc.) or algae, crustaceans, mollusks, fish and salpids in coastal waters (Marquez et al., 1996; Pritchard and Plotkin, 1995).

Figure 1. Morphological characteristics of the Olive Ridley.



1.1.2.2 Reproduction

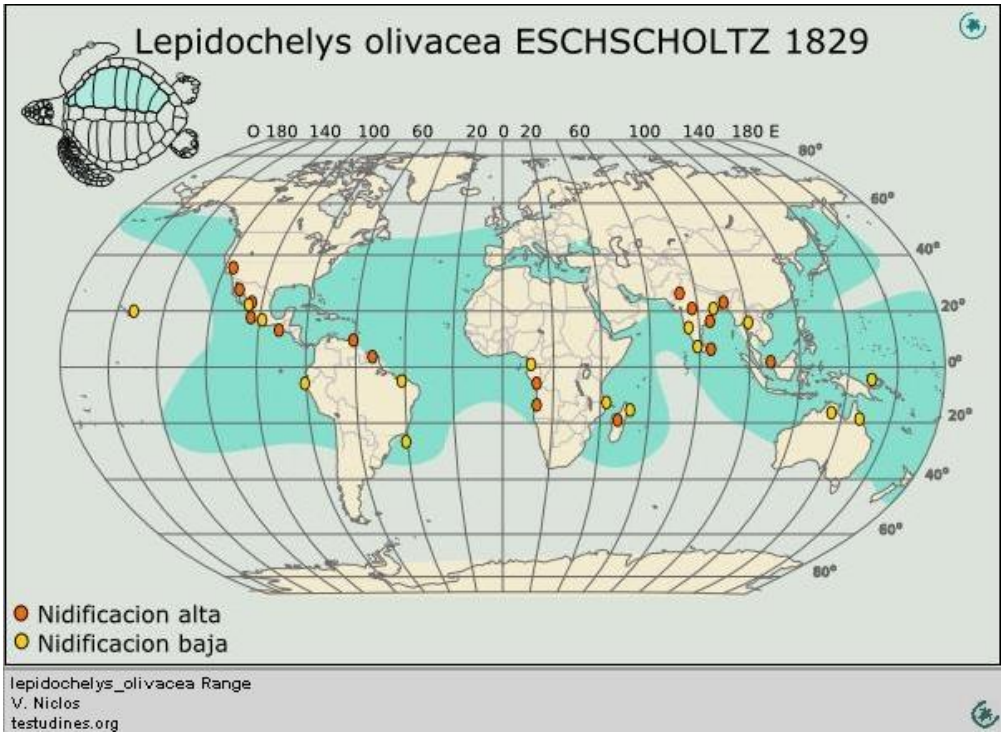
Marine turtles of the genus *Lepidochelys* reach sexual maturity between the ages of 7 and 9 years (Marquez et al., 1996). Olive Ridley turtles are one of two sea turtle species that have massive synchronous nesting emergences called *arribada.* Depending on the number of turtles, arribadas can last between 3 to 15 nights and take place between sunset and sunrise (when the sun is less strong and throughout the night) (Marquez et al., 1996). In general, the reproductive behavior of sea turtles is cyclic, of approximately 14 days for solitary nesting and 28 days for arribadas. Scientists have not yet found a determined signal for the start of this event, but the cues responsible for this reproductive synchrony seem to include wind, tide, cycle, lunar phase (generally during the waning quarters), rainfall patterns, temperature, and photoperiod as well as socially facilitated patterns (Beyer, 2002; Clusella Trullas and Paladino, 2007; Honarvar, 2007; Marquez et al., 1996; Pritchard and Plotkin, 1995). Usually, females lay two clutches of eggs per season, remaining near shore during the inter-nesting period (Abreu-Grobois

and Plotkin, 2008; Pritchard and Plotkin, 1995). It has been suggested that Olive Ridleys are capable of retaining oviductal eggs longer than other sea turtle species and they will retain the eggs until they receive the appropriate cues to nest (Honarvar, 2007; Márquez and Van Dissel, 1982; Plotkin et al., 1995). The number of eggs *per* nest can vary depending on the age, health status, number of seasons in the season, *inter alia*. Thus, the number of eggs can range from 30 to more than 150, with an average of 111. The incubation nest is made in the sand and has an average depth and diameter of 21 and 40 cm. The incubation period lasts from 41 to 51 days, depending on the temperature and humidity of the nest (Márquez, 1990; Vega and Robles, 2005). The arribada nesting strategy results in a large number of nesting turtles on the beach at a given time and consequently a large number of hatchlings (Márquez and Van Dissel, 1982).

1.1.2.3 Distribution and habitat

The Olive Ridley turtle is a pantropical species, with no morphological differences between its populations. The feeding areas of this species are in several coastal and pelagic environments within the turtles' migration patterns (Figure 2):

Figure 2. Olive Ridley world distribution and nesting areas. Taken from: testudines.org.



• In the Atlantic they are distributed along the West coast of Africa, occasionally in the Caribbean Sea, Puerto Rico, coasts of Brazil, French Guiana, Guyana, Suriname and Venezuela in South America.
• In the eastern Pacific Ocean they are observed from the northwest of the Peninsula of Baja California and the Gulf of California to Chile, with areas of concentration in Southwestern Baja California, South of Sinaloa, Michoacán, Guerrero and Oaxaca in Mexico; also in Guatemala, El Salvador, Nicaragua and Costa Rica. Between Panama and Colombia, most of the colonies converge in a very important feeding area.
• In the Indian Ocean, Micronesia, Japan, India, southern Arabia, northern Australia and southern Africa.

1.1.2.4 Ecological Importance

These turtles play a very important role in trophic chains, especially considering how numerous their populations have been historically. One of the roles of this species and its massive nesting is the transport of nutrients from the ocean to the beaches (through eggs). These are usually sandy habitats deficient in nutrients, and this nutrient transfer allows for the development of a greater quantity and diversity of flora and fauna (Silman et al., 2002). Ridleys also play an important role in ocean carbon transport, since their feeding system allows the transfer of large amounts of carbon to abyssal zones through the consumption of diverse organisms in shallow or pelagic surface waters (Buitrago, 2009).

Another of the important functions of this species is the regulation of the species that constitute its prey, for example the control of populations of certain species of jellyfish (Buitrago, 2009). Turtles themselves (especially as eggs and hatchlings) also play a vital role in the trophic chain as prey to many species, such as small mammals, birds, crabs and a large variety of fish (CONANP, 2011b). In summary, the role of the Olive Ridley is essential for the health and balance of important ecosystems, both marine and coastal.

1.1.2.5 Threats and biomarkers

As noted above, marine turtle species are currently on the International Union for Conservation of Nature's Red List of Threatened Species (IUCN, 2012). They are also all included in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). They are listed as species of mandatory conservation by the *Convention on Migratory Species* and in the *Inter-American Convention for the Protection and Conservation of Sea Turtles*.

The threats to the different species of sea turtles vary according to region, but generally speaking the most important are the following: (1) incidental capture of young specimens and adults during fishing seasons, (2) looting (for the use of eggs and meat as food or other products), (3) coastal development and anthropization of the coast which makes nesting sites undesirable for turtles, (4) exposure to pathogens, (5) climate change, and (6) pollutants (Mast et al., 2005; Rees et al., 2016; Wallace et al., 2011).

With regards to pollutants, marine turtles are able to accumulate diverse contaminants over many years due to their longevity and extensive migration areas and consequently they represent interesting biomarker species for marine ecosystem pollution (Andreani et al., 2008b; Godley et al., 1999a; Sakai et al., 2000). There are many different kinds of pollutants, with inorganic elements (metals, metalloids, trace elements) among the most studied due to their toxicity (see Chapter I). Bioaccumulation of these elements varies depending on several factors including geographic location, exposure to diverse pollutants, diet, species and tissues analyzed (Alava et al., 2006; Guirlet et al., 2008).

Oxidative stress is an unavoidable aspect of aerobic life. It is the result of an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defenses in the organisms. Therefore, this oxidative stress is produced by the cellular accumulation of ROS, molecules with strong oxidative properties (Valdivia et al., 2007), which can occur due to many factors. Among them, exposure to a variety of environmental stressors such as pollutants produces increased production of ROS and reduced antioxidant potential leading to oxidative stress (Labrada-Martagon et al.,

2011a). It has been demonstrated, in *in vivo* and *in vitro* works, that ROS have the potential to damage proteins, lipids and nucleic acids, and cause tissue damage, metabolic dysfunction and apoptosis cell death (Labrada-Martagon et al., 2011a; Morcillo et al., 2016; Valdivia et al., 2007). Additionally, stress oxidative biomarkers are very useful to all kind of studies in pollutant monitoring and their effects on marine organisms and ecosystems.

Although environmental pollutants in sea turtles have been assessed by several authors across the world (see Chapter I), very few studies have tried to document biomarkers or health effects derived from these exposures from a biochemical point of view (Camacho et al., 2013b; Keller and McClellan-Green, 2004; Keller et al., 2004; Keller et al., 2006a; Labrada-Martagon et al., 2011a), and only two of them have been performed on *L. olivacea* (Santillana, 2013; Santoro and Meneses, 2007). For sea turtle conservation purposes, it is essential to determine how these reptiles respond to different types of anthropogenic contaminants (Camacho et al., 2013b). In order to do this, a database on normal clinical parameters of health for species and for population is needed, in addition to regular pollutant biomonitoring in the feeding areas.

Serum biochemistry is another commonly used diagnostic tool for assessing health in domestic and wild animals. These parameters can vary depending on species, gender, age, diet, pathological process and analysis techniques (Anderson et al., 2011; Casal et al., 2009; Fazio et al., 2011). Due to all these variations it is not easy to establish normal parameters in marine turtles and the construction of a database by species and by populations (nesting/feeding areas) is recommended. This database would then provide indications of health, disease, and nutrition and even evaluate habitat quality (Camacho et al., 2013b). All this information would help to make a better assessment of the health of different populations and also provide an important tool to allow for better diagnosis and veterinary care in rescue animals.

Finally, the influence of pollutants on developmental instability and one of its consequences, the asymmetry of individuals, has been demonstrated in several species

(Beasley et al., 2013). The Olive Ridley is characterized by remarkable morphological variability in the number and shape of scutes, the origin of which is thought to be based on a permissive genetic background. However, the use of this asymmetry as a biomarker of contamination in adult individuals has never been explored.

1.1.3 La Escobilla beach

Mexico is perhaps the most important country in the world in terms of the production of Ridley hatchlings, only comparable to the number produced on the beaches of Gahimartha in India. Nancite and Ostional in Costa Rica are other beaches where a similar number of eggs are nested during the arribadas, but due to the topographic conditions, especially in Nancite, from the same number of nesting eggs fewer than half of the hatchlings are produced compared to Mexican beaches (Marquez et al., 1996).

In Mexico, all marine turtles are considered Endangered Species and are included within the NOM-ECOL-059-2010, as well as being considered *Priority Species for Conservation*. Mexico also has 3 important arribada beaches (Mexiquillo in Michoacán and Morro Ayuta and Escobilla in Oaxaca). The largest and most important is *La Escobilla*, which is located in the state of Oaxaca, Southwest Mexican Pacific (Figure 3). This beach has "Natural Sanctuary" status, held in the law as "areas where the habitat of a species is protected" (CONANP, 2011c). It has a length of 25 km, covering 615 km^2 (Peñaflores et al., 1998). The beach is mostly of fine sand with pieces of shells, pebbles and minerals. However, near to the outfall of the Cozoaltepec river the sand is thicker, containing shells and stones carried out by the current (Ocana, 2010). The tidal regime is semi-diurnal with two high tides and two low tides during each period, and it is a strong wave beach (Ávila and Meraz, 2008). After the complete ban by the Mexican Government in 1994 in the use, extraction, or manufacture of products derived from any turtle species, the population from La Escobilla has been regularly increasing. Thus, over the last decade, more than one million turtles have come out to nest on this beach every year (CONANP, 2011b).

Arribadas on this beach last from June to January, although nesting in solitary and in smaller numbers can occur all year long (Ocana, 2010). *La Escobilla* also presents a problem that has not been reported on any other beach at this level: a beetle (*Omorgus suberosus*) that depredates the eggs of the turtles that nest here, and can predate up to 70% of nests in some seasons (López-Reyes and Olivera, 1996; Muñoz, 2010).

Figure 3. Nesting beaches distribution in Mexico. Arribada beaches are denoted in red, La Escobilla is N° 1. Map taken from conanp.gob.mx

References

Abreu-Grobois, A., Plotkin, P.T., 2008. *Lepidochelys olivacea*, The IUCN Red List of Threatened Species.

Aguirre, A.A., Balazs, G.H., Zimmerman, B., Galey, F.D., 1994. Organic contaminants and trace metals in the tissues of green turtles (*Chelonia mydas*) afflicted with fibropapillomas in the Hawaiian Islands. Marine Pollution Bulletin 28, 109-114.

Alava, J.J., Keller, J.M., Kucklick, J.R., Wyneken, J., Crowder, L., Scott, G.I., 2006. Loggerhead sea turtle (*Caretta caretta*) egg yolk concentrations of persistent organic pollutants and lipid increase during the last stage of embryonic development. Science of the Total Environment 367, 170-181.

Anderson, E.T., Minter, L.J., Clarke, E.O., 3rd, Mroch, R.M., 3rd, Beasley, J.F., Harms, C.A., 2011. The Effects of Feeding on Hematological and Plasma Biochemical Profiles in Green (Chelonia mydas) and Kemp's Ridley (Lepidochelys kempii) Sea Turtles. Vet Med Int 2011, 890829.
Andreani, G., Santoro, M., Cottignoli, S., Fabbri, M., Carpene, E., Isani, G., 2008. Metal distribution and metallothionein in Loggerhead (*Caretta caretta*) and Green (*Chelonia mydas)* sea turtles. Science of the Total Environment 390, 287-294.
ATSDR, 2015. Support document to the 2015 priority list of hazardous substances, in: Sciences, A.f.T.S.a.D.R.D.o.T.a.H.H. (Ed.). ATSDR, Atlanta, GA.
Ávila, J., Meraz, J., 2008. Metodología de una marcación de nidos *in situ* de *Lepidochelys olivacea* en La Escobilla, Oaxaca, México. Ciencia y Mar XII, 25-28.
Barbieri, E., 2009. Concentration of heavy metals in tissues of Green turtles (*Chelonia mydas*) sampled in the Cananéia Estuary, Brazil. Brazilian Journal of Oceanography 57, 243-248.
Beasley, D.A.E., Bonisoli-Alquati, A., Mousseau, T.A., 2013. The use of fluctuating asymmetry as a measure of environmentally induced developmental instability: A meta-analysis. Ecological Indicators 30, 218-226.
Beyer, K., 2002. Investigations into the reproductive biology and ecology of Olive Ridley Turtles (*Lepidochelys olivacea*) Eschscholtz, 1829 at Old Ningo Beach, Ghana, West Africa. University of Bremen, Bremen, Germany, p. 119.
Bilandzic, N., Sedak, M., Ethokic, M., Ethuras Gomercic, M., Gomercic, T., Zadravec, M., Benic, M., Prevendar Crnic, A., 2012. Toxic element concentrations in the bottlenose (*Tursiops truncatus*), striped (*Stenella coeruleoalba*) and Risso's (*Grampus griseus*) dolphins stranded in eastern Adriatic Sea. Bulletin of Environmental Contamination and Toxicology 89, 467-473.
Buitrago, J.n., 2009. El Rol de las Tortugas Marinas en los Ecosistemas. Fundacion La Salle de Ciencias Naturales, Venezuela.
Camacho, M., Oros, J., Boada, L.D., Zaccaroni, A., Silvi, M., Formigaro, C., Lopez, P., Zumbado, M., Luzardo, O.P., 2013. Potential adverse effects of inorganic pollutants on clinical parameters of Loggerhead sea turtles (*Caretta caretta*): Results from a nesting colony from Cape Verde, West Africa. Marine Environmental Research 92, 15-22.
Casal, A.B., Camacho, M., Lopez-Jurado, L.F., Juste, C., Oros, J., 2009. Comparative study of hematologic and plasma biochemical variables in Eastern Atlantic juvenile and adult nesting loggerhead sea turtles (Caretta caretta). Vet Clin Pathol 38, 213-218.
Caurant, F., Amiard, J.C., Amiard-Triquet, C., Sauriau, P.G., 1994. Ecological and biological factors controlling the concentrations of trace-elements (as, Cd, Cn, Hg, Se, Zn) in delphinids *Globicephala melas* from the North-Atlantic Ocean. Marine Ecology-Progress Series 103, 207-219.
Clusella Trullas, S., Paladino, F.V., 2007. Micro-environment of olive ridley turtle nests deposited during an aggregated nesting event. Journal of Zoology 272, 367-376.
CONANP, 2011a. Ficha de la tortuga golfina. Dirección para especies prioritarias para la conservación, in: Marinas, P.N.p.l.C.d.l.T. (Ed.). Comisión Nacional Areas Naturales Protegidas, Mexico.

CONANP, C.N.d.A.N.P., 2011b. Ficha de la tortuga golfina, Programa Nacional para la Conservacion de las Tortugas Marinas. Direccion para especies prioritarias para la conservacion, Mexico, D.F., p. 7.
da Silva, C.C., Klein, R.D., Barcarolli, I.F., Bianchini, A., 2016. Metal contamination as a possible etiology of fibropapillomatosis in juvenile female green sea turtles *Chelonia mydas* from the southern Atlantic Ocean. Aquatic Toxicology 170, 42-51.
Dyc, C., Far, J., Gandar, F., Poulipoulis, A., Greco, A., Eppe, G., Das, K., 2016. Toxicokinetics of selenium in the slider turtle, Trachemys scripta. Ecotoxicology 25, 727-744.
Fazio, E., Liotta, A., Medica, P., Bruschetta, G., Ferlazzo, A., 2011. Serum and plasma biochemical values of health loggerhead sea turtles (Caretta caretta). Comparative Clinical Pathology 21, 905-909.
Finlayson, K.A., Leusch, F.D., van de Merwe, J.P., 2016. The current state and future directions of marine turtle toxicology research. Environment International 94, 113-123.
Fowler, S.L., 2005. Sharks, rays and chimaeras: the status of the Chondrichthyan fishes: status survey. IUCN.
Frias-Espericueta, M.G., Osuna-Lopez, J.I., Ruiz-Telles, A., Quintero-Alvarez, J.M., Lopez-Lopez, G., Izaguirre-Fierro, G., Voltolina, D., 2006. Heavy metals in the tissues of the sea turtle *Lepidochelys olivacea* from a nesting site of the northwest coast of Mexico. Bulletin of Environmental Contamination and Toxicology 77, 179-185.
García-Fernández, A.J., Gómez-Ramírez, P., Martínez-López, E., Hernández-García, A., María-Mojica, P., Romero, D., Jiménez, P., Castillo, J.J., Bellido, J.J., 2009. Heavy metals in tissues from loggerhead turtles (*Caretta caretta*) from the southwestern Mediterranean (Spain). Ecotoxicology and Environmental Safety 72, 557-563.
Gardner, S.C., Fitzgerald, S.L., Vargas, B.A., Rodriguez, L.M., 2006. Heavy metal accumulation in four species of sea turtles from the Baja California peninsula, Mexico. Biometals 19, 91-99.
Gelcich, S., Buckley, P., Pinnegar, J.K., Chilvers, J., Lorenzoni, I., Terry, G., Guerrero, M., Castilla, J.C., Valdebenito, A., Duarte, C.M., 2014. Public awareness, concerns, and priorities about anthropogenic impacts on marine environments. Proc Natl Acad Sci U S A 111, 15042-15047.
Godley, B.J., Broderick, A.C., Moraghan, S., 1999. Short-term effectiveness of passive integrated transponder (PIT) tags used in the study of mediterranean marine turtles. Chelon Conserv Biol 3, 477-479.
Guirlet, E., Das, K., Girondot, M., 2008. Maternal transfer of trace elements in leatherback turtles (*Dermochelys coriacea)* of French Guiana. Aquatic Toxicology 88, 267-276.
Honarvar, S., 2007. Nesting ecology of olive ridley (*Lepidochelys olivacea*) turtles on arribada nesting beaches. Drexel University, Philadelphia, Pennsylvania, p. 101.
IUCN, 2012. Red List of Threatened Species, Version 2012.1. International Union for Conservation of Nature, http://www.iucnredlist.org/.
Jakimska, A., Konieczka, P., Skóra, K., Namieśnik, J., 2011. Bioaccumulation of Metals in Tissues of Marine Animals, Part II: Metal Concentrations in Animal Tissues. Polish Journal of Environmental Studies 20.
Jerez, S., Motas, M., Canovas, R.A., Talavera, J., Almela, R.M., Bayón del Rio, A., 2010. Accumulation and tissue distribution of heavy metals and essential elements in

loggerhead turtles (*Caretta caretta*) from Spanish Mediterranean coastline of Murcia. Chemosphere 78, 256-264.
Keller, J., McClellan-Green, P., 2004. Effects of organochlorine compounds on cytochrome P450 aromatase activity in an immortal sea turtle cell line. Marine Environmental Research 58, 347-351.
Keller, J.M., Kucklick, J.R., Stamper, M.A., Harms, C.A., McClellan-Green, P.D., 2004. Associations between organochlorine contaminant concentrations and clinical health parameters in loggerhead sea turtles from North Carolina, USA. Environ Health Perspect. 112, 1074-1079.
Keller, J.M., McClellan-Green, P.D., Kucklick, J.R., Keil, D.E., Peden-Adams, M.M., 2006. Effects of organochlorine contaminants on loggerhead sea turtle immunity: Comparison of a correlative field study and in vitro exposure experiments. Environmental Health Perspectives 114, 70-76.
Labrada-Martagon, V., Rodriguez, P.A., Mendez-Rodriguez, L.C., Zenteno-Savin, T., 2011. Oxidative stress indicators and chemical contaminants in East Pacific green turtles (*Chelonia mydas*) inhabiting two foraging coastal lagoons in the Baja California peninsula. Comparative Biochemistry and Physiology Part C 154, 65-75.
Lamborg, C., Bowman, K., Hammerschmidt, C., Gilmour, C., Munson, K., Selin, N., Tseng, C.-M., 2014. Mercury in the Anthropocene Ocean. Oceanography 27, 76-87.
López-Reyes, E., Olivera, A., 1996. Control del escarabajo que destruye los huevos de la tortuga marina. Memorias 1er. Encuentro regional sobre investigación y desarrollo costero: Guerrero, Oaxaca y Chiapas. UMAR, POECO, CONACYT, SEMARNAP, CODE, 14-16.
Márquez, M.R., 1990. Sea Turt[es of the World. An annotated and ilustrated catalogue of sea turtles species known to date, FAO Species Catalogue. Food and Agriculture Organization of the United Nations, Rome, Italia, p. 81.
Marquez, M.R., Peñaflores, C., Vasconcelos, J.C., 1996. Olive ridley turtles (*Lepidochelys olivacea*) show signs of recovery at La Escobilla, Oaxaca. Marine Turtle Newsletter 73, 5-7.
Márquez, M.R., Van Dissel, H.G., 1982. A method for evaluating the number of massed nesting olive ridley sea turtles, *Lepidochelys olivacea*, during an arribazon, with comments on arribazon behaviour. Netherlands Journal of Zoology 32, 419-425.
Mast, R.B., Hutchinson, B.J., Howgate, E., Pilcher, N.J., 2005. MTSG update: IUCN/SSC marine turtle specialist group hosts the second burning issues assessment workshop. Marine Turtle Newsletter 110, 13-15.
Morcillo, P., Esteban, M.A., Cuesta, A., 2016. Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. Chemosphere 144, 225-233.
Muñoz, A., 2010. Control del escarabajo *Omorgus suberosus* en el santuario de La Ecobilla. Selva Negra, A.C., Oaxaca, México.
Musick, J.A., Limpus, C.J., 1997. Habitat utilization and migration in juvenile sea turtles, in: Musick, J.A., Lutz, P.L. (Eds.), The Biology of Sea Turltes. CRC Press, Boca Raton, Florida, pp. 137-163.
Musick, J.A., Lutz, P.L., 1997. The Biology of Sea Turtles. CRC Press, Boca Raton.
Ocana, M.A.S., 2010. Arribada nesting of olive ridley sea turtles (Lepidochelys olivacea) at La Escobilla, Mexico. Oregon State University, p. 89.

Peñaflores, C., Vasconcelos, J., Albavera, E., Márquez, R., 1998. Twenty five years nesting of olive ridley sea turtle Lepidochelys olivacea in Escobilla Beach, Oaxaca, Mexico, Eighteenth International Sea Turtle Symposium, p. 27.
Plotkin, P.T., Byles, R.A., Rostal, D.C., Owens, D.W., 1995. Independent versus socially facilitated oceanic migrations of the olive ridley, *Lepidochelys olivacea*. Marine Biology 122, 137-143.
Pritchard, P., Mortimer, J., 2000. Taxonomía, morfología externa e identificación de las especies. En: Eckert, KL, 23-41.
Pritchard, P.C.H., Plotkin, P.T., 1995. Olive ridley sea turtle, *Lepidochelys olivacea*, in: Plotkin, P.T. (Ed.), National Marine Fisheries Service and U.S. Fish and Wildlife Service Status Reviews for Sea Turtles Listed under the Endangered Species Act of 1973. National Marine Fisheries Service, Silver Spring, Maryland, pp. 123-139.
Rees, A.F., Alfaro-Shigueto, J., Barata, P.C.R., Bjorndal, K.A., Bolten, A.B., Bourjea, J., Broderick, A.C., Campbell, L.M., Cardona, L., Carreras, C., Casale, P., Ceriani, S.A., Dutton, P.H., Eguchi, T., Formia, A., Fuentes, M., Fuller, W.J., Girondot, M., Godfrey, M.H., Hamann, M., Hart, K.M., Hays, G.C., Hochscheid, S., Kaska, Y., Jensen, M.P., Mangel, J.C., Mortimer, J.A., Naro-Maciel, E., Ng, C.K.Y., Nichols, W.J., Phillott, A.D., Reina, R.D., Revuelta, O., Schofield, G., Seminoff, J.A., Shanker, K., Tomás, J., van de Merwe, J.P., Van Houtan, K.S., Vander Zanden, H.B., Wallace, B.P., Wedemeyer-Strombel, K.R., Work, T.M., Godley, B.J., 2016. Are we working towards global research priorities for management and conservation of sea turtles? Endangered Species Research 31, 337-382.
Sakai, H., Saeki, K., Ichihashi, H., Suganuma, H., Tanabe, S., Tatsukawa, R., 2000. Species-specific distribution of heavy metals in tissues and organs of Loggerhead turtle (*Caretta caretta*) and Green turtle (*Chelonia mydas*) from Japanese Coastal Waters. Marine Pollution Bulletin 40, 701-709.
Santillana, P.R., 2013. Valores Hematológicos y bioquímicos sanguíneos de tortugas anidantes de Golfina (*Lepidochelys olivacea*) en El Salvador. BIOMA, 11-17.
Santoro, M., Meneses, A., 2007. Haematology and plasma chemistry of breeding olive ridley sea turtles (*Lepidochelys olivacea*). The Veterinary Record 161, 818-819.
Silman, R., Vargas, I., Troëng, S., 2002. Tortugas Marinas. Guía Educativa, in: Corporation, C.C. (Ed.), 2a ed.
Valdivia, P.A., Zenteno-Savin, T., Gardner, S.C., Aguirre, A.A., 2007. Basic oxidative stress metabolites in eastern Pacific green turtles (*Chelonia mydas agassizii*). Comparative Biochemistry and Physiology, Part C 146, 111-117.
van de Merwe, J.P., Hodge, M., Olszowy, H.A., Whittier, J.M., Lee, S.Y., 2010. Using blood samples to estimate persistent organic pollutants and metals in green sea turtles (*Chelonia mydas*). Marine Pollution Bulletin 60, 579-588.
Vega, A.J., Robles, Y., 2005. Descripción del proceso de anidación y biometría de hembras, huevos y nidos en tortuga golfina Lepidochelys olivacea (Eschscholtz, 1829) en Isla de Cañas, Pacífico panameño. Tecnociencia 7, 43-55.
Venancio, L.P.R., Silva, M.I.A., da Silva, T.L., Moschetta, V.A.G., de Campos Zuccari, D.A.P., Almeida, E.A., Bonini-Domingos, C.R., 2013. Pollution-induced metabolic responses in hypoxia-tolerant freshwater turtles. Ecotoxicology and Environmental Safety 97, 1-9.
Vos, J.G., Bossart, G.D., Fournie, M., 2003. Toxicology of marine mammals.
Wallace, B.P., DiMatteo, A.D., Bolten, A.B., Chaloupka, M.Y., Hutchinson, B.J., Abreu-Grobois, F.A., Mortimer, J.A., Seminoff, J.A., Amorocho, D., Bjorndal, K.A., Bourjea, J.,

Bowen, B.W., Dueñas, R.B., Casale, P., Choudhury, B.C., Costa, A., Dutton, P.H., Fallabrino, A., Finkbeiner, E.M., Girard, A., Girondot, M., Hamann, M., Hurley, B.J., Lopez-Mendilaharsu, M., Marcovaldi, M.A., Musick, J.A., Nel, R., Pilcher, N.J., Troëng, S., Witherington, B., Mast, R.B., 2011. Global conservation priorities for marine turtles. PLoS One 6, e24510.
Wyneken, J., 2001. The anatomy of sea turtles. NMFS, US Department of Commerce, Miami, Florida.

1.2 The Art State

CHAPTER I. The current situation of inorganic elements in marine turtles: A general review and meta-analysis

1.2.1 Introduction

Inorganic pollutants are present in aquatic ecosystems worldwide from natural sources but also from their extensive anthropic use in agriculture and industry (such as Pb, Cd, Hg, Al, As, Cr, Cu, Fe, Mn, Ni, Se and Zn). Coastal and marine contamination is increasing around the world, but the environmental levels of many contaminants which can elicit adverse effects is largely unknown for marine megafauna (Hansen et al., 2016; Kunito et al., 2008; López et al., 2005; Reijnders, 2003; Von et al., 2003). Bioaccumulation of these toxic substances has become a concern due to the possibility of their transfer to the food chain and its impact on diverse species of marine wildlife, including marine turtles (Camacho et al., 2014a; Ley-Quiñónez et al., 2011; Storelli and Marcotrigiano, 2003). A better understanding of pollution of marine ecosystems and the consequences for fauna is one of the priorities highlighted by sea turtle specialists from 13 countries in a recent synthesis of threats (Rees et al., 2016).

The accumulation of inorganic elements, particularly non-essential ones (such as Cd, Hg and Pb), may alter normal immune functions and increase the incidence of infectious illnesses in different species such as humans and marine turtles (D'Ilio et al., 2011; Finlayson et al., 2016; Rana, 2014). Several authors mention the importance of investigating the toxicological consequences of the marine accumulation of these elements, as well as the effects of bioaccumulation from a spatial and temporal perspective to understand the variations and to provide baseline concentrations for further studies (D'Ilio et al., 2011; da Silva et al., 2016a; Day et al., 2007). Furthermore, other essential and trace inorganic elements (such as Al, As, Co, Cr, Cu, Se and Zn) have crucial roles in many essential physiological pathways in most vertebrates, but when their concentrations reach more than these physiological requirements, they can become (Keller et al., 2006b).

Due to their longevity, marine turtles can bioaccumulate diverse contaminants (such as metals) through the food chain and direct exposure. With this in mind, sea turtles represent a growing interest species as a potential marine ecosystem pollution bioindicator (Aguirre and Lutz, 2004; Andreani et al., 2008b; Godley et al., 1999b; Sakai

et al., 2000). It has been hypothesized that the accumulation of pollutants varies among the species depending on different factors, including geographic location, life-stages, diet, species, sex, inter-species variance and intrinsic factors (i.e. toxicokinetics and toxicodynamics particular to each species) (Alava et al., 2006; Guirlet et al., 2008; Rainbow, 2002). However, few studies have tested for differences among species or populations (Anan et al., 2001; Andreani et al., 2008b; Camacho et al., 2014a; Saeki et al., 2000), which implies a very low power of detection of association between pollutant and life-history traits. This small monophyletic group of animals inhabiting all oceans has been extensively studied, but no meta-analysis to search for any global inter-or intra-species tendency has been undertaken as of yet.

There are 7 extant marine turtle species that are globally distributed (Figure 1). They utilize several ecological niches in marine estuarine systems and forage across different trophic levels (Wallace et al., 2011; Wallace et al., 2010): Olive Ridley turtles (*Lepidochelys olivacea*) are the smallest of the 7 species (35-55 cm of curved carapace length -CCL- in their adult stage), are carnivorous and individuals usually live in oceanic and neritic zones (MTSG, 2007); Leatherback turtles (*Dermochelys coriacea*) are the largest sea turtle (130–180 cm adults CCL) and their diet is mainly comprised of jellyfish and they are truly pelagic animals (Stewart and Johnson, 2006). On the other hand, some species change their feeding habits throughout their life. For instance, juvenile Green turtles (*Chelonia mydas*) switch from an omnivorous to a primarily herbivorous diet (Kubis et al., 2009). Organisms that feed higher on the food chain are generally exposed to higher concentrations of metals (Peakall and Burger, 2003). This varies, however, because some plants can also bioaccumulate high levels of some metals, such some seaweed (Peakall and Burger, 2003; Zhou et al., 2008). The indicatory properties of living organisms are used to determine the rate, concentration and range of present and future changes in the natural environment. Bioindicators are organisms that accumulate toxic substances, providing the basis for estimating the concentration of environmental pollution with these substances (Gadzała-Kopciuch et al., 2004). Thus, marine turtles can be useful as sentinel species and bioindicators for diverse pollutants; they can help us to understand the risk to the species themselves but also to the ecosystem.

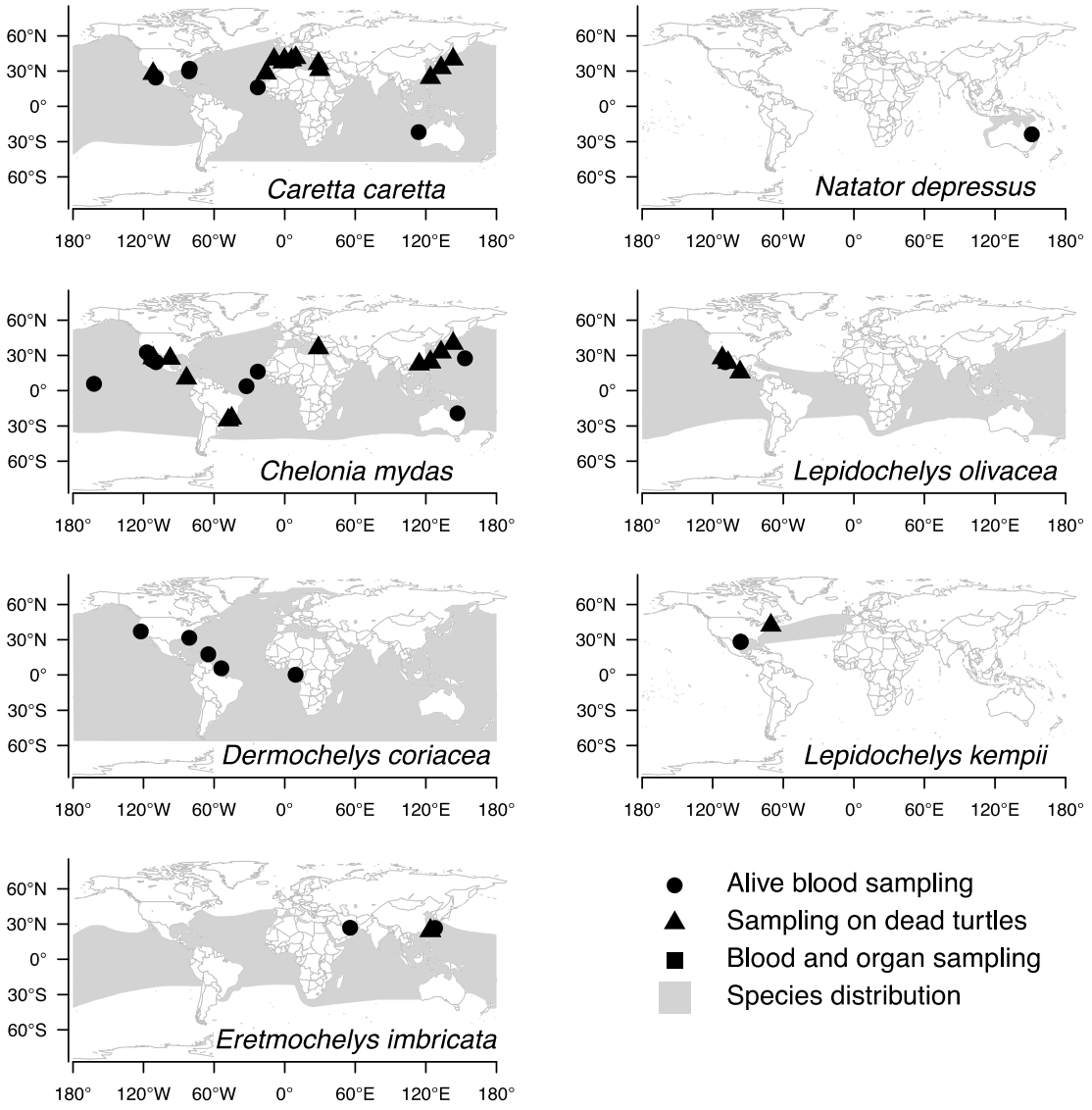
The aims of this work were: (i) to review international information on essential and non-essential inorganic elements (Pb, Cd, Hg, Al, As, Cr, Cu, Fe, Mn, Ni, Se and Zn) in different tissues of marine turtles around the world, and (ii) to perform a meta-analysis using all the relevant existing information in the seven species to characterize the presence of the inorganic elements worldwide. We have included all the representative-existing information about the seven-existent species of marine turtles (super-family Chelonioidea). Six species belong to the family Cheloniidae: Loggerhead (*Caretta caretta*), Green turtle (*Chelonia mydas*), Olive Ridley (*Lepidochelys olivacea*), Kemp's Ridley (*Lepidochelys kempii*), Hawksbill (*Eretmochelys imbricata*), and Flatback marine turtles (*Natator depressus*) and one to the family Dermochelyidae: Leatherback (*Dermochelys coriacea*).

1.2.2 Material and methods

1.2.2.1 Data collection

To collect the available literature about inorganic elements in sea turtles around the world, we used 4 different search engines: Google Scholar (https://scholar.google.com), PubMed (http://www.ncbi.nlm.nih.gov/pubmed), Bibliovie (http://bibliovie.inist.fr) and Scopus (https://www.scopus.com). We always used different combinations of all the 7 species names (scientific or common name) with "inorganic elements", "metals", "heavy metals", "trace elements", "metalloids" and also the elements' common names one by one. To avoid publication bias, both published and unpublished trials were included, such as conference abstracts and thesis works. For trials with several treatment groups, the eligibility of each individual group was assessed and only those relevant were included. Also, when results were represented for age or gender, adults and females were chosen to make the meta-analysis more homogenous. A total of 58 works in the different species and regions were included in this review and the most recent was published in April 2017. One of the works (Sakai et al., 2000), presented very high Hg concentrations reported in µg g^{-1} ww, we contacted the author and he confirmed that this was a mistake in the table and concentrations of Hg were in ng g^{-1} ww.

FIGURE 1. Distribution of the 7 species of marine turtles (grey shadow areas) denoting the location of the works included in this review. Different symbols were used to represent works done with blood samples (live turtles), tissues samples (dead turtles) and both (studies in alive and dead turtles at the same place).



"Heavy metals" is a term often used as a group name for metals and semimetals (metalloids) that have been associated with contamination and potential toxicity or ecotoxicity. However, many authors debate the appropriate use of this terminology, arguing that it has no geochemical, biological, chemical or toxicological basis (Appenroth, 2010; Duffus, 2002; Hodson, 2004). We will use the term "inorganic elements" to define all the elements included in this review to avoid any ambiguity; "non-essential" for those non-essential elements with a high toxicity risk at low doses

(Pb, Cd and Hg); and “essential” for those trace elements which are essential, but potentially toxic at high concentrations (Al, As, Cr, Cu, Fe, Mn, Ni, Se and Zn).

1.2.2.2 Analysis and statistics

For the analysis of this dataset, two challenges were faced: (i) studied characteristics were a mix of quantitative and qualitative variables, and (ii) not all the same inorganic elements were analyzed in all studies and therefore, many data are missing in the dataset. The first point was resolved with multiple factorial analysis (MFA), using mixed data (qualitative and quantitative). MFA is a principal component method used to explore data with both continuous and categorical variables (Pagès, 2004). In summary, the continuous variables were scaled to unit variance and the categorical variables were transformed into a disjunctive data table and then scaled. This ensures a balance of the influence of both continuous and categorical variables in the analysis. Becue-Bertaut and Pagès (2008) have enhanced MFA using grouping of variables. Missing data have been treated using the procedure described in Josse and Husson (2012) and Audigier et al. (2016) implemented in the R package missMDA (Josse and Husson, 2016). Missing values were imputed with a multiple factor analysis model without the supplementary variables (tissue, species and oceanic basin) to ensure that these factors could be further tested. This procedure ensures that missing data do not influence any conclusions but do not reduce power of analysis.

Meta-analysis of the load of inorganic elements (Pb, Cd, Hg, As, Cr, Cu, Mn, Ni, Se, Zn) has been performed taking into account tissue origin, species and oceanic basin as qualitative variables and average curved carapace length (CCL) for the pool of individuals (see below about standardization of CCL, named CCLs). Inorganic element concentrations were log transformed to normalize the data and homogenize the variances. No collinearity between variables was detected and all variables were retained for the analysis. Six groups were defined: 1) CCLs, 2) Non-essential inorganic elements, 3) Essential inorganic elements, 4) Species, 5) Oceanic basin (see below), and 6) Tissue. Tissue, species and oceanic basin were treated as supplementary variables. Supplementary variables were not included in the initial analysis but their effect was

tested *a posteriori*. This procedure ensures that the conclusion about tissue, species and sea effect was independent of the initial classification and can be interpreted as being significant or not.

To avoid species size bias, curved carapace length (CCL) was standardized for sub-adults as CCLs=CCL/maxCCL, with maxCCL being the max of CCL for each species in the dataset and CCLs was set to 1 for adults. The standardized CCL was used as a measure of stages (CCLs<1 for juvenile stage and CCLs=1 for adult stage), but independent of the species (all adult CCLs being equal to 1). We transformed SCL (straight carapace length) into CCL when required, using the following formulas: *Lepidochelys olivacea (Lo)*= (SCL-9.244)/0.818 (Whiting et al., 2007); *Lepidochelys kempii (Lk)*= (SCL-0.346)/0.948 (Wang, 2005); *Caretta caretta (Cc)*= 1.388+1.053(SCL) (Bjorndal et al., 2000); *Chelonia mydas (Cm)*= -0.028+1.051(SCL) (Bjorndal and Bolten, 1989) and *Eretmochelys imbricata (Ei)*= (SCL-0.449)/0.935 (Wabnitz and Pauly, 2008). *Dermochelys coriacea* (Dc) size was already indicated as CCL and *Natatus depresus* (Nd) was indicated as being adult stage. All statistical analyses were performed using R 3.4.0 (R Core Team, 2017) and missMDA 1.11, and FactoMineR 1.35 packages.

We also perform a Generalized Linear Model (GLM) with the 3 most important potentially toxic metals (Pb, Cd and Hg). We have used the original data (not the reconstructed ones using missMDA procedure, see above). The link between each metal load (log transformed) and the tissue, oceanic basin, species and standardized CCLs (see above) of the turtles was assessed using a Gaussian distribution and an identity link. Selection of variable linked with metals was performed using Akaike Information Criterion (AIC). AIC is a measure of quality of fit of a model penalized by the number of variables in the model (Burnham and Anderson, 2002).

For the analysis, the ocean/sea zones were delimited in accordance with the regional management units (RMU) defined for marine turtles by Wallace et al. (2010): Northeastern Pacific (NEP), for the Pacific coasts of Mexico and USA; Northwestern Pacific (NWP), in Japan and China; Southwestern Pacific (SWP), in Eastern Australia;

Northwestern Atlantic (NWA), for the Atlantic coasts of Mexico, USA and French Guiana; Mexican Gulf (GM), for Mexico and USA; Persian Gulf (GP), for Iran; the Caribbean (CAR), for Costa Rica; Northeastern Atlantic (NEA), for Portugal, Cape Verde, Canary Islands and Gabon; Southwestern Atlantic (SWA), for Brazil; the Mediterranean Sea (MED), for Spain, Italy, Turkey and Egypt; and the Eastern Indian Ocean (EIO), for Western Australia.

1.2.3 Results and Discussion

1.2.3.1 Sea turtle species: geographical distribution, habitat and biology.

Marine turtles belong to 2 distinct families: *Dermochelyidae* (leatherback turtles) and *Cheloniidae* (the 6-other species). Distribution for all species is shown in Figure 1 and is described here briefly with some information about their diet.

Loggerhead turtles (*Caretta caretta*) are distributed worldwide in tropical and temperate waters (Watanabe et al., 2010). It is carnivorous and its diet is based on fish, cephalopods and benthic and pelagic prey (Tomás et al., 2001).

Green turtles (*Chelonia mydas*) are long-lived, slow-growing marine turtle that may take as many as 25 to 50 years to reproduce; they are omnivorous as hatchlings, and become herbivores during the juvenile stage, feeding mainly on algae and seagrass (Cardona et al., 2009; Kubis et al., 2009). One dark subspecies is present in the Northeastern Pacific (*Chelonia mydas agassizii*).

Olive Ridley turtles (*Lepidochelys olivacea*) are a pantropical species, with no morphological differences among the populations. Their migratory routes include feeding areas in diverse coastal and pelagic areas. The Olive Ridley diet depends on their phase of growth and location; they are omnivorous and opportunistic. In adulthood, their diet consists of pelagic organisms in oceanic waters (Márquez, 1990; Montenegro and Bernal, 1982).

Kemp's Ridley turtles (*Lepidochelys kempii*) are endemic to the Gulf of Mexico; they are the most endangered marine turtle species due to their limited distribution compared with the other species. Their principal nesting beach is Rancho Nuevo Tamaulipas in Mexico. *Lepidochelys* are the only two species (Olive and Kemp's Ridleys) that present the unique phenomenon of "arribadas", when thousands of turtles come out to nest to the same beach at the same time (Marquez et al., 1996). These turtles are benthic

carnivores and as such would be expected to use the habitats of their prey (Schmid and Witzell, 2006).

Eretmochelys imbricata turtles, both juvenile and adult, forage in a variety of coral and sponge reefs, reef walls, and other hard-bottom habitats throughout the tropics, and they feed almost exclusively on sponges (Leon and Bjorndal, 2002; Stringell et al., 2016).

Natator depressus turtles live in inshore waters restricted to the continental shelf of Australia, eastern Indonesia and southern Papua New Guinea; they feed especially on soft bodied invertebrates, such as soft corals, sea cucumbers, jellyfish and sea pens (Ikonomopoulou et al., 2011 ; Limpus, 2007).

Leatherbacks (*Dermochelys coriacea*) are the largest of all the species and the one with the most extensive movements and longest migration routes. The Leatherback diet is mainly comprised of jellyfish and they are pelagic animals (Stewart and Johnson, 2006).

All marine turtle species also play an important role in food chains; for example, they transport different nutrients from the ocean to the beaches at the egg stage and back into the ocean as juveniles. Also of note is their role as prey, especially as hatchlings, and as predators, taking into account all the ecosystems they inhabit, and their different levels in the food chain (Richardson et al., 2009a; Silman et al., 2002).

For all exposited above, we can see the utility and advantage that marine turtles have over other marine species as bioindicators of etiology of presence of inorganic elements in marine turtles and their habitat because they are present in all oceanic basins and occupy different trophic levels, from the herbivorous to the carnivorous. With regard to the objective of this study (etiology of presence of inorganic elements in marine turtles), the most studied sites in this review were the Northwestern Pacific and the Mediterranean Sea; yet most of the oceans are represented in this paper. With regard to the 7-turtle species, *C. caretta* and *C. mydas* were the most studied species, with an important bias when compared with the other 5 species. Finally, we consider it important to highlight that accumulation in most tissues (except blood and in some cases liver) tends to reflect foraging sites and not necessarily nesting sites where many investigators achieve the greatest number of their samples. Given the length of the migration routes of some marine turtle species, the accumulated levels of inorganic

elements could even reflect other RMU depending on the migratory or nomadic life history of the species.

1.2.3.2 Inorganic elements

Information about inorganic element concentrations was separated according to the state of individuals, alive or dead. For live turtles, we found papers concerning blood samples, with Pb, Cd, Hg, Al, As, Cr, Cu, Mn, Ni, Se and Zn being the most analyzed elements (Table 1). The work with the largest number of samples was published by Camacho et al. (2013b), with 201 *C. caretta* samples; in total we found 9 works on this species. In green turtles, McFadden et al. (2014a) studied 87 turtles in the Central Pacific and there are also 9 papers on this species. In French Guiana, Guirlet et al. (2008) sampled 78 leatherback turtles, and there are 5 papers in total on this species. For Olive Ridleys, the higher number of sampling was undertaken by Cortés-Gómez et al. (2014), with 41 samples, and there are 5 papers published in total on this species. There are 3 works in hawksbills, one in Japan with 25 individuals (Suzuki et al., 2012). Kemp's Ridley has 4 publications, one with 110 and other with 106 samples (Kenyon et al., 2001; Wang, 2005); Flatbacks had just one work, with 20 individuals (Ikonomopoulou et al., 2011). Eleven of these 35 works were carried out in the USA, in different nesting and feeding areas; eight in Mexico; four in Australia; three in the Mediterranean Sea and Cape Verde; two in Brazil; and one in Gabon, Japan, Iran and French Guiana (Table 1).

Due to the difficulty of accessing carcasses in the wild, the number of organ samples is usually limited, although several authors, in collaboration with rehabilitation centers, have achieved a greater number of samples, such as Torrent et al. (2004). However, most of the analyzed papers have between 2 and 30 samples. Results were classified by tissue, from the most to the less frequently analyzed (Tables 2 to 7). Here it is important to take into account the mobility that some elements have in sick, moribund or stranded turtles, which could result in a concentration variance between sick and healthy turtles; this has been demonstrated for As and Ni in *Caretta caretta* by Camacho et al. (2014b).

1.2.3.3 Inorganic elements: Meta-analysis

The analysis of the representative papers found for this study allows us to obtain a unique database of inorganic element content for all the marine turtle species in all the seas and oceans in different tissues. It offers a unique opportunity to analyze marine turtle accumulation of contaminants across different oceanic basins and in different developmental stages, which was impossible when only one study was analyzed at a time.

The first three axes of the multiple factor analysis explain 63% of the total variance. All the 10 inorganic elements are significantly positively correlated with the first axis (28% of the variance). Among the category variables, only the tissue is significantly linked with the first axis. CCLs is strongly correlated with the second axis (r=0.98) and well as Se concentration (r=0.73). All three non-essential elements are located on the same side of this second axis, on the opposite side of CCLs, whereas the 7 essential elements are located on both sides. Oceanic basin and Species are linked significantly with this second axis. Pb, Hg, Cr and Ni are linked with the third axis. It should be noted that contributions of Ni and Pb are always very close on the 3 first dimensions and this is confirmed with significant correlation between load of Ni and Pb (r=0.6, $p=10^{-7}$).

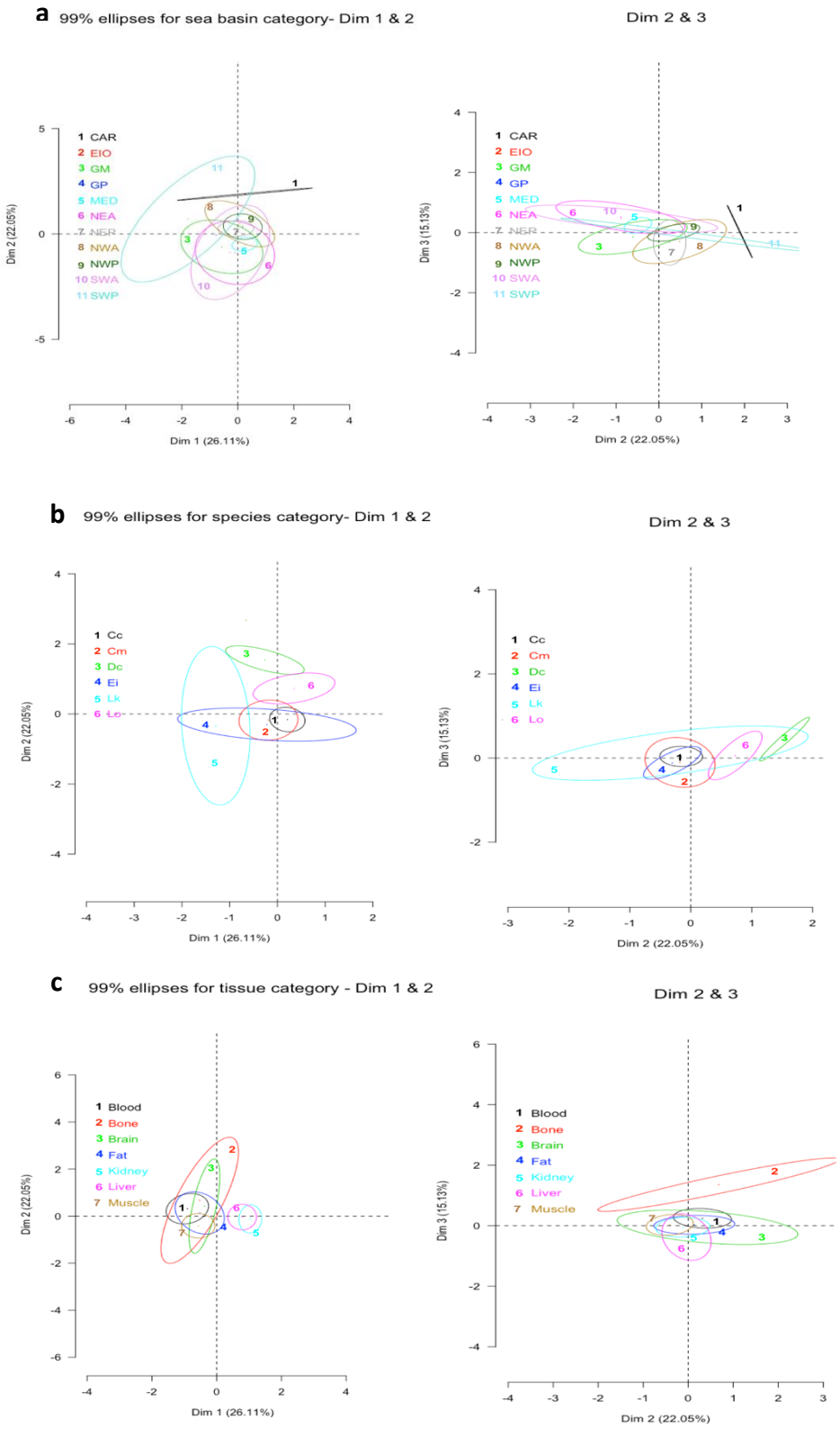
The repartition of the elements based on the basin categories, species and tissue analyzed are represented in ellipses graphs (Figure 2): Figure 2a refers to all the elements by basin categories. Most of the ellipses are overlapped and no structuration can be clearly seen.

In Figure 2b we observe that *C. caretta, C. mydas* and *L. olivacea* overlap. *C. caretta* and *L. olivacea* overlap almost completely and both of them around 80% with *C. mydas.* These results lead us to think about the feeding habits of the species: *C. caretta* and *L. olivacea* are carnivorous (and both ellipses are very related), *C. mydas* are omnivorous in the first part of their life (with a prevalence of animal prey), becoming herbivorous during their late juvenile stage (Cardona et al., 2009). Additionally, a recent study has found that *C. mydas* may remain omnivorous longer than thought (Vélez-Rubio et al., 2016); this could be the reason why *C. mydas* ellipse is related with the carnivorous

species much more than expected. In this review, *C. mydas* ranged from juveniles to adults (from 39 to 101 cm CCL), but most of them were studies on juveniles and sub-adults, and this could explain, in part, the overlap with the known carnivores. The *E. imbricata* ellipse is very large and is also overlapped with *C. caretta, C. mydas* and *L. olivacea*. This large ellipse could be due to the few references regarding this species (*n*=5). However, *D. coriacea* has the same number of references, but it appears less spread out and well separated from the others. *E. imbricata* and *D. coriacea* have very different feeding habits compared with the other species, sponges and crustaceans being the base of the *E. imbricata* diet, and jellyfish being the base for *D. coriacea* (Stringell et al., 2016). With 4 references *L. kempii* also has a large ellipse, similar *to E.* imbricata. Finally, since *N. depresus* had just one reference, the information was not enough to establish their dispersion with the ellipses.

Lastly, in Figure 2c we can see that dispersion for a given tissue in general is very low, except for the bone and brain. It may be because only 4 works have studied these tissues. We also observe that kidney and liver tend to have the same behavior regarding the accumulation of the elements included in this review; different from muscle, blood and brain which share also the same tendency. Blood, brain and muscle also overlap each other almost completely, meaning that they have a similar accumulation tendency; however, number of brain samples were very low. This difference between kidney and liver with the other tissues could be related to metallothioneins (MT), a family of cysteine-rich, low molecular weight proteins (Guirlet and Das, 2012; Ngu and Stillman, 2009). These proteins have the capacity to bind both essential and non-essential elements and they are implicated in the detoxification of toxic elements (i.e. Cd, Hg and As) and homeostasis of essential elements (i.e. Zn, Cu and Se) (Andreani et al., 2008b; Guirlet and Das, 2012). MT are bound to these elements in liver, to then either be stored in liver, or be redistributed to the kidney (Andreani et al., 2008b; Bondía et al., 2013; Klaassen et al., 2009). Bone ellipse, on the other hand, seems to point that this tissue has a very different pathway of elements accumulation than the other tissues. This could be explained by the fact that bone is the main tissue in Pb accumulation (García-Fernández et al., 2009).

Figure 2. Ellipses graphs, dimensions 1 & 2 and 2 & 3, from factorial analysis for inorganic elements in: a) sea basins (CAR=Caribbean, GM=Gulf of Mexico, GP=Persian Gulf, MED=Mediterranean Sea, NEA= Northeastern Atlantic, NWA=Northwest Atlantic, NWP=Northwest Pacific, SWA=Southwest Atlantic, SWP=Southwest Pacific); b) all species of sea turtles (Cc=*Caretta caretta*, Cm=*Chelonia mydas,* Dc=*Dermochelys coriacea*, Ei=*Eretmochelys imbricata*, Lk=*Lepidochelys kempii*, Lo=*Lepidochelys olivacea* and Nd=*Natator depressus*; and c) tissues.



1.2.3.4 Inorganic elements: data review

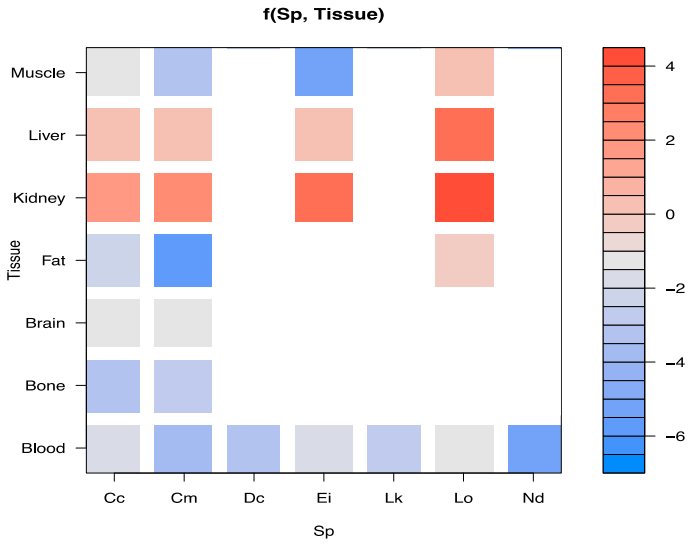
Pb, Cd, Hg, Se, Mn, Cu, Zn, Ni, As, Cr and Al were the most often investigated elements in marine turtles. The articles presented in this review (Tables 1 to 7), from the most commonly to the least commonly analyzed elements, were: Cd (was included in 79% of the total papers), Pb (73%), Cu (70%), Zn (67%), Hg (53%), Ni (45%), Mn (44%), As (37%), Se (36%), Fe (22%), Al (22%) and finally Cr was included in only 19% of the papers. Cu, Cr, Mn, Ni, Se, Al, Fe and Zn play a significant role in diverse animal metabolisms and growth pathways (D'Ilio et al., 2011; Ley-Quiñónez et al., 2011). On the other hand, Cd, Hg, Pb and As are widely distributed in the environment and they can be introduced to marine organisms by ingestion of food, seawater and maternal transfer (Von et al., 2003). However, the most common route for these elements is their dietary intake (Bilandzic et al., 2012; Ikonomopoulou et al., 2011; Storelli et al., 2005). The result of the pollution of the oceans is that these metal and trace elements can accumulate in organs and tissues of consumers across various trophic levels, such as sea turtles, which could alter the natural physiological balance of organisms (Camacho et al., 2013b; Haynes and Johnson, 2000). It is the principal reason why these elements are the most studied by different researchers around the globe in different species. If not all sea turtles are high in the food chain (*C. mydas* as adults*, D. coriacea* and *E. imbricata* are placed low), they have other important features regarding bioaccumulation: their long lifespan and their high conversion of prey to biomass (Perrault, 2012).

1.2.3.4.1 Blood

Concentrations of inorganic elements in blood samples analyzed in published papers greatly varies depending on the species, location, resources, place or target population (Table 1). In general, Cd showed the highest blood concentration among the most toxic metals (Pb, Cd and Hg). Ley-Quiñónez et al. (2011) and Abdallah and Abd-Allah (2011) reported the highest concentration in *C. caretta* from the Mexican Pacific and the Mediterranean, with a mean of 1.80±0.63 and 1.32±0.5 $\mu g\ g^{-1}$ ww respectively. We did not find a significant relationship between Cd and any particular sea basin. Even if blood had very low concentrations compared with other tissues, *C. caretta* is one of the species with the highest concentrations (Figure 3). These high concentrations in *C. caretta* from

the Mediterranean and Mexican Pacific may be due to a high availability of Cd near the sampling areas. For instance, the concentration of Cd in biomonitoring programs in the Spanish Mediterranean had reported medium and high concentrations of this element in sediments (between 40 and 873 µg kg^{-1}dry weight) (Benedicto et al., 2012). Here it is important to mention the high and fast elimination rate of Cd from the blood relative to the very long half-life of Cd in the liver and kidney (Burger, 2008; Guirlet and Das, 2012). With regards to this, Guirlet and Das (2012) showed that in freshwater turtles (*Trachemys scripta*), even a high Cd diet resulted in low blood concentrations, indicating that blood Cd levels were a poor bioindicator of exposure. Recent publications have found higher levels of Cd in the sea basin where these samples were taken, than those recommended by the World Health Organization for water quality (Pérez-Moreno et al., 2016; Wahbi and El-Greisy, 2016). The rest of the works about Cd in turtle blood reported concentrations ranking from 0.01 to 0.99 µg g^{-1}.

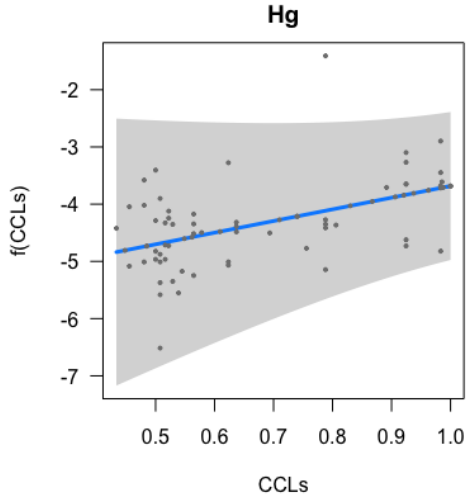
Figure 3. Cd concentrations for species and tissue. Red boxes denotes higher and blue lower concentrations. White spaces denotes not available information.



On the other hand, Hg showed the lowest values in most of the papers, ranging from 0.004 to 0.66 µg g^{-1} ww. Abdallah and Abd-Allah (2011) reported the highest concentration, and Jerez et al. (2010b) the lowest, both for *C. caretta* in the Mediterranean (Egypt and Spain respectively). Ikonomopoulou et al. (2011) reported the 3 studied non-essential metals (Cd, Pb and Hg) below the detection limit (<0.1 µg g^{-}

[1]) in *N. depressus*. Even if works made in the Egypt basin showed that water and sediments do not have very high concentrations of Hg (Hamed et al., 2013), it is well known that Hg can bioaccumulate and biomagnify in the food chain, including marine turtles (García-Fernández et al., 2009; Perrault, 2012). With this in mind, another important factor to consider is the size of the turtles, for example, turtles sampled by Abdallah and Abd-Allah (2011) were almost 2 times larger than those sampled by Jerez et al. (2010b), 75.5 vs 48.7 cm). Additionally, in this factorial analysis which included CCL as a variable, we observed a positive tendency of Hg accumulating in larger CCL individuals for all the species included (Figure 4). Furthermore, *C. caretta* tends to accumulate more Hg than the other species (Figure 3).

Figure 4. Relationship between CCLs and Hg concentrations in all tissues and all species.



Regarding Pb, Komoroske et al. (2012) and Abdallah and Abd-Allah (2011) reported the highest concentrations in *C. mydas* from the US Pacific basin (1.26±0.22 µg g^{-1} ww) and *C. caretta* from the Egypt Mediterranean basin (0.49±0.09 µg g^{-1} ww) respectively. These concentrations are far higher compared with other studies (Table 1). Komoroske et al. (2012) This could be because her work was done with turtles from an urbanized estuarine in San Diego Bay (USA). Thus, these turtles are in a particularly highly polluted environment compared with turtles inhabiting open oceans (or at the very least they had been in this environment for some time when samples were collected). Another interesting fact about Pb was with samples taken in 2005 by Páez-Osúna et al. (2010a), who reported a concentration of 0.19 µg g^{-1} in Olive Ridleys from the Mexican Pacific, a

population from Oaxaca. However, in another publication with population from the same place, in samples taken 7 years later (2012), Cortés-Gómez et al. (2014) reported that Pb concentration in blood from those turtles was among the lowest in sea turtles around the world (0.02 μg g^{-1}). Here it is important to keep in mind the different methodologies used in both studies (graphite furnace atomic absorption spectrophotometry vs inductively coupled plasma optical emission spectrophotometry). Furthermore, 3 of the 25 papers found for Pb analysis presented values BDL, 2 of them in the Northeast Pacific (DL=0.01 μg g^{-1}) and 1 in the Southwestern Pacific (DL=0.1 μg g^{-1}). This could indicate that the concentration of this element in the Pacific Ocean is lower than in other areas (at least those available for turtles). In all the other cases, the concentration of Pb was generally low (from 0.0008 to 0.19 μg g^{-1}ww). This decrease in Pb in different locations worldwide has been previously reported in diverse biomonitoring studies using different species such as mussels (Solaun et al., 2013), shrimps (Nascimento et al., 2016), marine mammals (Von et al., 2003), seabirds (Summers et al., 2014), turtles (Cortés-Gómez et al., 2014) and humans (Orisakwem et al., 2014). The principal explanation for this tendency is the prohibition of leaded gasoline, increase of wastewater treatment and diversity of dischargers to oceans (Orisakwem et al., 2014; Solaun et al., 2013; Von et al., 2003); these actions were implemented in the 1990's and the early 2000's in many countries. Finally, an important point was that (Abdallah and Abd-Allah, 2011) had the highest reports of these 3 non-essential metals in loggerhead turtles from the Egyptian Mediterranean, which should be an alarm signal regarding the pollution of these area.

Other elements commonly analyzed were Zn, Cu, Se, As and Ni; less frequently Cr, Al and Mn were studied. All these 8 elements were detected in all the samples (except As in *D. coriacea* in Gabon), probably due to the fact that these elements are physiologically important in all species, including marine turtles (Frye, 1995; Ikonomopoulou et al., 2011).

Arsenic is present in various chemical forms, some of these forms can be very toxic in low levels (Kunito et al., 2008). This element usually had low concentrations in blood of

the turtles, ranging from 0.007 to 1.40 µg g^{-1} ww, but there were 2 studies showing more concerning concentrations, one with 4.06 (Ley-Quiñónez et al., 2011) and another with 11.17 (Register, 2011), both in *C. caretta*. Concentrations found by Register (2011) are very high, even compared with tissues such as muscle where this element tends to accumulate (Table 4). There are some works demonstrating high concentrations of As in mollusk prey of loggerheads in Japan (Saeki et al., 2000), but this study was undertaken on the US Atlantic coast. Additionally, other loggerheads from the same area do not show these high concentrations (Table 1). Therefore, we could consider either a limited polluted area or a one-off event that could have raised As levels in the area these turtles inhabit when samples for the Register (2011) work were taken.

Another essential element that can be harmful in high concentrations in many species is Se. Living animals mainly accumulate Se through their diet organically (i.e selenomethionine (SeMet) or inorganically (selenite and selenate). SeMet is considered the primary organic selenium form relevant for bioaccumulation and toxicity in wildlife (Dyc et al., 2016). In an experiment in freshwater turtles Dyc et al. (2016) found that SeMet accumulation is from dietary sources and in a dose-dependent manner, but any adverse effect was associated with Se exposure in the SeMet turtle group. However, the highest concentration reached in blood in this experiment was 6.88 µg g^{-1} ww. The highest concentration found in marine turtles in this review were 9.98 and 10.92 µg g^{-1} ww (in *D. coriacea* from French Guiana and *L. olivacea* from the Mexican Pacific respectively). Neither species nor localization seem to have any influence on these high concentrations (Table 1). It is worth mentioning the positive relationship between Se and CCLs seen on the second axis of the MFA. This observation is in accordance with an accumulation of Se throughout life.

In blood, Zn presented the highest concentration, although with very wide ranges: the highest value was reported in *N. depressus* sampled in Australia, with 151.15 ± 1.45 µg g^{-1} ww (Ikonomopoulou et al., 2011), and the lowest in *C. mydas* from Cape Verde with 1.04 ± 0.45 µg g^{-1} ww (Camacho et al., 2014a). This difference could be influenced by several circumstances, such as: 1) the size of the individuals: while Camacho et al.

(2014a) sampled juveniles of both sexes (CCL=25-66 cm), Ikonomopoulou et al. (2011) sampled only adult females (CCL=90-100 cm); 2) the feeding habits: while Green turtles are mostly herbivorous (Kubis et al., 2009), Flatback turtles are carnivorous (Limpus, 2007); 3) the species: while there is more information available for Green turtles, this is the only work found on Flatbacks, so we have no other comparison for this species; 4) the habitat: Camacho et al. (2014a) turtles were found in open waters around the Cape Verde archipelago, while Ikonomopoulou et al. (2011) worked with nesting turtles from Curtis Island. This island is heavily developed for coal seam gas facilities (QGC, 2011); sonesting sites (as in many cases) are unlikely to be the source of the accumulated contaminants. The number of samples was the same in both studies (Table 1); consequently, all these factors (size, feeding habits, species or habitat), should not influence the concentration of Zn found in both studies.

1.2.3.4.2 Liver

Regarding non-essential metals, Cd in liver was determined in 25 populations (Table 2), the most frequently analyzed, followed by Pb with 23 and finally, Hg, reported in 11 papers (13 populations). Hg showed the lowest concentrations in this tissue (from 0.08 to 0.50 µg g^{-1}ww), concentration for species are very similar in this organ and the NEA had one of the highest Hg concentrations in general (Figure 5). In general, *C. caretta* presented the higher concentrations of Hg; this could be explained by the loggerhead carnivorous diet. It is also important to mention that many areas have not been studied regarding Hg in turtles (Figure 5b).

The highest concentration of Pb was reported by Frias-Espericueta et al. (2006) in the north of the Mexican Pacific in *L. olivacea* (3.32 µg g^{-1}ww). However, these results contrast with those of Gardner et al. (2006) in the same year, which did not detect Pb in any of the 3 species analyzed (*L. olivacea, C. caretta* and *C. mydas*; detection limit <0.006 µg g^{-1} dw) in the same zone (Mexican Pacific). Both studies used a similar analysis technique (flame atomic absorption spectrophotometry). These differences in *L. olivacea* could be related then, to: 1) the size: turtles from Frias-Espericueta et al. (2006) were bigger than those studied for Gardner et al. (2006), and 2) the year(s) when the

Table 1. Heavy and metalloid concentrations (mean ± SD, µg g^{-1} wet weight) in blood of marine turtles from different areas.

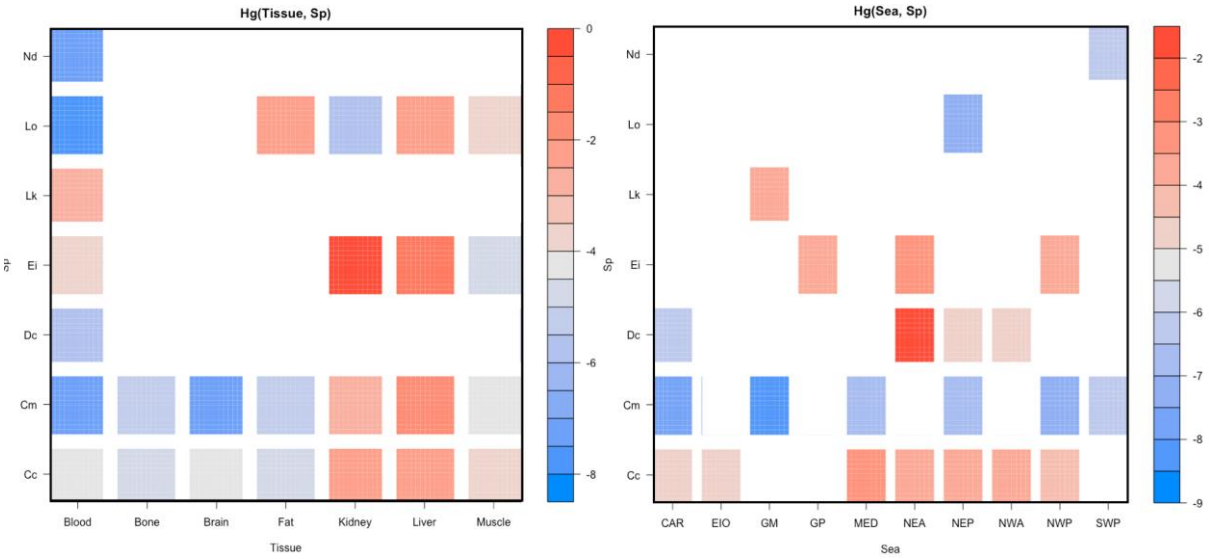
Source	Sp	*n*	Country	CCL	Pb	Cd	Hg	Al	As	Cr	Cu	Mn	Ni	Se	Zn
Páez-Osuna *et al.* 2010a, 2010b and 2011*	*Lo*	25	Mexico (Pacific)	66.4	0.19±0.03	0.09±0.04	0.001±0.000	-	-	-	0.47±0.08	-	0.56 ± 0.26	-	11.68 ± 0.94
Zavala-Norzag *et al.* 2014	*Lo*	17	Mexico	63.15	BLD	0.55	-	-	1.87	-	1.09	2.47	2.17	10.92	37.19
Cortés-Gómez *et al* 2014	*Lo*	41	Mexico (Pacific)	65.50	0.02±0.01	0.17±0.10	-	2.18±1.59	1.19±0.70	1.81±0.06	0.60±0.11	0.59±0.09	0.04±0.02	5.74±2.50	10.43±4.12
Kenyon, 2001	*Lk*	106	USA (Gulf of Mex.)	40b	0.01	-	0.02	-	-	-	0.52	-	-	-	7.50
Wang 2005	*Lk*	91	USA (Atlantic)	37.7b	0.03±0.03	0.01±0.005	0.01±0.01	-	-	0.02±0.05	0.41±0.11	-	-	-	6.71±4.46
	Lk	19	Mexico (Atlantic)	68.2b	0.05±0.02	0.01±0.01	0.06±0.04	-	-	0.005±0.00	0.40±0.09	-	-	-	22.70±12.6
Innis *et al*., 2008	*Lk*	29	USA (Atlantic)	26	-	-	0.02±0.009	-	-	-	0.69±0.68c	-	-	0.49±0.06c	2.29±3.35c
Day, 2003	*Cc*	33	USA (Gulf of Mex.)	77.3b	-	-	0.03±0.01	-	-	-	-	-	-	-	-
Deem *et al*., 2009	*Cc*	23	USA (Atlantic)	72.5	0.0008	-	0.02±0.00	-	0.007±0.00	-	-	-	-	-	-
Jerez et al., 2010*	*Cc*	5	Spain	48.7b	0.06±0.06	0.02±0.04	0.004±0.002	-	1.40±1.86	-	-	-	-	0.56±0.26	1.41±0.57
Register, 2011	*Cc*	81	USA (Atlantic)	77.3b	0.01±0.007	0.04±0.06	-	0.06±0.4	11.17±6.01	0.04±0.06	0.58±0.09	0.03±0.02	0.005±0.005	3.49±1.59	10.50±2.00
Abdallah et al., 2011*	*Cc*	8	Egypt (Mediter)	75.6	0.49±0.09	1.32±0.5	0.66±0.13	-	0.74±0.34	-	-	-	-	-	-
Ley-Quiñónez *et al*., 2011	*Cc*	22	Mexico (Pacific)	73.9b	BLD	1.80±0.63	-	-	4.09±2.56	-	2.83±0.62	0.61±0.55	1.159±2.42	6.14±3.58	44.81±17.53
Ancora *et al.* 2012*	*Cc*	55	Italia		0.06±0.07	0.11±0.09	0.06±0.05	-	-	-	-	-	-	-	-
Trocini, 2013	*Cc*	98	Australia	96	0.02±0.01	0.30±0.30	0.02±0.01	-	2.77±3.46	0.03±0.02	0.67±0.13	0.04±0.03	0.01±0.02	2.83±5.01	11.54±1.77
Camacho et al., 2013	*Cc*	201	Cape Verde	95.51	0.06±0.02	0.29±0.25	0.04±0.04	1.07±1.21	0.58±0.95	0.26±1.02	1.27 ± 8.46	0.03±0.03	1.41 ± 6.66	2.53±2.21	4.97 ± 2.9
Labrada *et al,* 2011	*Cm*	42	Mexico (Pacific)	64	-	0.06±0.00	-	-	-	-	-	-	76.47±11.02	1.59±0.19	13.92±0.49

Van de Merwe *et al*., 2010	*Cm*	13	Australia	57 e	0.02±0.05	0.03±0.09	0.002±0.005	-	4.36±1.41	-	1.09±0.99	-	-	2.44±0.63	7.92±0.66
Komoroske *et al.* 2012	*Cm*	27	USA (Pacific)	90.50	1.26±0.22	0.01±0.04	0.001±0.00	0.14±0.03	0.15±0.02	-	0.75±0.04	0.46±0.09	-	0.77±0.25	-
Ley-Quiñónez *et al*., 2013	*Cm*	12	Mexico (Pacific)	67.2b	-	0.99±0.35	-	-	-	-	1.71±0.73	1.22±0.99	1.03±1.01	7.66±3.19	63.58±17.06
Camacho et al., 2014 (d)	*Cm*	21	Cape Verde	45.49	0.07±0.02	0.30±0.07	0.03±0.01	1.95±2.35	0.44±0.10	0.04±0.01	0.25±0.12	0.03±0.02	2.76±0.54	0.61±0.25	1.04±0.45
McFadden *et al*, 2014	*Cm*	87	USA (central Pacific)	70.4b	0.02±0.01	0.02±0.02	0.001±0.003	1.05±0.93	-	0.03±0.03	0.43±0.17	0.02±0.02	0.02±0.02	1.57±3.52	7.50±2.34
Setim Prioste *et al*., 2015	*Cm*	31	Brazil	72	0.03 a	0.01 a	-	0.08 a	0.20 a	-	0.78 a	-	-	0.28 a	0.01 a
da Silva et al., 2016	*Cm*	13	Brazil	37.0	0.98±0.15	0.08±0.01	-	-	-	-	0.95±0.10	-	9.15±1.45	-	0.66±0.11
Villa *et al*., 2017	*Cm*	35	Australia	75	0.02±0.01	0.003±0.00	-	-	0.18±0.25	0.001±0.00	0.64±0.17	0.06±0.04	0.01±0.007	0.15±0.13	11.0±2.50
Deem *et al*., 2006	*Dc*	9	Gabon	150	0.08±0.03	-	0.20±0.20	-	BDL	-	-	-	-	-	-
Guirlet et al., 2008	*Dc*	78	French Guiana	160	0.18±0.05	0.08±0.03	0.011±0.003	-	-	-	1.34±0.28	-	-	9.98±0.05	11.10±0.28
Innis *et al* 2010	*Dc*	16	USA (Atlantic)	145	-	0.07±0.02f	0.01±0.00f	-	-	-	7.55±2.83f	-	-	-	-
Harris *et al*., 2011	*Dc*	23	USA (Pacific)	158	0.18a	0.065a	0.015a	-	-	-	-	-	-	-	-
Perrault *et al*., 2013	*Dc*	70	USA (Atlantic)	154	-	-	0.003	-	-	-	-	-	-	2.65	-
Susuki *et al*., 2012 (d)	*Ei*	25	Japan	77.7b	0.13a,c	-	-	8.33a,c	0.02a,c	0.03a,c	0.51a,c	0.01a,c	0.001a,c	0.25a,c	1.35a,c
Ehsanpour *et al.* 2014	*Ei*	12	Iran	63.54	0.11±0.05	0.07±0.01	0.03±0.01	-	-	-	0.37±0.15	-	-	-	7.52±0.79
Camacho et al., 2014	*Ei*	13	Cape Verde	54.55	0.03±0.02	0.32±0.06	0.04±0.01	0.78±0.29	0.48±0.23	0.06±0.03	0.22±0.15	0.04±0.02	0.02±0.005	0.59±0.4	2.6±2.11
Ikonomopoulou *et al*., 2011	*Nd*	20	Australia	-	BDL	BDL	BDL	-	-	-	7.74±0.09	-	BDL	-	151.15±1.45

* Results originally published in dry weight transformed into wet weight using the humidity percentage reported by the authors, in case any percentage was reported by the authors, we used 80% of humidity reported by Guirlet et al. (2008). BDL = below the detection limit (<0.1 µg g^{-1}); *Lo= Lepidochelys olivacea; Lk=Lepidochelys kempii; Cc= Caretta caretta; Cm=Chelonia mydas*;

c= Dermochelys coriacea; Ei= Eretmochelys imbricata; *Nd= Natator depressus*. **a =** median; **b=** Original publication in straight carapace long (SCL), transform into CCL; **c=**plasma; **d=** turtles in captivity; **e=** range transform into mean with the following formula: ((max-min)/2+min).

Figure 5. Hg concentrations related with species and tissue (a) and with geographic area and species (b). Red boxes denotes higher and blue lower concentrations. White spaces denotes not available information.



samples were taken: the work of Frias-Espericueta et al. (2006) was in 2005, whereas Gardner et al. (2006) did not report the years of their sampling, perhaps the sampling period of the latter was prior to Frias-Espericueta et al. (2006).

Lastly, Cd concentrations in liver were usually low in turtles from the Atlantic, Mediterranean and Pacific oceans in all the species (among 0.20 and 3.36 μg g^{-1}ww), medium in Spain (Mediterranean Sea) and Japan (Pacific) with 5.85 and 9.74 μg g^{-1} ww respectively (García-Fernández et al., 2009; Sakai et al., 2000), both in *C. caretta*. However, Cortés-Gómez et al. (2014) reported a mean of 82.87 ± 36.65 mg g^{-1} ww in *L. olivacea* from the southern Mexican Pacific, the highest report from any sea turtle species. Cd is one of the most toxic metals with unknown physiological function for most vertebrates, it tends to accumulate (especially in kidney) and has a high persistence in the environment (Ruangsomboon and Wongrat, 2006; Wang and Wang, 2009). Liver was the second most important tissue for accumulated Cd in the species where it has been studied (Figure 3).

With regard to the essential elements, all of them were present in all samples analyzed. Cu was the most frequently analyzed, being determined in 24 different populations. The lowest concentrations of Cu were reported by Kaska et al. (2004), with 0.42 and 0.75 μg g^{-1} in *C. mydas* and *C. caretta* respectively. However, among the rest of the papers, Green turtles presented higher Cu concentrations (from 11 to 37 μg g^{-1} ww) than Loggerheads (from 4 to 8 μg g^{-1}) or Ridleys (from 8 to 16 μg g^{-1}). These results showed an important species influence in Cu accumulation (except for *C. mydas* from the coast of Turkey). Here it is important to point out that Cu has been increasingly accumulating in marine algae due to its use in antifouling paints for boats and ships (Turner et al., 2009), perhaps this is the reason why green turtles, which feed principally on algae and seagrass, is the species with the higher Cu concentration. Fe on the other hand, was the element that reached the highest concentrations in liver (Table 2), once again Kaska et al. (2004) had the lowest concentrations (3.2 for *C. mydas* and 3.39 μg g^{-1} ww for *C. caretta*) in the Mediterranean Sea; for the rest, Fe reached as high as 760 μg g^{-1} ww in

loggerhead turtles from Japan (Sakai et al., 2000). In this case, the accumulation of Fe must be related to the availability of this element in the turtles' environment, since *C. caretta* had the lowest and highest concentrations, with *C. mydas* and *L. olivacea* also presenting high concentrations. This could be related, in part, to the presence of organic matter in coastal waters, which has been described with high affinity for iron in seawater in Japan (Takata, 2005). Another important influence of this high difference in Fe concentrations could be the size (age) of the turtles, because turtles with higher concentrations were also the largest in *C caretta* and *C. mydas* (Andreani et al., 2008b; Kubota et al., 2002).

Arsenic presented very wide concentration ranges in this organ, from 0.23 (*C. caretta* from Egypt), 0.44 (*C. mydas* from Japan) and 0.48 μg g^{-1} ww (*D. coriacea* from the Atlantic USA); to 27.9 (Italy) and 17.07 μg g^{-1} ww (Canary Islands), both in *C. caretta*. The rest of the works were around 1 to 4 μg g^{-1} ww. Saeki et al. (2000) mentioned that high concentrations of As are expected in Green and Loggerheads turtles feeding on algae (which sometime contains As at 1000-50000 times the level in their ambient seawater) and mollusks, which accumulate high concentrations of As. With these different concentrations in different species we can suspect that along with the feeding habits of turtles, the availability of this metal varies a lot in different locations.

Another element with high concentrations in this organ (compared with the other tissues) was Se. The higher concentrations were found in *E. imbricata* from Japan (12.25±9.25), *L. olivacea* from the Mexican Pacific (8.24±2.47) and *D. coriacea* from Atlantic USA (8.23±1.91); the rest of the works range from 0.77 to 5.23 μg g^{-1} ww. One of the roles of Se in liver is Hg detoxification, but it also can be toxic itself at increased concentrations (Dyc et al., 2016; Tinggi, 2003). In this regard, *D. coriacea* has been reported with a very high Se:Hg ratio (Perrault, 2012). This detoxification role is consistent with the findings of this review, where liver was the organ with the highest concentrations of Hg and Se. Moreover, both Hg and Se showed a positive relationship in all the species with CCL and Hg concentrations (S2).

Regarding Zn, it was determined in 16 studies (Table 2), with concentrations from 7.55 (Jerez et al., 2010b) in *C. caretta* to 46.65 mg g^{-1} (Cortés-Gómez et al., 2014) in *L. olivacea*. An investigation performed by Andreani et al. (2008b), identified significant correlations between accumulated Cu, Zn and Cd with metallothioneins (MT); a widely recognized group of proteins known for their role in element storage and elimination.

1.2.3.4.3 Kidney

Twenty-one papers on 29 different populations include kidney data (Table 3). Cadmium was the element most frequently studied in kidney (n=25), followed by Pb and Cu (n=23), Zn (n=20), Mn (n=15), Ni (n=14), Hg and As (n=12), Se (n=10) and Fe (n=9), and finally, Cr with 4 publications.

High concentrations of Cd were reported in the Northeastern Pacific (Cortés-Gómez et al., 2014; Faust et al., 2014a; Gardner et al., 2006; Kampalath et al., 2006), ranging from 20.41 to 150.9 µg g^{-1} ww, although there was one report of 5.28 µg g^{-1} (Frias-Espericueta et al., 2006). Even so, the highest concentration of Cd was found in the South Mexican Pacific, specifically in Olive Ridleys from "La Escobilla" beach, with 150.9 µg g^{-1}ww (Cortés-Gómez et al., 2014). In the Mediterranean Sea, sea turtles presented low Cd concentrations, ranging from 1.97 g g^{-1} (Andreani et al., 2008b) to 10.5 µg g^{-1}ww.

Table 2. Metal and metalloids concentration (mean ± SD, µg g^{-1} wet weight) in liver of marine turtles from different areas.

Source	Sp	*n*	Country	CCL	Pb	Cd	Hg	As	Cr	Cu	Fe	Mn	Ni	Se	Zn
Gardner *et al.* 2006*	*Lo*	6	Mexico	62.1 b	BLD	4.47	-	-	-	9.18	182.7	0.025	0.14	-	11.78
Frías-Esperic. *et al.*, 2006	*Lo*	7	Mexico	80	3.32	3.28	-	-	-	8.20	-	-	-	-	-
Kampalath *et al*., 2006	*Lo*	6	Mexico	59.2	-	-	0.213	-	-	-	-	-	-	-	-
Cortés-Gómez *et al.* 2014	*Lo*	13	Mexico	65.5	0.11±0.08	82.87±36.65	-	3.34±1.50	-	16.37±10.34	-	3.36±1.34	0.08±0.05	8.24±2.47	46.65±16.38
Sakai et al 2000	*Cc*	7	Japan	88.7 b	0.08 ±0.03	9.74±3.37	0.40±0.15	-	-	17.7±8.93	760	2.18±0.40	<0.03	-	28.1±4.73
Saeki *et al*., 2000*	*Cc*	4	Japan	-	-	-	-	1.58±0.39	-	-	-	-	-	-	-
Kubota *et al.* 2002*	*Cc*	5	Japan	77	-	-	-	3.36±0.6	-	-	-	-	-	-	-
Kaska *et al*. 2004*	*Cc*	32	Turkey	67.43	0.88±0.33	2.71±0.97	-	3.55±1.90	0.67±0.23	0.75±0.22	3.93±1.19	-	2.89±0.77	3.22±1.49	-
Torrent *et al.*, 2004	*Cc*	78	Canarias Islands	43.5 b,c	2.94±0.59	2.53±0.45	-	17.07±2.9	-	15.02±2.07	-	-	2.88±0.35	-	13.48±1.70
Franzellitti *et al.*, 2004	*Cc*	30	Italy	73.1 b	-	2.84±0.72	-	27.9±6.5	-	7.4±3.9	377.4±211.2	6.23±2.8	4.38±1.43	-	27.9±6.5
Storelli *et al.* ,2005	*Cc*	19	Italy	46.1 b	0.16±0.05	3.36±1.94	0.43±0.29	-	-	7.69±4.63	-	-	-	3.54±1.49	29.3±7.71
Maffucci *et al.*, 2005*	*Cc*	22	Italy	61.1	-	4.82±8.55	0.27±0.42	-	-	9.32±2.17	-	-	-	2.45±1.32	16.5±10.6
Gardner *et al.* 2006*	*Cc*	5	Mexico	61.4 b	BLD	0.43	-	-	-	8.48	75.25	-	0.09	-	17.28
Kampalath *et al*., 2006	*Cc*	11	Mexico	59.8	-	-	0.091	-	-	-	-	-	-	-	-
García-Fdz et al., 2009	*Cc*	16	Spain	-	0.69±0.41	5.85±13.42	-	-	-	5.40±2.01	-	-	-	-	26.8±20.6
Jerez *et al.* 2010*	*Cc*	13	Spain	48.7 b	0.05±0.02	0.20±0.12	0.09±0.10	3.18±3.25	-	-	-	-	-	0.77±0.36	7.55±3.05
Abdallah et al., 2011*	*Cc*	8	Egypt (Mediter)	75.6	1.53±0.30	1.61±0.98	0.29±0.19	0.23±0.01	-	-	-	-	-	-	-
Nicolau *et al.,* 2017	*Cc*	38	Portugal	50	0.10±0.01	5.03±0.54	0.30±0.03	4.49±0.35	-	5.99±0.48	-	1.78±0.09	0.14±0.02	5.23±0.20	24.01±0.94
Andreani *et al*., 2008*	*Cc*	34	Italy	58.5 c	0.025±0.02	0.6±0.1	-	-	-	4.37±0.61	308±101	1.99±0.35	-	-	25.75±3.5
	Cm	11	Costa Rica (Carib)	101.5 c	0.02±0.003	2.65±0.27	-	-	-	25±2.75	620.5±71.5	2.24±0.23	-	-	20.62±1.22

Sakai *et al.*, 2000	*Cm*	2	Japan	99.8 b	0.12	8.0	0.188	-	-	11.11	135.5	1.88	0.06	-	58.3
Saeki *et al*., 2000*	*Cm*	19	Japan	-	-	-	-	0.44±0.23	-	-	-	-	-	-	-
Kubota *et al.* 2002*	*Cm*	5	Japan	48	-	-	-	1.09±0.61	-	-	-	-	-	-	-
Lam *et al.* 2004*	*Cm*	2	China	-	0.03±0.01	0.27±0.24	0.19±0.05	1.16±0.99	0.27	33.25±37.15	-	4.06±3.45	0.06±0.06	6.41±7.15	32.22±15.98
Gardner *et al.* 2006*	*Cm*	11	Mexico	65.2 b	BLD	0.82	-	-	-	15.01	3.58	-	0.002	-	15.72
Kampalath *et al*., 2006	*Cm*	4	Mexico	57.3	-	-	0.501	-	-	-	-	-	-	-	-
Talavera *et al.*, 2007	*Cm*	8	Mexico	65.1 b,c	0.00	4.23	-	-	-	19.13	-	0.06	0.00	-	22.73
Barbieri, et al., 2009*	*Cm*	15	Brazil	-	-	0.23±0.07	-	-	-	9.97±0.48	-	1.08±0.17	0.07±0.02	-	-
Da Silva *et al.* 2014*	*Cm*	29	Brazil	39	1.12±0.12	1.47±0.22	-	-	-	25.22±3.97	-	-	-	-	10.12±0.72
Faust *et al*., 2014	*Cm*	12	USA (Pacific)	52.4 b	0.10±0.02	0.90±0.12	0.08±0.01	2.71±0.25	4.25±0.11	37.1±7.3	-	2.31±0.18	0.15±0.03	1.65±0.15	35.0±3.3
Kaska *et al*. 2004*	*Cm*	22	Turkey	53.6	0.61±0.30	1.84±0.68	-	2.42±1.26	0.61±0.30	0.42±0.20	3.12±0.83	-	2.31±0.48	2.66±1.56	-
Anan *et al*., 2001*	*Cm*	26	Japan	53.7 b	0.12±0.10	4.55±2.42	0.10±0.04	-	0.55±0.15	34.75±21.5	-	1.18±0.51	-	1.27±0.57	21.8±7.65
	Ei	22	Japan	49.1 b	0.04±0.03	1.76±1.59	0.21±0.46	-	0.21±0.17	13.75±29	-	2.07±1.06	-	12.25±9.25	27.25±13.5
Saeki *et al*., 2000*	*Ei*	4	Japan	-	-	-	-	3.82±2.19	-	-	-	-	-	-	-
Perrault, 2012	*Dc*	14	USA (Atlantic)	147.3	-	-	0.48±0.38	-	-	-	-	-	-	8.32±1.91	-

*Results originally published in dry weight transformed into wet weight using the humidity percentage reported by the authors, in case any percentage was reported by the authors, we used 75% of humidity reported by Garcia-Fernandez et al. (2009). BDL = below the detection limit; *Lo*= *Lepidochelys olivacea; Cc*= *Caretta caretta; Cm*=*Chelonia mydas*; *Ei*= *Eretmochelys imbricata*. **a =** median; **b=** Original publication in straight carapace long (SCL), transform into CCL using the following formulas: *Lo*= (SCL - 9.244) / 0.818 (Whiting et al., 2007); *Cc* = 1.388 + 1.053 (SCL) (Bjorndal et al., 2000); *Cm* = -0.028+1.051 (SCL) (Bjorndal et al., 1989) and *Ei* = (SCL - 0,449) / 0,935 (Wabnitz et al., 2008); **c=** range transform into mean with the following formula: ((max-min)/2+min).

.

(García-Fernández et al., 2009), compared with the North Pacific, both studies were in *C. caretta*; the Atlantic Ocean (5 publications) showed similar levels with a minimum of 0.78 µg g^{-1}ww (Barbieri, 2009) from the coast of Brazil in *C. mydas* and a maximum of 13.32 g^{-1}ww in Costa Rica (Andreani et al., 2008b) in the same species; giving *C. mydas* higher concentrations than *C. caretta* in this area. This should be related to their very different dietary habits.

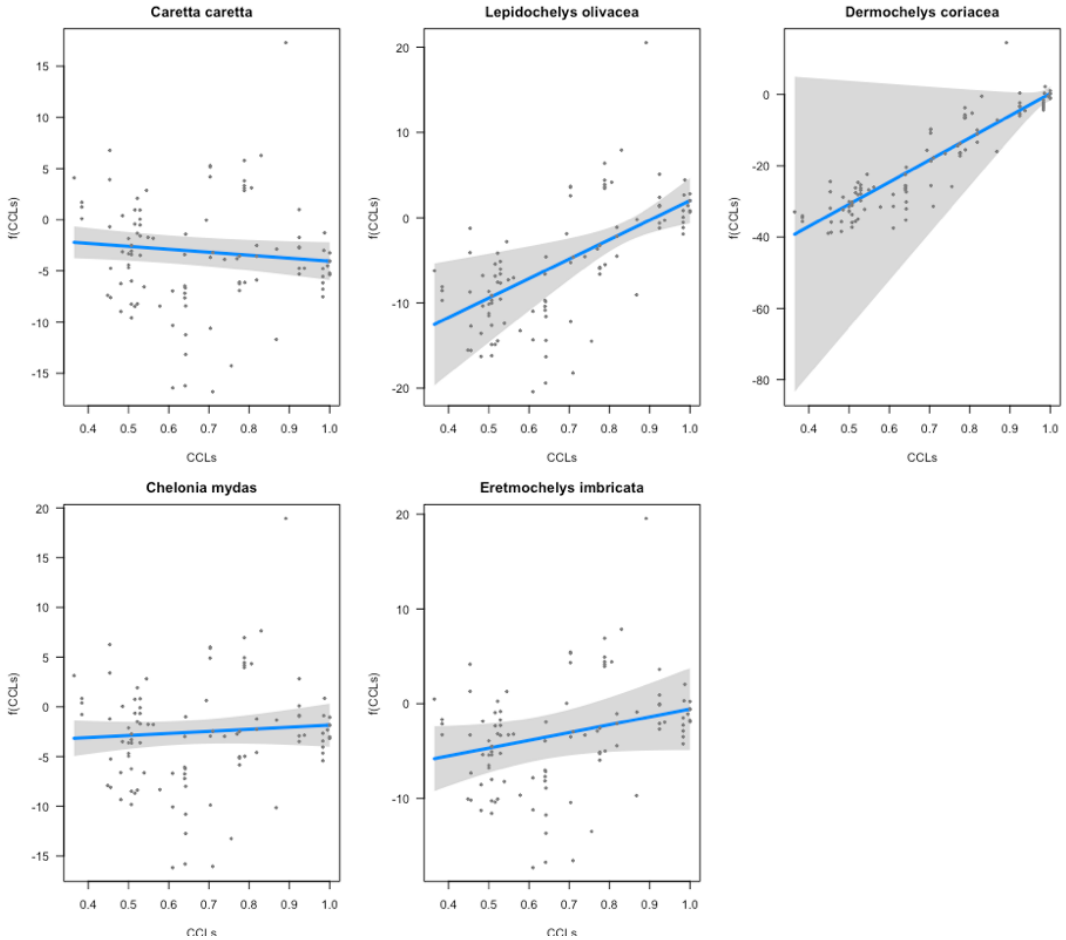
In the Eastern Pacific Ocean, there were 3 publications (with 5 different populations including *L. olivacea, C. caretta and C. mydas*) about Cd, with an important difference with Japan (4 different populations including Loggerhead, Green and Hawksbill turtles) and China (just 1 publication in Green turtles). Sea turtles from Japan had concentrations from 33 to 51 µg g^{-1}ww (Anan et al., 2001; Sakai et al., 2000), and in China (Lam et al., 2004) reported just 0.89 µg g^{-1} in Green turtles. Here it is important to note that Lam et al. (2004) had just 2 individuals in their study, so a larger study is needed to know if this difference is just because the few individuals sampled are not representative or because Chinese nesting turtles are less exposed to Cd than Japanese. *L. olivacea* is the species that seems to accumulate higher concentrations compared with the other species studied (Figure 3). Inorganic element concentration patterns that arise by phylogenetic grouping are likely to reflect the diet, trophic level, and feeding strategies of the species (Hansen et al., 2016). Besides, a recent work has pointed out that the concentration of Cd in some sea basins has increased more than 100 times within a period of 10 years (Schifter et al., 2015). However, Cd concentration in *L. olivacea* is still very high compared with other high trophic species, such as marine mammals (Bilandzic et al., 2012; Caceres-Saez et al., 2013; Polizzi et al., 2013).

In this meta-analysis, kidney is clearly the most favorable tissue for monitoring this metal. According to some authors, this accumulation is primarily due to the binding of this metal with MT, regulatory proteins and detoxicants of some metals, which form Cd-MT complexes and have very limited excretion (Andreani et al., 2008b; García-Fernández et al., 2009; Guirlet and Das, 2012). MT is synthesized in the liver, where it also forms complexes with other metals (such as Zn and Cu) to then either migrate and

accumulate in the kidney (i.e. MT-Cd) or accumulate in the liver (i.e. MT-Cu) (Andreani et al., 2008b; Caceres-Saez et al., 2016; Klaassen et al., 2009; Ruttkay-Nedecky et al., 2013).

On the other hand, Pb had positive relationships with 5 species and their CCLs (Figure 6), meaning that older turtles present higher Pb concentration in kidney (bioaccumulation) in most of the marine turtle species. This relationship of CCL and Pb was more expected in bone (target accumulation tissue) than in kidney, but all the species showed the same tendency, with an especially strong relationship in *L. olivacea*. Mercury concentrations were generally low and similar in all the studies, ranging from 0.02 (*C. mydas* from the Atlantic coast of USA) to 0.46 µg g^{-1} ww (*E. imbricata* from Japan).

Figure 6. Relationships between CCLs and Pb concentration in kidney by species.



Once again, all essential trace elements were found above the detection limit but many of them in low concentrations: Mn from 0.11 to 4.12 (n= 16), Ni from 0.01 to 5.81 (n= 14), As from 0.09 to 11.80 (n=10) and Cr from 0.38 to 4.28 (n=3) all in μg g^{-1} ww. Zinc presented a more important range among the studies, from 2.32 (*L. olivacea* from the Mexican Pacific) to 60.84 μg g^{-1} ww (*C. mydas* from Japan). Any tendency for species or size was found. Zn does not show any particular tropism, as in some studies the highest concentrations were found in liver while in others they were found in kidney (Table 3).

The highest concentrations of Fe in kidney were found in a study from Italy (*C. caretta*), with 191 μg g^{-1} (Andreani et al., 2008b); and the lowest in a study in Turkey *(C. mydas*), with 4.53 μg g^{-1}ww (Kaska et al., 2004); both studies were carried out on juvenile and sub-adult turtles in the Mediterranean Sea. Thus, the species or age (size) of the individuals does not seem to be related to the accumulation of Fe; and so it must be influenced by the bioavailability of this element in the feeding areas of every turtle's population. This Fe bioavailability could be related to anthropogenic pollution but also to natural sources, such as magmatic origins (Nuccio, 2015).

Selenium was detected in a range of 0.88 in *C. mydas* to 10 μg g^{-1} ww in *E. imbricata* (n=10 papers). In reptiles, this element in particular was found to be accumulating in concentrations greatly exceeding established toxicity thresholds for other vertebrates, especially in kidney and liver (Grillitsch and Schiesari, 2010; Hopkins et al., 2005b). Se accumulation has also been found to be influenced by tissue type (especially high in liver and kidney) and sex (higher in females) (Hopkins et al., 2005b). Grillitsch and Schiesari (2010) mention that high concentrations (up to 14.1 μg g^{-1} Se dry weight) can cause reproductive toxicity in fish and birds (10 μg g^{-1} ww is around 29 μg g^{-1} in dw). Despite these high concentrations (compared with other reptiles), in other experiments in freshwater turtles (*T. scripta*), with concentrations as high as 56.74 μg g^{-1} in dw, Dyc et al. (2016) did not find any adverse effects. Thus, we arelead to think that Se toxicity most likely occurred through different pathways in different species of reptiles.

Table 3. Metal and metalloid concentration (mean ± SD, µg g^{-1} wet weight) in kidney of marine turtles from different areas.

Source	Sp	*n*	Country	CCL	Pb	Cd	Hg	As	Cr	Cu	Fe	Mn	Ni	Se	Zn
Gardner *et al.* 2006*	*Lo*	6	Mexico	62.1 b	0.01	20.41	-	-	-	1.65	69.4	1.80	0.54	-	2.32
Frías-Esperic *et al.*, 2006	*Lo*	7	Mexico	80	4.46	5.28	-	-	-	6.40	-	-	-	-	-
Cortés-Gómez *et al.* 2014	*Lo*	13	Mexico	65.5	0.06±0.03	150.9±110.1	-	1.38±0.85	-	1.41±0.68	-	2.70±1.19	0.07±0.04	1.66±0.77	40.61±22.61
Sakai *et al* 2000	*Cc*	7	Japan	88.7 b	0.16±0.05	38.3±17.5	0.27	0.23±0.14	-	1.30±0.25	20.7	1.50±0.51	0.22±0.09	-	25.4±4.39
Saeki *et al.*, 2000*	*Cc*	4	Japan	-	-	-	-	3.40±1.93	-	-	-	-	-	-	-
Kaska *et al.* 2004*	*Cc*	20	Turkey	67.43	1.43±0.76	6.10±3.53	-	6.83±3.68	-	0.75±0.21	5.51±1.39	-	3.46±0.87	2.79±1.28	-
Torrent *et al.*, 2004	*Cc*	78	Islas Canarias	43.5 b,c	2.44±0.48	5.01±1.02	-	13.80±2.40	-	4.60±0.97	-	-	5.81±1.10	-	9.09±0.96
Storelli *et* al., 2005	*Cc*	19	Italy	46.1 b	0.12±0.07	8.35±4.83	0.16±0.07	-	-	1.21±0.54	-	-	-	-	23.1±4.53
Maffucci *et al.*, 2005*	*Cc*	19	Italy	61.1	-	20.59±12.45	0.32±0.25	-	-	0.93±0.25	-	-	-	5.58±3.27	34.9±11.4
Gardner *et al.* 2006*	*Cc*	5	Mexico	61.4 b	0.01	26.31	-	-	-	1.56	85.32	2.16	0.01	-	11.68
García-Fdz et al., 2009	*Cc*	19	Spain	-	0.17±0.16	10.5±23.6	-	-	-	1.26±1.17	-	-	-	-	9.29±8.92
Jerez *et al.* 2010*	*Cc*	7	Spain	48.7 b	0.41±0.29	2.33 ±2.12	0.16±0.18	11.80±10.81	-	-	-	-	-	1.90±1.96	12.46±4.14
Abdallah et al., 2011*	*Cc*	8	Egypt (Mediter)	75.6	2.34±0.76	15.29±4.14	0.31±0.12	0.09±0.01	-	-	-	-	-	-	-
Nicolau *et al.*, 2017	*Cc*	38	Portugal	50.1	0.08±0.03	1.86±0.37	0.34±0.22	5.71±0.49	-	4.72±0.24	-	2.09±0.13	0.06±0.01	5.46±1.23	30.5±1.49
Andreani *et al.*, 2(	*Cc*	9	Italy	58.5 c	0.003±0.02	1.97±0.47	-	-	-	1.89±0.32	191±40	2.38±0.44	-	-	40.46±2.72
	Cm	33	Costa Rica	101 c	0.02±0.003	13.32±1.05	-	-	-	2.83±0.59	75±6.25	1.95±0.09	-	-	26.31±0.64
Sakai *et al.*, 2000	*Cm*	2	Japan	99.8 b	0.07	41.2	0.04	-	-	1.51	13.9	17.17	0.51	-	34
Saeki *et al.*, 2000*	*Cm*	19	Japan	-	-	-	-	2.05±1.07	-	-	-	-	-	-	-
Lam *et al.* 2004*	*Cm*	2	China	-	0.11±0.06	0.89±0.62	0.12±0.01	2.50±0.02	0.38±0.14	5.47±2.59		4.12±1.72	0.05±0.04	2.12±0.45	51.51±4.47

Gardner *et al.* 2006*	*Cm*	11	Mexico	65.2 b	0.003	43.56	-	-	-	2.04	15.87	0.11	0.41	-	46.08
Kampalath *et al*., 2006	*Cm*	10	Mexico	57.3	-	-	0.089	-	-	-	-	-	-	-	-
Talavera *et al*.2007	*Cm*	8	Mexico	65.1 b,c	0.02	37.4	-	-	-	1.98	-	0.51	1.08	-	64.26
Barbieri, 2009*	*Cm*	15	Brazil	-	-	0.78±0.09	-	-	-	4.93±0.41	-	1.50±0.30	0.06±0.01	-	-
Da Silva *et al.* 2014*	*Cm*	29	Brazil	39	1.94±0.14	10.18±0.82	-	-	-	4.39±0.39	-	-	-	-	19.54±1.47
Faust *et al*., 2014	*Cm*	12	USA (Atlantic)	52.4 b	0.21±0.11	3.97±0.61	0.023±0.003	0.20±0.08	4.28±0.18	3.24±1.11	-	1.21±0.17	0.06±0.007	0.88±0.08	27.9±5.70
Kaska *et al.* 2004*	*Cm*	14	Turkey	53.6	0.70±0.54	5.69±2.32	-	4.95±1.95	-	0.63±0.24	4.53±1.19	-	3.74±1.02	2.81±1.11	-
Anan *et al*., 2001*	*Cm*	8	Japan	53.7 b	0.29±0.19	51.12±23	0.10±0.05	-	0.79±0.25	2.97±1.46	-	2.01±0.49	-	1.9±0.86	60.84±21.96
	Ei	6	Japan	43.4 b	0.09±0.08	33.7±27.4	0.46±0.43	-	0.57±0.28	2.53±1.0	-	4.75±0.99	-	10±6.8	43.2±11.5
Saeki *et al*., 2000*	*Ei*	4	Japan	-	-	-	-	10.2±3.53	-	-	-	-	-	-	-

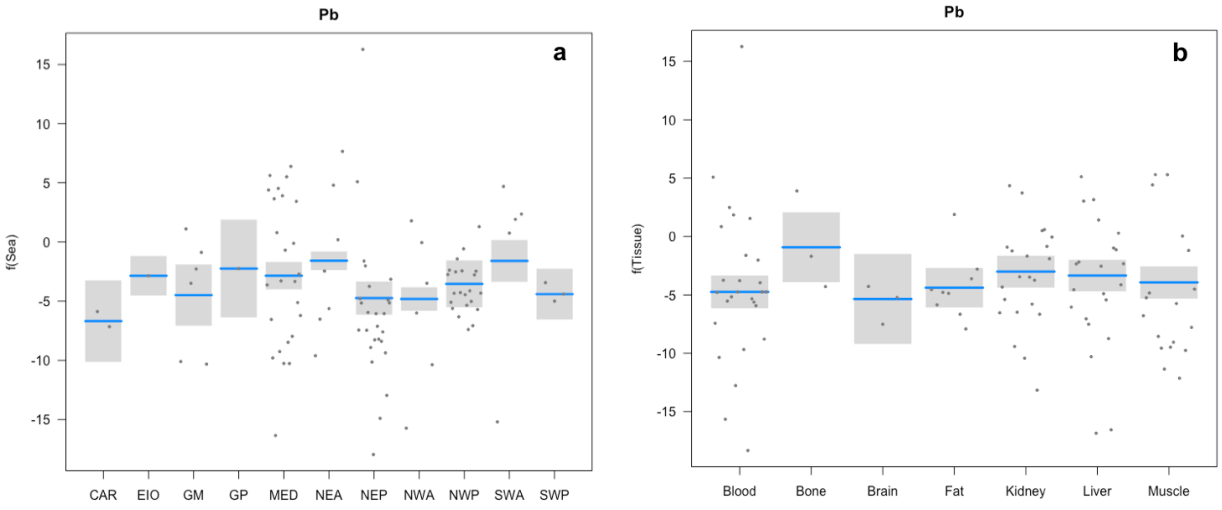
*Results originally published in dry weight transformed into wet weight using the humidity percentage reported by the authors, in case any percentage was reported by the authors, we used 64% of humidity reported by Garcia-Fernandez et al. (2009). BDL = below the detection limit; *Lo*= *Lepidochelys olivacea; Cc*= *Caretta caretta; Cm=Chelonia mydas*; *Ei*= *Eretmochelys imbricata*. **a =** median; **b=** Original publication in straight carapace long (SCL), transform into CCL using the following formulas: *Lo*= (SCL - 9.244) / 0.818 (Whiting et al., 2007); *Cc* = 1.388 + 1.053 (SCL) (Bjorndal et al., 2000); *Cm* = -0.028+1.051 (SCL) (Bjorndal et al., 1989) and *Ei* = (SCL - 0,449) / 0,935 (Wabnitz et al., 2008); **c=** range transform into mean with the following formula: ((max-min)/2+min).

1.2.3.4.4 Muscle

Seventeen publications (on 23 different populations) for this tissue were included in this review (Table 4). Cd was the most analyzed element (n=20), followed by Pb (n=18), Cu and Zn (n=17), Hg (n=12), Ni and Mn (n=11), Fe (n=9), Se (n=8) and finally, As and Cr with 6 studies. In general, concentrations of these elements in muscle were low. Hg concentrations in this tissue were generally low, from 0.008 in *C. mydas* and *E. imbricata* to 0.18 µg g^{-1}ww in *C. caretta*. In general, *C. caretta* and *E. imbricata* had very high concentrations of this metal (Figure 5). Moreover, the same tendency was found when we analyzed the concentration for sea basin and species (Figure 5). As we mentioned previously, patterns that arise through phylogenetic grouping are likely to reflect the diet, trophic level, and feeding strategies of the species (Hansen et al., 2016).

Cadmium and Pb also had low concentrations in this tissue; Frias-Espericueta et al. (2006) reported one of the highest Cd and Pb concentrations (2.6 and 1.78 µg g^{-1} ww respectively) in *L. olivacea* from Mexico. Torrent et al. (2004) Abdallah and Abd-Allah (2011) had the highest report for Pb with 2.26 (Canary Islands) and 2.24 (Mediterranean) µg g^{-1} ww respectively, both in *C. caretta*. In the Figure 7a the NEA area (Canary Islands included) has a slight upward trend compared with other RMUs.

Figure 7. Pb concentration by geographic zone (a) and tissue accumulation (b).



Zinc was the element that presented the highest concentrations, for all the species, ranging between 0.25 and 30 µg g^{-1} ww. The highest concentrations of this element were found in the Mediterranean Sea in *C. caretta* (Andreani et al., 2008b; Franzellitti et al., 2004; Jerez et al., 2010b; Storelli et al., 2005) and in the Northwestern Pacific in *C. mydas* (Anan et al., 2001; Lam et al., 2004; Sakai et al., 2000). On the other hand, the lowest concentrations were found in the Atlantic and Western Pacific, especially in *L. olivacea* from the Mexican Pacific (Frias-Espericueta et al., 2006; Gardner et al., 2006).

In this tissue As showed the highest concentrations, especially remarkable was *E. imbricata* from Japan with 30.06±13.1 µg g^{-1} ww (Saeki et al., 2000), followed by *C. caretta* from Portugal with 14.7±1.47 µg g^{-1} ww (Nicolau et al., 2017); the rest of the works were between 2.89 (Lam et al., 2004) and 7.35 µg g^{-1}ww (Torrent et al., 2004). Generally, in marine food webs, arsenic is bioconcentrated but not biomagnified, and the accumulations are larger in lower species (Saeki et al., 2000). Thus, it is interesting that relatively higher animals, like Hawksbill and Loggerhead turtles, have higher arsenic concentration than lower species. It is important also to point out that the number of individuals sampled by Saeki et al. (2000) was very low (n=4), and that Hawksbills from that region have not been studied again regarding this element. However, the publication of Nicolau et al. (2017) is recent and with 38 individuals. This being said, we consider that this large accumulation of As in these specific works may be due to these turtles feeding on prey containing a very high amount of this element.

Like in liver, Fe showed a very important range in its concentrations, with a range between 4.12 (Kaska et al., 2004) and 60.9 µg g^{-1} ww (Franzellitti et al., 2004). Living organisms are protected from Fe toxic effects by two mechanisms: 1) proteins sequester iron; or 2) Fe binds to plasma transferrin, and accumulates within cells in the form of ferritin; to prevent an excess of storage, the absorption of Fe is finely regulated (Andreani et al., 2008b; Emerit et al., 2001). Eighty percent of the body's Fe content is incorporated into hemoglobin in developing erythroid precursors and mature red cells. Ten to fifteen percent is present in muscle fibers (myoglobine) and in other tissues (cytochromes and enzymes) (Emerit et al., 2001).

1.2.3.4.5 Fat

Although it may seem unusual to focus on fat as a matrix for elemental analysis, many inorganic elements bind to proteins which make them soluble in lipids (i.e. Me-Hg). Important concentrations in this tissue for some of these elements were reported in eight papers and in 12 different turtle populations (Table 5). The most analyzed element was Cd (n=9), followed by Pb, Cu, Mg and Zn (n=8), Hg, Fe and Ni (n=7), As (n=2) and finally Cr and Se with only one report. Pb, Cd, Se, Cu, As and Cr presented very low concentrations in most places and species in this tissue. Unlike marine mammals, sea turtles present low Hg concentrations (Bilandzic et al., 2012; Hansen et al., 2016; Lailson-Brito et al., 2012; Lavery et al., 2009). These findings are surprising but they could be easily linked to the low concentrations that all turtles present in general, making it seem that marine turtles are less exposed to Hg than marine mammals. This low body burden of Hg, especially in species occupying a high trophic level (e.g. *C. caretta, L. olivacea, L. kempii*), has been plausibly explained by other authors, and it is mainly attributed to the nature of their diet. Basically, all carnivorous sea turtle species feed upon benthic mollusks, crustaceans, small fish, sponges or jellyfish; these prey organisms are in low trophic levels and thus, less exposed to mercury contamination (Godley et al., 1999b; Storelli et al., 2005). This should account for the low bioaccumulation, but especially the biomagnification of Hg in marine turtles.

1.2.3.4.6 Brain

Only three publications (4 different populations) about metals in brain from two species (*C. caretta* and *C. mydas*) were found in this review (Table 6). The number of publications and samples (from 2 to 7) for this tissue was reduced due to 1) the difficulty of opening the skull in the field, and 2) the fast liquefaction of this organ after death. Two loggerheads from Spain, one from Japan and one *C. mydas* also from Japan were included in these studies (García-Fernández et al., 2009; Jerez et al., 2010b; Sakai et al., 2000). From the 10 elements analyzed, only Pb, Cd and Zn were studied in the 4 populations. Concerning Pb, both populations from Japan (Sakai et al., 2000) were under the detection limit (<0.03 μg g^{-1} ww) and turtles from the Mediterranean showed low

levels (García-Fernández et al., 2009; Jerez et al., 2010b). This should be due to the brain not being a target organ in Pb accumulation.

Mercury was analyzed in 3 populations; all concentrations were low, 0.002 μg g^{-1} ww in *C. mydas* and 0.007 μg g^{-1} ww in *C. caretta,* both from Japan; and 0.03 μg g^{-1} ww in *C. caretta* from the Mediterranean. This element is one of the most widely studied non-essential elements worldwide regarding human risk, but the information existing in marine turtles is very limited (Table 6 and Figure 7). It is well known that brain is highly susceptible to environmental Hg exposure; this is due to organic-Hg (Me-Hg; a highly neurotoxic form) crosses the blood-brain barrier mediated by molecular mimicry (Lohren et al., 2015). Naturally, Hg is present in trace levels in the ocean, but human activity has dramatically altered the global mercury cycle, resulting in loadings to the ocean that have increased, by at least a factor of three from natural sources (Lamborg et al., 2014). To our knowledge, there are no studies about the accumulation or effects of Hg in turtles' brain, but in fish and mammals it has been found that the brain has an important role in Hg magnification processes, and that more than 77% of the Hg found in those brains was MeHg (Mieiro et al., 2009; Pereira et al., 2013). These authors have also documented that the brain is highly susceptible to environmental Hg exposure, reporting neurotoxic damage. Despite the low concentrations of Hg found in all tissues, a significant relationship was found for all the tissues in all the species for CCLs and Hg (Figure 4), meaning that older turtles present higher Hg concentrations (bioaccumulation). Essentially, all these low concentrations of Hg, in all tissues and in all species, are consistent with the explanation given in the fat section regarding their feeding habits based on low trophic level organisms (Storelli et al., 2005). Thus, Hg does not appear to be an element of high concern for turtles so far. However, it is important to keep in mind the significant lack of information regarding this metal in many species and in different areas worldwide.

Table 4. Metal and metalloids concentration (mean ± SD, µg g^{-1} wet weight) in muscle of marine turtles from different areas.

Fuente	Sp	*n*	Country	CCL	Pb	Cd	Hg	As	Cr	Cu	Fe	Mn	Ni	Se	Zn
Gardner *et al.* 2006*	*Lo*	6	Mexico	62.1 b	BLD	0.09	-	-	-	0.25	18.61	0.15	0.002	-	0.25
Frías-Espericue *et al.*, 2006	*Lo*	7	México	80	1.78	2.6	-	-	-	-	-	-	-	-	3.10
Kampalath *et al*., 2006	*Lo*	6	Mexico	59.2	-	-	0.050	-	-	-	-	-	-	-	-
Sakai *et al.*, 2000	*Cc*	7	Japan	88.7 b	0.02±0.03	0.06±0.02	0.09±0.03	-	-	0.81±0.27	19.8±8.71	0.28±0.11	0.08±0.02	-	25.0±3.49
Saeki *et al*., 2000*	*Cc*	4	Japan	-	-	-	-	4.12±2.62	-	-	-	-	-	-	-
Kaska *et al.* 2004*	*Cc*	32	Turkey	67.4	0.48±0.65	0.71±0.68	-	4.16±1.99	0.30±0.15	0.31±0.16	4.12±0.51	-	2.04±0.74	1.41±0.68	-
Torrent *et al.*, 2004	*Cc*	78	Canarias Islands	43.5 b,c	2.26±0.51	1.14±0.28	-	7.35±1.37	-	2.85±0.52	-	-	1.74±0.29	-	6.70±0.96
Franzellitti *et al*., 2004	*Cc*	17	Italy	55.4 b	-	0.36±0.11	-	-	-	1.5±0.4	60.9±38.3	2.7±1.2	2.76±0.60	-	30.9±8.0
Storelli *et al.* 2005	*Cc*	19	Italy	46.1 b	-	0.07±0.03	0.18±0.21	-	-	0.59±0.41	-	-	-	1.65±0.58	27.9±4.85
Maffucci *et al*., 2005*	*Cc*	26	Italy	61.1	-	0.04±0.04	0.08±0.06	-	-	0.54±0.28	-	-	-	2.24±0.98	21.4±5.2
Gardner *et al.* 2006*	*Cc*	5	Mexico	61.4 b	0.002	0.02	-	-	-	0.08	4.19	0.16	0.002	-	6.22
Kampalath *et al*., 2006	*Cc*	4	Mexico	59.8	-	-	0.026	-	-	-	-	-	-	-	-
García-Fdz *et al*., 2009	*Cc*	20	Spain	-	0.05±0.05	0.04±0.03	-	-	-	1.01±0.39	-	-	-	-	13.08±5.66
Jerez *et al.* 2010 *	*Cc*	13	Spain	48.7 b	0.04±0.05	0.02±0.01	0.03±0.04	-	-	-	-	-	-	0.65±0.33	22.65±53.4
Andreani *et al*., 2008*	*Cc*	10	Italy	67.5 c	BLD	0.16±0.01	-	-	-	0.48±0.05	17.08±1.98	0.27±0.05	-	-	21±2.8
Abdallah et al., 2011*	*Cc*	8	Egypt (Mediter)	75.6	2.24±0.42	0.09±0.01	0.09±0.01	0.05±0.01	-	-	-	-	-	-	-
Nicolau *et al*., 2017	*Cc*	38	Portugal	50.1	0.01±0.00	0.16±0.01	0.05±0.01	14.7±1.47	-	0.55±0.04	-	0.14±0.01	0.08±0.03	2.34±0.09	19.79±0.82
Novillo *et al.*, 2017	*Cc*	25	Spain	43.7	0.08±0.03	0.05±0.03	0.04±0.02	-	-	-	-	-	-	-	-
Sakai *et al.*, 2000	*Cm*	2	Japan	99.8 b	<0.03	0.02	0.0028	-	-	0.25	11.3	0.26	0.05	-	9.67

Saeki *et al*., 2000*	*Cm*	19	Japan	-	-	-	-	4.82±2.62						
Lam *et al*. 2004*	*Cm*	2	China	-	0.02±0.02	BLD-0.02 f	0.08±0.04	2.89±0.97	0.54±0.57	0.74±0.33	-	0.25±0.10	0.40±0.40	0.89±0.21	29.54±2.52
Gardner *et al.* 2006*	*Cm*	11	México	65.2 b	0.002	0.002	-	-	-	0.006	4.19	0.0006	0.006	-	7.65
Kampalath *et al*., 2006	*Cm*	10	Mexico	57.3	-	-	0.072	-	-	-	-	-	-	-	-
Da Silva *et al.* 2014*	*Cm*	29	Brazil	39	0.84±0.06	0.14±0.02	-	-	-	0.24±0.04	-	-	-	-	3.32±0.26
Faust *et al*., 2014	*Cm*	12	USA (Atlantic)	52.4 b	BLD	BLD	0.012	6.86±1.07	4.80±0.12	0.65±0.09	-	0.24±0.04	BLD	0.86±0.08	16±2.2
Kaska *et al*. 2004*	*Cm*	22	Turkey	53.6	0.28±0.06	0.29±0.09	-	3.09±1.38	0.44±0.34	0.42±0.17	3.49±0.94	-	1.94±0.75	1.26±0.43	-
Anan *et al*., 2001*	*Cm*	8	Japan	54.7 b	0.02±0.01	0.04±0.03	0.008±0.01	-	0.28±0.04	0.17±0.08	-	0.09±0.02	-	0.64±0.2	9.54±3.72
	Ei	6	Japan	43.4 b	0.008±0.01	0.01±0.007	0.008±0.006	-	0.22±0.22	0.19±0.06	-	0.13±0.10	-	2.2±2	9.72±5.22
Saeki *et al*., 2000*	*Ei*	4	Japan	-	-	-	-	30.6±13.1	-	-	-	-	-	-	-

*Results originally published in dry weight transformed into wet weight using the humidity percentage reported by the authors, in case any percentage was reported by the authors, we used 80% of humidity reported by Garcia-Fernandez et al. (2009). BDL = below the detection limit; *Lo*= *Lepidochelys olivacea; Cc*= *Caretta caretta; Cm*=*Chelonia mydas*; *Ei*= *Eretmochelys imbricata*. **a =** median; **b=** Original publication in straight carapace long (SCL), transform into CCL using the following formulas: *Lo*= (SCL - 9.244) / 0.818 (Whiting et al., 2007); *Cc* = 1.388 + 1.053 (SCL) (Bjorndal et al., 2000); *Cm* = -0.028+1.051 (SCL) (Bjorndal et al., 1989) and *Ei* = (SCL - 0,449) / 0,935 (Wabnitz et al., 2008); **c=** range transform into mean with the following formula: ((max-min)/2+min).

Table 5. Metal and metalloids concentration (mean ± SD, µg g^{-1} wet weight) in fat of marine turtles from different areas.

Fuente	Sp	*n*	Country	CCL	Pb	Cd	Hg	As	Cr	Cu	Fe	Mn	Ni	Se	Zn
Gardner *et al.* 2006*	*Lo*	6	Mexico	62.1 b	BLD	0.69	-	-	-	0.83	27.91	2.10	0.03	-	3.70
Kampalath *et al*., 2006	*Lo*	6	Mexico	59.2	-	-	0.180	-	-	-	-	-	-	-	-
Sakai *et al.*, 2000	*Cc*	7	Japan	88.7 b	<0.03	0.06±0.03	0.005±0.003	-	-	0.11±0.03	9.92±5.61	0.12±0.09	<0.03	.	96.1±18.8
Franzellitti *et al*., 2004	*Cc*	7	Italy	55.4 b	-	2.33±0.52	-	-	-	3.4±1.7	132.3±121.0	8.4±2.5	18.77±4.40	-	68.2±34.7
Gardner *et al.* 2006*	*Cc*	5	Mexico	61.4 b	BLD	0.50	-	-	-	0.69	1.33	1.82	0.17	-	12.66
Kampalath *et al*., 2006	*Cc*	5	Mexico	59,8	-	-	0.039	-	-	-	-	-	-	-	-
Andreani *et al*., 2008*	*Cc*	28	Italy	67.5 c	0.063±0.031	0.113±0.02	-	-	-	0.446±0.087	45.2±6.1	0.826±0.13	-	-	62.1±8.4
Abdallah et al., 2011*	*Cc*	8	Egypt (Mediter)	75.6	0.50±0.10	0.09±0.02	0.01±0.002	0.06±0.003							
Novillo *et al.*, 2017	*Cc*	25	Spain	43.7	0.09±0.05	0.04±0.02	0.03±0.02	-	-	-	-	-	-	-	-
Sakai *et al.*, 2000	*Cm*	2	Japan	99.8 b	<0.03	0.06	0.0026	-	-	0.33	18.91	0.2	0.06	-	51.3
Lam *et al.* 2004*	*Cm*	2	China	-	0.08±0.026	BLD	0.005±0.003	1.27±0.199	0.90±0.239	0.99±0.383	-	0.19±0.057	0.15±0.047	0.72±0.114	105±17.48
Gardner *et al.* 2006*	*Cm*	11	Mexico	65.2 b	0.03	0.002	-	-	-	0.01	2.63	0.003	0.02	-	49.82
Kampalath *et al*., 2006	*Cm*	6	Mexico	57.3	-	-	0.018	-	-	-	-	-	-	-	-

*Results originally published in dry weight transformed into wet weight using the humidity percentage reported by the authors, in case any percentage was reported by the authors, we used 75% of humidity reported by Lam et al. (2004). BDL = below the detection limit; *Lo= Lepidochelys olivacea; Cc= Caretta caretta; Cm=Chelonia mydas*. **a =** median; **b=** Original publication in straight carapace long (SCL), transform into CCL using the following formulas: *Lo*= (SCL - 9.244) / 0.818 (Whiting et al., 2007); *Cc* = 1.388 + 1.053 (SCL) (Bjorndal et al., 2000) and *Cm* = -0.028+1.051 (SCL) (Bjorndal et al., 1989); **c=** range transform into mean with the following formula: ((max-min)/2+min).

Table 6. Metal and metalloid concentration (mean ± SD, µg g^{-1} wet weight) in brain of marine turtles from different areas.

Source	Sp	*n*	Country	CCL	Pb	Cd	Hg	As	Cu	Fe	Mn	Ni	Se	Zn
Sakai *et al.*, 2000	*Cc*	7	Japan	88.7b	<0.03	2.27±0.06	0.007±0.002	-	2.05±1.11	21.2±12.9	0.35±0.14	0.050	-	8.78±1.72
	Cm	2	Japan	99.8b	<0.03	0.13	0.0028	-	0.84	12.05	0.47	0.12	-	8.78
García-Fernández et al., 2009	*Cc*	3	Spain	-	0.17±0.12	0.06±0.06	-	-	0.86±0.22	-	-	-	-	2.76±2.81
Jerez *et al.* 2010 *	*Cc*	3	Spain	48.7b	0.05±0.05	0.03±0.002	0.03±0.03	9.26±3.23	-	-	-	-	1.05±0.1	18.29±6.05

*Results originally published in dry weight transformed into wet weight using 75% of humidity reported by Garcia-Fernandez et al. (2009); BDL = below the detection limit; *Cc*= *Caretta caretta; Cm=Chelonia mydas*; **b=** Original publication in straight carapace long (SCL), transform into CCL using the following formulas: *Cc* = 1.388 + 1.053 (SCL) (Bjorndal et al., 2000); *Cm* = -0.028+1.051 (SCL) (Bjorndal et al., 1989).

Table 7. Metal and metalloid concentration (mean ± SD, µg g^{-1} wet weight) in bone of marine turtles from different areas.

Source	Species	*n*	Country	CCL	Pb	Cd	Hg	As	Cu	Fe	Mn	Ni	Se	Zn
Sakai *et al.*, 2000	*Cc*	7	Japan	88.7b	3.63±1.62	0.13±0.03	0.007±0.002	-	0.20±0.05	6.70±1.12	10.8±4.96	0.14±0.04	-	197±26.5
	Cm	2	Japan	99.8b	2.35	0.03	0.002	-	0.25	5.99	6.56	0.05	-	176.5
García-Fernández et al., 2009	*Cc*	3	Spain		0.99±1.40	BLD	-	-	BLD	-	-	-	-	15.34±21.55
Jerez *et al.* 2010 *	*Cc*	12	Spain	48.7b	1.48±0.67	0.04±0.02	0.04±0.05	1.17±0.51	-	-	-	-	0.24±0.11	115.6±55.2

*Results originally published in dry weight transformed into wet weight using 20% of humidity reported by Garcia-Fernandez et al. (2009); BDL = below the detection limit; *Cc*= *Caretta caretta; Cm=Chelonia mydas*; **b=** Original publication in straight carapace long (SCL), transform into CCL using the following formulas: *Cc* = 1.388 + 1.053 (SCL) (Bjorndal et al., 2000) and *Cm* = -0.028+1.051 (SCL) (Bjorndal et al., 1989).

Selenium and arsenic were published in only one study; Fe, Mn and Ni in 2 papers and Cu in 3 different populations (Table 6). The concentration of these elements was variable, and the means of Mn, Ni and Cu were very low. Zn concentrations were between 2.76 (García-Fernández et al., 2009) and 18.29 µg g^{-1}ww (Jerez et al., 2010b), both reported in the Mediterranean Sea in *C. caretta*. Arsenic was reported with a high concentration compared with other tissues analyzed, with a mean of 9.26 µg g^{-1}. Finally, Fe in this organ was analyzed only in the paper of Sakai et al. (2000) from Japan, with a maximum concentration of 21.2 µg g^{-1}ww in *C. caretta*.

1.2.3.4.7 Bone

As well as in brain, 3 papers about bone with 4 different populations were found in this review (Table 7). In comparison with other tissues summarized here, Cd concentrations were low (from <0.01 to 0.13 µg g^{-1} ww). On the other hand, Pb showed the highest concentration, with a minimum of 0.99 µg g^{-1}ww (García-Fernández et al., 2009) in the Mediterranean, and a maximum of 3.63 µg g^{-1}(Sakai et al., 2000) in the Pacific Japanese, both in *C. caretta*. However, after the meta-analysis, it was observed that the distribution of this metal was very similar at a global scale, except for the Caribbean zone, which had the lowest concentrations (Figure 7a). It has also been found that tropical waters present higher Pb concentrations than Northern (and colder) waters; and pelagic waters have higher Pb concentrations than deeper zones (Echegoyen et al., 2014). All turtles spend time in pelagic waters since they need to breath regularly, and many populations nest in Tropical zones. Hence, marine turtles are very susceptible species for this metal. Finally, Pb has a clear tendency to accumulate in bone tissue (Figure 7b), a known target organ in different species, such as humans (ATSDR, 2007b; Pemmer et al., 2013) and marine mammals (Caurant et al., 2006; Lavery et al., 2009; Vighi et al., 2016). This tendency is explained due to the kinetics of Pb following those of Ca in bones, so Pb storage is related to calcitropic factors (Guirlet et al., 2008).

Similarly to brain, the values of Hg in the Spanish Mediterranean (Jerez et al., 2010b) were higher than in the Japanese Pacific (Sakai et al., 2000) , both in *C. caretta,* with 0.04±0.05 and 0.007±0.002 μg g^{-1} ww respectively. However, loggerheads from Japan reported the highest concentrations of Zn, Mn and Fe. Zn was the element with the highest concentrations (ranging from 15.43 to 197 μg g^{-1}). Ni was the element with the lowest values in this tissue.

1.2.4 Chemical analytical techniques

For the analysis of Pb, Cd, Mn, Cu, Zn, Ni, As, Cr and Fe, 6 techniques were used in the 58 papers included in this review. The Atomic Absorption Spectrophotometer (AAS) was the most commonly used, with 3 different variations: flame, graphite and air-acetylene flame (n=30, 50%), followed by the Inductively Coupled Plasma Mass Spectrophotometer (ICP-MS) (n=20; 34%), the Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES) and the Inductively Coupled Plasma Atomic Emission Spectrophotometer (ICP-AES) (both with n=4; 7%). Finally, the Anodic Stripping Voltammetry (ASV) and the particle induced X-ray emission analysis (PIXE) were used only in one published work.

Mercury was determined in a total of 28 papers (in the 7 tissues included in this review). The AAS technique (cold vapor and hybrid generation) was the most used (n=15; 50%). Cold vapor ICP-MS was the second most used in 5 papers (18%); cold-vapor atomic fluorescence spectrometry (CV-AFS) was used in 4 papers (14%). All the other studies used different techniques: the ultrasound nebulizer ICP-OES, the hybrid generation ICP-AES, the cold vapor UV spectrophotometer, the cold vapor atomic absorption mercury analyzer and the direct mercury analyzer.

The preference for one technique over another seems to be largely due to the availability of the equipment. At the same time, because through the years more elements can be assessed with smaller sample quantities, researchers take into account the optimization of

the information and so the number of elements that can be quantified is crucial in selecting the technique. Thus, the spectrophotometer is the most chosen method. On the other hand, some researchers still make reference to the sensibility of the equipment, preferring a technique that might take longer and be able to determine a smaller number of elements, but that has a more sensitive detection limit, for instance the use of the Voltammetry technique (García-Fernández et al., 2009). This last method may be used due either to 1) the cost or 2) the ease of which one can obtain element speciation data relative to the other methods (Buffle and Tercier-Waeber, 2005).

1.2.5 Conclusions

Among non-essential metals, Cd was found in all reported tissues for the investigated species in different parts of the world. The highest concentrations were found in kidney, in *L. olivacea* and *C. mydas* from the Western Pacific, which suggests that this area was highly contaminated for this metal, at least in the moment of the sampling. Pb shows a general decreasing concentration through time in different tissues. As expected, bone had the highest Pb concentrations as it can accumulate for several years in this tissue. In general, Hg presented low concentrations. Although highly toxic, Hg was one of the least reported elements. This may be attributed to the specific analytical requirements for its determination. Among trace essential elements, all of them were found in widely varying concentrations; especially Zn and Cu. The meta-analysis showed some features of contamination: Leatherbacks (*Dermochelys coriacea*) show a distinct pattern of contamination as compared to other species, possibly as a consequence of its pelagic habitat and jellyfish diet. Liver and kidney on one side and bone on the other show a very specific pattern of contaminants. No clear oceanic basin effect is observed, which is not a very good sign for the state of the planet: contamination is ubiquitous. Finally, we encourage all authors to report the moisture percentage in their samples, this simple action may improve the databases information.

References

Abdallah, M.A.M., Abd-Allah, M.A.M., 2011. Bioaccumulation of toxic metals in Loggerhead turtles from Mediterranean Sea coast, Egypt, in: Özhan, E. (Ed.), 10th International Conference on the Mediterranean Coastal, Environment. MEDCOAST 11, Rhodes, Greece, pp. 570-579.

Aguirre, A.A., Lutz, P.L., 2004. Marine turtles as sentinels of ecosystem health: Is fibropapillomatosis an indicator? EcoHealth 1, 275-283.

Alava, J.J., Keller, J.M., Kucklick, J.R., Wyneken, J., Crowder, L., Scott, G.I., 2006. Loggerhead sea turtle (*Caretta caretta*) egg yolk concentrations of persistent organic pollutants and lipid increase during the last stage of embryonic development. Science of the Total Environment 367, 170-181.

Anan, Y., Kunito, T., Watanabe, I., Sakai, H., Tanabe, S., 2001. Trace element eccumulation in Hawksbill turtles (*Eretmochelys imbricata*) and Green Turtles (*Chelonia mydas*) from Yaeyama Islands, Japan. Environmental Toxicology and Chemistry 20, 2802-2814.

Ancora, S., Casini, S., Bianchi, N., Fanello, E., Fossi, M.C., Leonzio, C., 2012. Non lethal samples for trace elements and porphyrins investigation, Mediterranean *C. caretta*. Workshop of biology and ecotoxicology of large marine vertebrates: potential sentinels of good environmental status of marine environment, implication on European Marine Strategy Framework Directive., Siena, Italy, p. 38.

Andreani, G., Santoro, M., Cottignoli, S., Fabbri, M., Carpene, E., Isani, G., 2008. Metal distribution and metallothionein in Loggerhead (*Caretta caretta*) and Green (*Chelonia mydas)* sea turtles. Science of the Total Environment 390, 287-294.

Appenroth, K.-J., 2010. Definition of "heavy metals" and their role in biological systems, Soil heavy metals. Springer, pp. 19-29.

ATSDR, 2007. Toxicological profile for lead. U.S. Departament of Health and Human Services. Agency for Toxic Substances and Disease Registry, Atlanta, Georgia.

Audigier, V., Husson, F., Josse, J., 2016. A principal component method to impute missing values for mixed data. Advances in Data Analysis and Classification 10, 5-26.

Barbieri, E., 2009. Concentration of heavy metals in tissues of Green turtles (*Chelonia mydas*) sampled in the Cananéia Estuary, Brazil. Brazilian Journal of Oceanography 57, 243-248.

Becue-Bertaut, M., Pagès, J., 2008. Multiple factor analysis and clustering of a mixture of quantitative, categorical and frequency data. Computational Statistics and Data Analysis 52, 3255-3268.

Benedicto, J., Campillo, J.A., Fernadez, B., Martinez-Gomez, C., Leon, V.M., 2012. Estrategias marinas: Evaluacion inicial, buen estado ambiental y objetivos ambientales, Estrategia Marina. Demarcacion marina levantino-balear, Madrid, p. 110.

Bilandzic, N., Sedak, M., Ethokic, M., Ethuras Gomercic, M., Gomercic, T., Zadravec, M., Benic, M., Prevendar Crnic, A., 2012. Toxic element concentrations in the bottlenose (*Tursiops truncatus*), striped (*Stenella coeruleoalba*) and Risso's (*Grampus griseus*) dolphins stranded in eastern Adriatic Sea. Bulletin of Environmental Contamination and Toxicology 89, 467-473.

Bjorndal, K.A., Bolten, A.B., 1989. Comparison of straight-line and over-the-curve measurements for growth rates of green turtles, *Chelonia mydas*. Bull. Mar. Sci. (1.333) 45, 189-192.
Bjorndal, K.A., Bolten, A.B., Martins, H.R., 2000. Somatic growth model of juvenile loggerhead sea turtles *Caretta caretta*: duration of pelagic stage. Marine Ecology-Progress Series 202, 265-272.
Bjorndal, K.A., Carr, A., 1989. Variation in clutch size and egg size in the green turtle nesting population at Tortuguero, Costa Rica. Herpetologica 45, 181-189.
Bondía, S., Ribas, B., De la Torre, A., Ruiz, A.S., 2013. Zinc and cadmium metalloprotein induced by cadmium administration, Nutrition, Digestion, Metabolism: Proceedings of the 28th International Congress of Physiological Sciences, Budapest, 1980. Elsevier, p. 71.
Buffle, J., Tercier-Waeber, M.L., 2005. Voltammetric environmental trace-metal analysis and speciation: from laboratory to in situ measurements. TrAC Trends in Analytical Chemistry 24, 172-191.
Burger, J., 2008. Assessment and management of risk to wildlife from cadmium. Science of the Total Environment 389, 37-45.
Burnham, K.P., Anderson, D.R., 2002. Model selection and multimodel inference: A practical information-theoretic approach. Springer-Verlag, New York.
Caceres-Saez, I., Polizzi, P., Romero, B., Dellabianca, N.A., Ribeiro Guevara, S., Goodall, R.N., Cappozzo, H.L., Gerpe, M., 2016. Hepatic and renal metallothionein concentrations in Commerson's dolphins (Cephalorhynchus commersonii) from Tierra del Fuego, South Atlantic Ocean. Mar Pollut Bull.
Caceres-Saez, I., Ribeiro-Guevara, S., Dellabianca, N., Goodall, N., Cappozzo, L., 2013. Heavy metals and essential elements in Commerson's dolphins (*Cephalorhynchus c. commersonii*) from the southwestern South Atlantic Ocean. Environmental Monitoring and Assessment 185, 5375-5386.
Camacho, M., Boada, L.D., Oros, J., Lopez, P., Zumbado, M., Almeida-Gonzalez, M., Luzardo, O.P., 2014a. Monitoring organic and inorganic pollutants in juvenile live sea turtles: Results from a study of *Chelonia mydas* and *Eretmochelys imbricata* in Cape Verde. Science of the Total Environment 481, 303-310.
Camacho, M., Oros, J., Boada, L.D., Zaccaroni, A., Silvi, M., Formigaro, C., Lopez, P., Zumbado, M., Luzardo, O.P., 2013. Potential adverse effects of inorganic pollutants on clinical parameters of Loggerhead sea turtles (*Caretta caretta*): Results from a nesting colony from Cape Verde, West Africa. Marine Environmental Research 92, 15-22.
Camacho, M., Oros, J., Henriquez-Hernandez, L.A., Valeron, P.F., Boada, L.D., Zaccaroni, A., Zumbado, M., Luzardo, O.P., 2014b. Influence of the rehabilitation of injured loggerhead turtles (Caretta caretta) on their blood levels of environmental organic pollutants and elements. Sci Total Environ 487, 436-442.
Capelli, R., Das, K., Pellegrini, R.D., Drava, G., Lepoint, G., Miglio, C., Minganti, V., Poggi, R., 2008. Distribution of trace elements in organs of six species of cetaceans from the Ligurian Sea (Mediterranean), and the relationship with stable carbon and nitrogen ratios. Science of the Total Environment 390, 569-578.

Cardellicchio, N., Giandomenico, S., Ragone, P., Di Leo, A., 2000. Tissue distribution of metals in striped dolphins (*Stenella coeruleoalba*) from the Apulian coasts, Southern Italy. Environmental Research 49, 55-66.
Cardona, L., Aguilar, A., Pazos, L., 2009. Delayed ontogenic dietary shift and high levels of omnivory in green turtles (*Chelonia mydas*) from the NW coast of Africa. Marine Biology 156, 1487-1495.
Caurant, F., Aubaij, A., Lahaye, V., Van Canneyt, O., Rogan, E., Lopez, A., Addink, M., Churlaud, C., Robert, M., Bustamante, P., 2006. Lead contamination of small cetaceans in European waters - The use of stable isotopes for identifying the sources of lead exposure. Marine Environmental Research 62, 131-148.
Cortés-Gómez, A.A., Fuentes-Mascorro, G., Romero, D., 2014. Metals and metalloids in whole blood and tissues of Olive Ridley turtles (L*epidochelys olivacea*) from La Escobilla Beach (Oaxaca, Mexico). Marine Pollution Bulletin 89, 367-375.
D'Ilio, S., Mattei, D., Blasi, M.F., Alimonti, A., Bogialli, S., 2011. The occurrence of chemical elements and POPs in loggerhead turtles (*Caretta caretta*): An overview. Marine Pollution Bulletin 62, 1606-1615.
da Silva, C.C., Klein, R.D., Barcarolli, I.F., Bianchini, A., 2016. Metal contamination as a possible etiology of fibropapillomatosis in juvenile female green sea turtles *Chelonia mydas* from the southern Atlantic Ocean. Aquatic Toxicology 170, 42-51.
da Silva, C.C., Varela, A.S., Jr., Barcarolli, I.F., Bianchini, A., 2014. Concentrations and distributions of metals in tissues of stranded green sea turtles (*Chelonia mydas*) from the southern Atlantic coast of Brazil. Science of the Total Environment 466-467, 109-118.
Day, R.D., 2003. Mercury in loggerhead sea turtles, *Caretta caretta*: Developing monitoring strategies, investigating factors affecting contamination, and assessing health impacts. College of Charleston, South Carolina.
Day, R.D., Segars, A.L., Arendt, M.D., Lee, A.M., Peden-Adams, M.M., 2007. Relationship of blood mercury levels to health parameters in the loggerhead sea turtle (*Caretta caretta*). Environmental Health Perspectives 115, 1421-1428.
Deem, S.L., Dierenfeld, E.S., Sounguet, G.P., Alleman, A.R., Cray, C., Poppenga, R.H., Norton, T.M., Karesh, W.B., 2006. Blood values in free-ranging nesting leatherback sea turtles (*Dermochelys coriacea*) on the coast of the Republic of Gabon. Journal of Zoo and Wildlife Medicine 37, 464-471.
Deem, S.L., Norton, T.M., Mitchell, M., Segars, A., Alleman, A.R., Cray, C., Poppenga, R.H., Dodd, M., Karesh, W.B., 2009. Comparison of blood values in foraging, nesting, and stranded loggerhead turtles (*Caretta caretta*) along the coast of Georgia, USA. Journal of Wildlife Diseases 45, 41-56.
Duffus, J.H., 2002. " Heavy metals" a meaningless term?(IUPAC Technical Report). Pure and applied chemistry 74, 793-807.
Dyc, C., Far, J., Gandar, F., Poulipoulis, A., Greco, A., Eppe, G., Das, K., 2016. Toxicokinetics of selenium in the slider turtle, Trachemys scripta. Ecotoxicology 25, 727-744.
Echegoyen, Y., Boyle, E.A., Lee, J.M., Gamo, T., Obata, H., Norisuye, K., 2014. Recent distribution of lead in the Indian Ocean reflects the impact of regional emissions. Proc Natl Acad Sci U S A 111, 15328-15331.

Ehsanpour, M., Afkhami, M., Khoshnood, R., Reich, K.J., 2014. Determination and maternal transfer of heavy metals (Cd, Cu, Zn, Pb and Hg) in the Hawksbill sea turtle (*Eretmochelys imbricata*) from a nesting colony of Qeshm Island, Iran. Bulletin of Environmental Contamination and Toxicology 92, 667-673.
Emerit, J., Beaumont, C., Trivin, F., 2001. Iron metabolism, free radicals, and oxidative injury. Biomed Pharmacother 55, 333-339.
Faust, D.R., Hooper, M.J., Cobb, G.P., Barnes, M., Shaver, D., Ertolacci, S., Smith, P.N., 2014. Inorganic elements in green sea turtles (*Chelonia mydas*): relationships among external and internal tissues. Environmental Toxicology and Chemistry 33, 2020-2027.
Finlayson, K.A., Leusch, F.D., van de Merwe, J.P., 2016. The current state and future directions of marine turtle toxicology research. Environment International 94, 113-123.
Franzellitti, S., Locatelli, C., Gerosa, G., Vallini, C., Fabbri, E., 2004. Heavy metals in tissues of loggerhead turtles (*Caretta caretta*) from the northwestern Adriatic Sea. Comp Biochem Physiol C Toxicol Pharmacol 138, 187-194.
Frias-Espericueta, M.G., Osuna-Lopez, J.I., Ruiz-Telles, A., Quintero-Alvarez, J.M., Lopez-Lopez, G., Izaguirre-Fierro, G., Voltolina, D., 2006. Heavy metals in the tissues of the sea turtle *Lepidochelys olivacea* from a nesting site of the northwest coast of Mexico. Bulletin of Environmental Contamination and Toxicology 77, 179-185.
Frye, F.L., 1995. Nutritional considerations, in: Warwick, C., Frye, F.L., Murphy, J.B. (Eds.), Health and Welfare of Captive Reptiles, Chapman and Hall, New York, pp. 82-112.
Gadzała-Kopciuch, R., Berecka, B., Bartoszewicz, J., Buszewski, B., 2004. Some considerations about bioindicators in environmental monitoring. Polish Journal of Environmental Studies 13, 453-462.
García-Fernández, A.J., Gómez-Ramírez, P., Martínez-López, E., Hernández-García, A., María-Mojica, P., Romero, D., Jiménez, P., Castillo, J.J., Bellido, J.J., 2009. Heavy metals in tissues from loggerhead turtles (*Caretta caretta*) from the southwestern Mediterranean (Spain). Ecotoxicology and Environmental Safety 72, 557-563.
Gardner, S.C., Fitzgerald, S.L., Vargas, B.A., Rodriguez, L.M., 2006. Heavy metal accumulation in four species of sea turtles from the Baja California peninsula, Mexico. Biometals 19, 91-99.
Godley, B.J., Thompson, D.R., Furness, R.W., 1999. Do heavy metal concentrations pose a threat to marine turtles from the Mediterranean sea? Marine Pollution Bulletin 38, 497-502.
Grillitsch, B., Schiesari, L., 2010. The Ecotoxicology of Metals in Reptiles. 337-448.
Guirlet, E., Das, K., 2012. Cadmium toxicokinetics and bioaccumulation in turtles: trophic exposure of *Trachemys scripta elegans*. Ecotoxicology 21, 18-26.
Guirlet, E., Das, K., Girondot, M., 2008. Maternal transfer of trace elements in leatherback turtles (*Dermochelys coriacea)* of French Guiana. Aquatic Toxicology 88, 267-276.
Hamed, M.A., Mohamedein, L.I., El-Sawy, M.A., El-Moselhy, K.M., 2013. Mercury and tin contents in water and sediments along the Mediterranean shoreline of Egypt. The Egyptian Journal of Aquatic Research 39, 75-81.

Hansen, A.M., Bryan, C.E., West, K., Jensen, B.A., 2016. Trace element concentrations in liver of 16 species of cetaceans stranded on Pacific Islands from 1997 through 2013. Archives of Environmental Contamination and Toxicology 70, 75-95.
Harris, H.S., Benson, S.R., Gilardi, K.V., Poppenga, R.H., Work, T.M., Dutton, P.H., Mazet, J.A.K., 2011. Comparative health assessment of Western Pacific Leatherback Turtles (*Dermochelys Coriacea*) foraging off the coast of California, 2005–2007. Journal of Wildlife Diseases 4, 321–337.
Haynes, D., Johnson, J.E., 2000. Organochlorine, heavy metal and polyaromatic hydrocarbon pollutant concentrations in the Great Barrier Reef (Australia): a review. Marine Pollution Bulletin 41, 267-278.
Hodson, M.E., 2004. Heavy metals—geochemical bogey men? Environmental Pollution 129, 341-343.
Hopkins, W.A., Snodgrass, J.W., Baionno, J.A., Roe, J.H., Staub, B.P., Jackson, B.P., 2005. Functional relationships among selenium concentrations in the diet, target tissues, and nondestructive tissue samples of two species of snakes. Environmental Toxicology and Chemistry 24, 344-351.
Ikonomopoulou, M.P., Olszowy, H., Hodge, M., Bradley, A.J., 2009. The effect of organochlorines and heavy metals on sex steroid-binding proteins in vitro in the plasma of nesting green turtles, *Chelonia mydas*. Journal of Comparative Physiology B 179, 653-662.
Ikonomopoulou, M.P., Olszowy, H., Limpus, C., Francis, R., Whittier, J., 2011. Trace element concentrations in nesting flatback turtles (*Natator depressus*) from Curtis Island, Queensland, Australia. Marine Environmental Research 71, 10-16.
Innis, C., Merigo, C., Dodge, K., Tlusty, M., Dodge, M., Sharp, B., Myers, A., McIntosh, A., Wunn, D., Perkins, C., Herdt, T.H., Norton, T., Lutcavage, M., 2010. Health evaluation of leatherback turtles (*Dermochelys coriacea*) in the Northwestern Atlantic during direct capture and fisheries gear disentanglement. Chelonian Conservation and Biology 9, 205-222.
Innis, C., Tlusty, M., Perkins, C., Holladay, S., Merigo, C., Weber, E.S., 2008. Trace Metal and Organochlorine Pesticide Concentrations in Cold-Stunned Juvenile Kemp's Ridley Turtles (*Lepidochelys kempii*) from Cape Cod, Massachusetts. Chelonian Conservation and Biology 7, 230-239.
Jerez, S., Motas, M., Canovas, R.A., Talavera, J., Almela, R.M., Bayón del Rio, A., 2010. Accumulation and tissue distribution of heavy metals and essential elements in loggerhead turtles (*Caretta caretta*) from Spanish Mediterranean coastline of Murcia. Chemosphere 78, 256-264.
Josse, J., Husson, F., 2012. Handling missing values in exploratory multivariate data analysis methods. Journal de la Société Française de Statistique 153, 79-99.
Josse, J., Husson, F., 2016. missMDA: A package for handling missing values in multivariate data analysis. Journal of Statistical Software 70.
Kampalath, R., Gardner, S.C., Mendez-Rodriguez, L.C., Jay, J.A., 2006. Total and methylmercury in three species of sea turtles of Baja California Sur. Marine Pollution Bulletin 52, 1816-1823.

Kaska, Y., Çelik, A., Bağ, H.s., Aureggi, M., Özel, K., Elçi, A., Kaska, A., Elçi, L., 2004. Heavy metal monitoring in stranded sea turtles along the mediterranean coast of Turkey. Fresenius Environmental Bulletin 13, 769-776.
Keller, J.M., Peden-Adams, M.M., Aguirre, A.A., Gardner, S., 2006. Immunotoxicology and implications for reptilian health, in: Gardner, S.C., Oberdörster, E. (Eds.), Toxicology of Reptiles. Taylor & Francis, Boca Raton, USA, pp. 199-240.
Kenyon, L.O., Landry, A.M., Gill, G.A., 2001. Trace metal concentration in blood of the Kemp's ridley sea turtle (*Lepidochelys kempii*). Chelonian Conservation and Biology 4, 128-135.
Klaassen, C.D., Liu, J., Diwan, B.A., 2009. Metallothionein protection of cadmium toxicity. Toxicology and Applied Pharmacology 238, 215-220.
Komoroske, L.M., Lewison, R.L., Seminoff, J.A., Deustchman, D.D., Deheyn, D.D., 2012. Trace metals in an urbanized estuarine sea turtle food web in San Diego Bay, CA. Science of the Total Environment 417-418, 108-116.
Kubis, S., Chaloupka, M., Ehrhart, L., Bresette, M., 2009. Growth rates of juvenile green turtles *Chelonia mydas* from three ecologically distinct foraging habitats along the east central coast of Florida, USA. Marine Ecology-Progress Series 389, 257–269.
Kubota, R., Kunito, T., Tanabe, S., 2002. Occurrence of Several Arsenic Compunds in the Liver of Birds, Cetacean, Pinnipeds and Sea Turtles. Environmental Toxicology and Chemistry 22, 1200-1207.
Kunito, T., Kubota, R., Fujihara, J., Agusa, T., Tanabe, S., 2008. Arsenic in marine mammals, seabirds, and sea turtles. Reviews of Environmental Contamination and Toxicology, 31-69.
Labrada-Martagon, V., Rodriguez, P.A., Mendez-Rodriguez, L.C., Zenteno-Savin, T., 2011. Oxidative stress indicators and chemical contaminants in East Pacific green turtles (*Chelonia mydas*) inhabiting two foraging coastal lagoons in the Baja California peninsula. Comparative Biochemistry and Physiology Part C 154, 65-75.
Lailson-Brito, J., Cruz, R., Dorneles, P.R., Andrade, L., Azevedo Ade, F., Fragoso, A.B., Vidal, L.G., Costa, M.B., Bisi, T.L., Almeida, R., Carvalho, D.P., Bastos, W.R., Malm, O., 2012. Mercury-selenium relationships in liver of Guiana dolphin: the possible role of Kupffer cells in the detoxification process by tiemannite formation. PLoS One 7, e42162.
Lam, J.C., Tanabe, S., Chan, S.K., Yuen, E.K., Lam, M.H., Lam, P.K., 2004. Trace element residues in tissues of green turtles (*Chelonia mydas*) from South China waters. Marine Pollution Bulletin 48, 174-182.
Lamborg, C., Bowman, K., Hammerschmidt, C., Gilmour, C., Munson, K., Selin, N., Tseng, C.-M., 2014. Mercury in the Anthropocene Ocean. Oceanography 27, 76-87.
Lavery, T.J., Kemper, C.M., Sanderson, K., Schultz, C.G., Coyle, P., Mitchell, J.G., Seuront, L., 2009. Heavy metal toxicity of kidney and bone tissues in South Australian adult bottlenose dolphins (*Tursiops aduncus*). Marine Environmental Research 67, 1-7.
Leon, Y.M., Bjorndal, K.A., 2002. Selective feeding in the hawksbill turtle, an important predator in coral reef ecosystems. Marine Ecology-Progress Series 245, 249-258.
Ley-Quiñónez, C., Zavala-Norzagaray, A.A., Espinosa-Carreon, T.L., Peckham, H., Marquez-Herrera, C., Campos-Villegas, L., Aguirre, A.A., 2011. Baseline heavy metals and metalloid values in blood of loggerhead turtles (*Caretta caretta*) from Baja California Sur, Mexico. Marine Pollution Bulletin 62, 1979-1983.

Ley-Quiñónez, C.P., Zavala-Norzagaray, A.A., Rendon-Maldonado, J.G., Espinosa-Carreon, T.L., Canizales-Roman, A., Escobedo-Urias, D.C., Leal-Acosta, M.L., Hart, C.E., Aguirre, A.A., 2013. Selected heavy metals and selenium in the blood of black sea turtle (*Chelonia mydas agasiizzi*) from Sonora, Mexico. Bull Environ Contam Toxicol 91, 645-651.
Limpus, C.J., 2007. A biological review of Australian Marine Turtles. 5 Flatback Turtle, *Natator depresus* (Garman). Environmental Protection Agency, Australia, p. 53.
Lohren, H., Bornhorst, J., Galla, H.-J., Schwerdtle, T., 2015. The blood–cerebrospinal fluid barrier–first evidence for an active transport of organic mercury compounds out of the brain. Metallomics 7, 1420-1430.
Márquez, M.R., 1990. Sea Turt[es of the World. An annotated and ilustrated catalogue of sea turtles species known to date, FAO Species Catalogue. Food and Agriculture Organization of the United Nations, Rome, Italia, p. 81.
Marquez, M.R., Peñaflores, C., Vasconcelos, J.C., 1996. Olive ridley turtles (*Lepidochelys olivacea*) show signs of recovery at La Escobilla, Oaxaca. Marine Turtle Newsletter 73, 5-7.
McFadden, K.W., Gomez, A., Sterling, E.J., Naro-Maciel, E., 2014. Potential impacts of historical disturbance on green turtle health in the unique & protected marine ecosystem of Palmyra Atoll (Central Pacific). Marine Pollution Bulletin 89, 160-167.
Mieiro, C.L., Pacheco, M., Pereira, M.E., Duarte, A.C., 2009. Mercury distribution in key tissues of fish (*Liza aurata*) inhabiting a contaminated estuary-implications for human and ecosystem health risk assessment. Journal of Environmental Monitoring 11, 1004-1012.
Montenegro, S.B., Bernal, G., 1982. Análisis del contenido estomacal de *Lepidochelys olivacea*. Universidad Nacional Autónoma de México, México.
MTSG, 2007. *Lepidochelys olivacea*, Marine Turtle Specialist Group- Red list ssessment
Nascimento, J.R., Bidone, E.D., Rolão-Araripe, D., Keunecke, K.A., Sabadini-Santos, E., 2016. Trace metal distribution in white shrimp (*Litopenaeus schmitti)* tissues from a Brazilian coastal area. Environmental Earth Sciences 75.
Ngu, T.T., Stillman, M.J., 2009. Metal-binding mechanisms in metallothioneins. Dalton Trans, 5425-5433.
Nicolau, L., Monteiro, S.S., Pereira, A.T., Marcalo, A., Ferreira, M., Torres, J., Vingada, J., Eira, C., 2017. Trace elements in loggerhead turtles (*Caretta caretta*) stranded in mainland Portugal: Bioaccumulation and tissue distribution. Chemosphere 179, 120-126.
Novillo, O., Pertusa, J.F., Tomas, J., 2017. Exploring the presence of pollutants at sea: Monitoring heavy metals and pesticides in loggerhead turtles (*Caretta caretta*) from the western Mediterranean. Science of the Total Environment 598, 1130-1139.
Nuccio, P.M., 2015. Pollution of waters and soils by contaminants of magmatic origin. Rendiconti Lincei 27, 21-28.
Orisakwem, O.E., Blum, J.L., Sujak, S., Zelikoff, J.T., 2014. Metal pollution in Nigeria: A biomonitoring update. Journal of Health and Pollution 4, 40-52.
Páez-Osúna, F., Calderon-Campuzano, M.F., Soto-Jiménez, M.F., Ruelas-Inzunza, J., 2011. Mercury in blood and eggs of the sea turtle *Lepidochelys olivacea* from a nesting colony in Oaxaca, Mexico. Marine Pollution Bulletin 62, 1320-1323.

Páez-Osúna, F., Calderón-Campuzano, M.F., Soto-Jiménez, M.F., Ruelas-Inzunza, J.R., 2010a. Lead in blood and eggs of the sea turtle, *Lepidochelys olivacea*, from the Eastern Pacific: concentration, isotopic composition and maternal transfer. Marine Pollution Bulletin 60, 433-439.
Páez-Osúna, F., Calderón-Campuzano, M.F., Soto-Jiménez, M.F., Ruelas-Inzunza, J.R., 2010b. Trace metals (Cd, Cu, Ni, and Zn) in blood and eggs of the sea turtle *Lepidochelys olivacea* from a nesting colony of Oaxaca, Mexico. Archives of Environmental Contamination and Toxicology 59, 632-641.
Pagès, J., 2004. Analyse factorielle de données mixtes. Revue de statistique appliquée 52, 93-111.
Peakall, D., Burger, J., 2003. Methodologies for assessing exposure to metals: speciation, bioavailability of metals, and ecological host factors. Ecotoxicology and Environmental Safety 56, 110-121.
Pemmer, B., Roschger, A., Wastl, A., Hofstaetter, J.G., Wobrauschek, P., Simon, R., Thaler, H.W., Roschger, P., Klaushofer, K., Streli, C., 2013. Spatial distribution of the trace elements zinc, strontium and lead in human bone tissue. Bone 57, 184-193.
Pereira, P., Raimundo, J., Canário, J., Almeida, A., Pacheco, M., 2013. Looking at the aquatic contamination through fish eyes – A faithful picture based on metals burden. Marine Pollution Bulletin 77, 375-379.
Pérez-Moreno, V., Ramos-López, M.Á., Zavala-Gómez, C.E., Rodríguez, M.Á.R., 2016. Heavy metals in seawater along the Mexican Pacific Coast. Interciencia 41, 419.
Perrault, J., Wyneken, J., Thompson, L.J., Johnson, C., Miller, D.L., 2011. Why are hatching and emergence success low? Mercury and selenium concentrations in nesting leatherback sea turtles (Dermochelys coriacea) and their young in Florida. Marine Pollution Bulletin 62, 1671-1682.
Perrault, J.R., 2012. Assessment of mercury and selenium concentrations in tissues of stranded leatherback sea turtles (*Dermochelys coriacea*). Journal of Herpetological Medicine and Surgery 22, 76-85.
Perrault, J.R., Miller, D.L., Garner, J., Wyneken, J., 2013. Mercury and selenium concentrations in leatherback sea turtles (*Dermochelys coriacea*): Population comparisons, implications for reproductive success, hazard quotients and directions for future research. Science of The Total Environment 463-464, 61-71.
Polizzi, P.S., Chiodi Boudet, L.N., Romero, M.B., Denuncio, P.E., Rodriguez, D.H., Gerpe, M.S., 2013. Fine scale distribution constrains cadmium accumulation rates in two geographical groups of Franciscana dolphin from Argentina. Marine Pollution Bulletin 72, 41-46.
QGC, G., 2011. The Queensland Curtis LNG poject, in: 7, R. (Ed.). http://www.bg-group.com/713/qgc, Australia.
R Core Team, 2017. R: A language and environment for statistical computing, 3.4.0 ed. R Foundation for Statistical Computing, Vienna, Austria.
Rainbow, P.S., 2002. Trace metal concentrations in aquatic invertebrates: why and so what? Environmental Pollution 120, 497-507.
Rana, S.V., 2014. Perspectives in endocrine toxicity of heavy metals-a review. Biological Trace Element Research 160, 1-14.

Rees, A.F., Alfaro-Shigueto, J., Barata, P.C.R., Bjorndal, K.A., Bolten, A.B., Bourjea, J., Broderick, A.C., Campbell, L.M., Cardona, L., Carreras, C., Casale, P., Ceriani, S.A., Dutton, P.H., Eguchi, T., Formia, A., Fuentes, M., Fuller, W.J., Girondot, M., Godfrey, M.H., Hamann, M., Hart, K.M., Hays, G.C., Hochscheid, S., Kaska, Y., Jensen, M.P., Mangel, J.C., Mortimer, J.A., Naro-Maciel, E., Ng, C.K.Y., Nichols, W.J., Phillott, A.D., Reina, R.D., Revuelta, O., Schofield, G., Seminoff, J.A., Shanker, K., Tomás, J., van de Merwe, J.P., Van Houtan, K.S., Vander Zanden, H.B., Wallace, B.P., Wedemeyer-Strombel, K.R., Work, T.M., Godley, B.J., 2016. Are we working towards global research priorities for management and conservation of sea turtles? Endangered Species Research 31, 337-382.

Register, A.L., 2011. Effects of Heavy Metal Pollution on the Loggerhead Sea Turtle, School of Science and Technology. Loma Linda University, Electronic Theses and Dissertations, p. 117.

Richardson, A.J., Bakun, A., Hays, G.C., Gibbons, M.J., 2009. The jellyfish joyride: causes, consequences and management responses to a more gelatinous future. Trends in Ecology and Evolution 24, 312-322.

Ruangsomboon, S., Wongrat, L., 2006. Bioaccumulation of cadmium in an experimental aquatic food chain involving phytoplankton (*Chlorella vulgaris*), zooplankton (*Moina macrocopa*), and the predatory catfish *Clarias macrocephalus* x *C. gariepinus*. Aquatic Toxicology 78, 15-20.

Ruttkay-Nedecky, B., Nejdl, L., Gumulec, J., Zitka, O., Masarik, M., Eckschlager, T., Stiborova, M., Adam, V., Kizek, R., 2013. The role of metallothionein in oxidative stress. Int J Mol Sci 14, 6044-6066.

Saeki, K., Sakakibaea, H., Sakai, H., Kunito, T., Tanabe, S., 2000. Arsenic accumulation in three species of sea turtles. Biometals 13, 241-250.

Sakai, H., Saeki, K., Ichihashi, H., Suganuma, H., Tanabe, S., Tatsukawa, R., 2000. Species-specific distribution of heavy metals in tissues and organs of Loggerhead turtle (*Caretta caretta*) and Green turtle (*Chelonia mydas*) from Japanese Coastal Waters. Marine Pollution Bulletin 40, 701-709.

Schifter, I., González-Macías, C., Salazar-Coria, L., Sánchez-Reyna, G., González-Lozano, C., 2015. Ecological and human risk assessment of long-term produced water discharge to the ocean at the Sonda de Campeche, Gulf of Mexico. Environmental Earth Sciences 74, 5813-5826.

Schmid, J.R., Witzell, W.N., 2006. Seasonal migrations of immature Kemp's ridley turtles (*Lepidochelys kempii* Garman) along the west coast of Florida. Gulf of Mexico Science 24, 28-40.

Setim Prioste, F.E., de Oliveira Souza, V.C., Ramos Queiroz, M., 2015. Chemical Element Concentrations in the Blood of Green Turtles (*Chelonia Mydas*) Captured at Fernando De Noronha Marine National Park, Brazil. Journal of Environmental & Analytical Toxicology 05.

Silman, R., Vargas, I., Troëng, S., 2002. Tortugas Marinas. Guía Educativa, in: Corporation, C.C. (Ed.), 2a ed.

Solaun, O., Rodriguez, J.G., Borja, A., Gonzalez, M., Saiz-Salinas, J.I., 2013. Biomonitoring of metals under the water framework directive: detecting temporal trends and abrupt changes, in relation to the removal of pollution sources. Mar Pollut Bull 67, 26-35.

Stewart, K., Johnson, C., 2006. *Dermochelys coriacea* – Leatherback Sea Turtle, in: Meylan, P.A. (Ed.), Biology and Conservation of Florida Turtles. Chelonian Research Foundation, pp. 144–157.
Storelli, M.M., Marcotrigiano, G.O., 2003. Heavy metal residues in tissues of marine turtles. Marine Pollution Bulletin 46, 397-400.
Storelli, M.M., Storelli, A., D'Addabbo, R., Marano, C., Bruno, R., Marcotrigiano, G.O., 2005. Trace elements in loggerhead turtles (*Caretta caretta*) from the eastern Mediterranean Sea: overview and evaluation. Environmental Pollution 135, 163-170.
Stringell, T.B., Clerveaux, W.V., Godley, B.J., Kent, F.E., Lewis, E.D., Marsh, J.E., Phillips, Q., Richardson, P.B., Sanghera, A., Broderick, A.C., 2016. Taxonomic distinctness in the diet of two sympatric marine turtle species. Marine Ecology.
Summers, C.F., Bowerman, W.W., Parsons, N., Chao, W.Y., Bridges, W.C., Jr., 2014. Lead and cadmium in the blood of nine species of seabirds, Marion Island, South Africa. Bulletin of Environmental Contamination and Toxicology 93, 417-422.
Suzuki, K., Noda, J., Yanagisawa, M., Kawazu, I., Sera, K., Fukui, D., Asakawa, M., Yokota, H., 2012. Relationships between carapace sizes and plasma major and trace element status in captive hawksbill sea turtles (*Eretmochelys imbricata*). Journal of Veterinary Medical Science 74, 1677-1680.
Takata, H., 2005. Comparative vertical distributions of iron in the Japan Sea, the Bering Sea, and the western North Pacific Ocean. Journal of Geophysical Research 110.
Talavera-Saenz, A., Gardner, S.C., Riosmena Rodriquez, R., Acosta Vargas, B., 2007. Metal profiles used as environmental markers of green turtle (*Chelonia mydas*) foraging resources. Science of the Total Environment 373, 94-102.
Tapiero, H., Tew, K.D., 2003. Trace elements in human physiology and pathology: zinc and metallothioneins. Biomedicine & Pharmacotherapy 57, 399-411.
Tinggi, U., 2003. Essentiality and toxicity of selenium and its status in Australia: a review. Toxicology Letters 137, 103-110.
Tiwari, M., Bjorndal, K.A., 2000. Variation in morphology and reproduction in loggerheads, *Caretta caretta*, nesting in the United States, Brazil, and Greece. Herpetologica 56, 343-356.
Tomás, J., Aznar, F.J., Raga, J.A., 2001. Feeding ecology of the loggerhead turtle *Caretta caretta* in the western Mediterranean. Journal of Zoology, London 255, 525-532.
Torrent, A., Gonzalez-Diaz, O.M., Monagas, P., Oros, J., 2004. Tissue distribution of metals in loggerhead turtles (*Caretta caretta*) stranded in the Canary Islands, Spain. Marine Pollution Bulletin 49, 854-860.
Trocini, S., 2013. Health assessment and hatching success of two Western Australian loggerhead turtle (*Caretta caretta*) populations. Murdoch University.
Turner, A., Pollock, H., Brown, M.T., 2009. Accumulation of Cu and Zn from antifouling paint particles by the marine macroalga, *Ulva lactuca*. Environmental Pollution 157, 2314-2319.
van de Merwe, J.P., Hodge, M., Olszowy, H.A., Whittier, J.M., Lee, S.Y., 2010. Using blood samples to estimate persistent organic pollutants and metals in green sea turtles (*Chelonia mydas*). Marine Pollution Bulletin 60, 579-588.

Vélez-Rubio, G.M., Cardona, L., López-Mendilaharsu, M., Martínez Souza, G., Carranza, A., González-Paredes, D., Tomás, J., 2016. Ontogenetic dietary changes of green turtles (*Chelonia mydas*) in the temperate southwestern Atlantic. Marine Biology 163.
Vighi, M., Borrell, A., Aguilar, A., 2016. Bone as a surrogate tissue to monitor metals in baleen whales. Chemosphere 171, 81-88.
Villa, C.A., Flint, M., Bell, I., Hof, C., Limpus, C.J., Gaus, C., 2017. Trace element reference intervals in the blood of healthy green sea turtles to evaluate exposure of coastal populations. Environmental Pollution 220, 1465-1476.
Von, J.G., Bossart, G.D., Fournie, M., 2003. Toxicology of marine mammals.
Wabnitz, C., Pauly, D., 2008. Length-weight relationships and additional growth parameters for sea turtles. Fish Cent Res Rep 16, 92-101.
Wahbi, O.M., El-Greisy, Z.A., 2016. Impact of Water Quality at Different Locations of Alexandria Mediterranean Coast on the Pituitary-ovarian Axis of Gilthead Seabream Sparus aurata. Journal of Fisheries and Aquatic Science 11, 244-254.
Wallace, B.P., DiMatteo, A.D., Bolten, A.B., Chaloupka, M.Y., Hutchinson, B.J., Abreu-Grobois, F.A., Mortimer, J.A., Seminoff, J.A., Amorocho, D., Bjorndal, K.A., Bourjea, J., Bowen, B.W., Dueñas, R.B., Casale, P., Choudhury, B.C., Costa, A., Dutton, P.H., Fallabrino, A., Finkbeiner, E.M., Girard, A., Girondot, M., Hamann, M., Hurley, B.J., Lopez-Mendilaharsu, M., Marcovaldi, M.A., Musick, J.A., Nel, R., Pilcher, N.J., Troëng, S., Witherington, B., Mast, R.B., 2011. Global conservation priorities for marine turtles. PLoS One 6, e24510.
Wallace, B.P., DiMatteo, A.D., Hurley, B.J., Finkbeiner, E.M., Bolten, A.B., Chaloupka, M.Y., Hutchinson, B.J., Abreu-Grobois, F.A., Amorocho, D., Bjorndal, K.A., Bourjea, J., Bowen, B.W., Dueñas, R.B., Casale, P., Choudhury, B.C., Costa, A., Dutton, P.H., Fallabrino, A., Girard, A., Girondot, M., Godfrey, M.H., Hamann, M., López-Mendilaharsu, M., Marcovaldi, M.A., Mortimer, J.A., Musick, J.A., Nel, R., Seminoff, J.A., Troëng, S., Witherington, B., Mast, R.B., 2010. Regional management units for marine turtles: a novel framework for prioritizing conservation and research across multiple scales. PLoS One 5, e15465.
Wang, H.-C., 2005. Trace metal uptake and accumulation pathways in Kemp's Ridley. Texas A&M University, p. 275.
Wang, M.J., Wang, W.X., 2009. Cadmium in three marine phytoplankton: accumulation, subcellular fate and thiol induction. Aquatic Toxicology 95, 99-107.
Watanabe, K.K., Hatase, H., Kinoshita, M., Omuta, K., Bando, T., Kamezaki, N., Sato, K., Matsuzawa, Y., Goto, K., Nakashima, Y., Takeshita, H., Aoyama, J., Tsukamoto, K., 2010. Population structure of the loggerhead turtle *Caretta caretta*, a large marine carnivore that exhibits alternative foraging behaviors. Marine Ecology-Progress Series.
Whiting, S.D., Long, J.L., Hadden, K.M., Lauder, A.D.K., Koch, A.U., 2007. Insights into size, seasonality and biology of a nesting population of the Olive Ridley turtle in northern Australia. Wildlife Research 34, 200-210.
Zavala-Norzagaray, A.A., Ley-Quiñónez, C.P., Espinosa-Carreon, T.L., Canizalez-Roman, A., Hart, C.E., Aguirre, A.A., 2014. Trace elements in blood of sea turtles *Lepidochelys olivacea* in the Gulf of California, Mexico. Bulletin of Environmental Contamination and Toxicology 93, 536-541.

Zhou, Q., Zhang, J., Fu, J., Shi, J., Jiang, G., 2008. Biomonitoring: an appealing tool for assessment of metal pollution in the aquatic ecosystem. Anal Chim Acta 606, 135-150.

2. OBJECTIVES

The principal objective of this Doctoral Thesis was the evaluation of *Lepidochelys olivacea* marine turtles regarding the presence and possible effects of inorganic elements (including heavy metals), in a population from Southern Pacific Mexican coast.

The specific objectives were:

1) Study the current situation about heavy metals and other inorganic elements in blood and tissues of Olive Ridley turtle (*Lepidochelys olivacea*) around the world.
2) Biomonitoring inorganic element concentrations in blood from live and different tissues from death turtles from *La Escobilla* beach (Oaxaca, Mexico), during 3 years (2012 to 2014).
3) Assess biochemical and molecular biomarkers related to inorganic elements.
4) Asses a possible morphological biomarker using the carapace of the turtles.

3. EXPERIMENTAL CHAPTERS

FIRST PART. BIOMONITORING

3.1 A First Approach

CHAPTER II. Metals and metalloids in whole blood and tissues of Olive Ridley turtles (*Lepidochelys olivacea*) from La Escobilla Beach (Oaxaca, Mexico)

3.1.1 Introduction

The accumulation of trace elements in marine turtles varies depending on several factors, including geographical location, exposure to environmental contamination, diet, species and type of tissue (Alava et al., 2006; Guirlet et al., 2008). Biomonitoring of long-living species has become an important tool in biotoxicology because it can serve both to provide baseline measurements for further studies into the health status of this population and to increase knowledge about the pollution levels in the areas where these turtles live. Furthermore, data regarding those contaminants are scarce and widely dispersed across the world, taken in regions such as Australia (Gordon et al., 1998), US (Aguirre et al., 1994a; Homer et al., 2000; Perrault et al., 2011), Japan (Saeki et al., 2000; Sakai et al., 2000), UK (Godley et al., 1998), Mediterranean Coast (Franzellitti et al., 2004; García-Fernández et al., 2009; Godley et al., 1999b; Jerez et al., 2010b; Kaska et al., 2004; Maffucci et al., 2005; Storelli et al., 1998; Storelli and Marcotrigiano, 2003; Storelli et al., 2005), African Atlantic (Camacho et al., 2013b; Torrent et al., 2004) and Brazil (da Silva et al., 2014).

Beaches in Mexico are important for the nesting of several species of marine turtles. All sea turtles are considered endangered species within the regulation norm NOM-ECOL-059-2010 and have been made a priority for conservation. The Olive Ridley turtle (*Lepidochelys olivacea*), a species with a large population in the Pacific Ocean, is classified as "vulnerable" by the International Union for Conservation of Nature (IUCN, 2012), and is also listed in Appendix I of the Convention of International Trade in Endangered Species (CITES). In the State of Oaxaca (Mexico), La Escobilla beach (EB hereafter), is a nature sanctuary and one of the most important nesting beaches globally for this species, with more than one million turtles arriving every year to nest (CONANP, 2011a; Fuentes-Mascorro et al., 2007; Marquez et al., 2005). All females remain in their feeding areas (usually in pelagic waters) until their next annual nesting migration, but their migratory routes can vary from year to year (Marquez et al., 2005). There are few studies on metal levels in blood and tissues of this species (Frías-Espericueta *et al*., 2006 in NW Mexico; Gardner *et al*., 2006 in Baja California;

Ley-Quiñónez, 2009 in Baja California Sur; Páez-Osuna *et al*., 2010a, 2010b and 2011 in Oaxaca) and no study has been undertaken on this beach regarding metal levels in either live Olive Ridley turtles or on tissues from cadavers in the same nesting season or *arribada*.

Heavy metals and metalloids such as Pb, Cd and As are important marine pollutants, and their effects in many species are well known. There are other trace pollutant elements, however, such as Cu, Mn, Ni, Se, and Zn, which play essential roles in tissue metabolism and growth, but their negative effects on sea turtles are still not well understood (Camacho et al., 2013b). Therefore, the purpose of this study was to evaluate the concentrations of Pb, Cd, Cu, Zn, Mn, Se, Ni and As in Olive Ridley turtles from EB, using blood samples as well as the tissues of dead turtles to establish the levels of these elements. This information can be used to monitor any changes in the bioavailability of potentially inorganic pollutants in the habitat of these sea turtles and to determine whether these elements are present in tissues at concentrations that could have an impact on the sea turtles health.

3.1.2 Material and methods

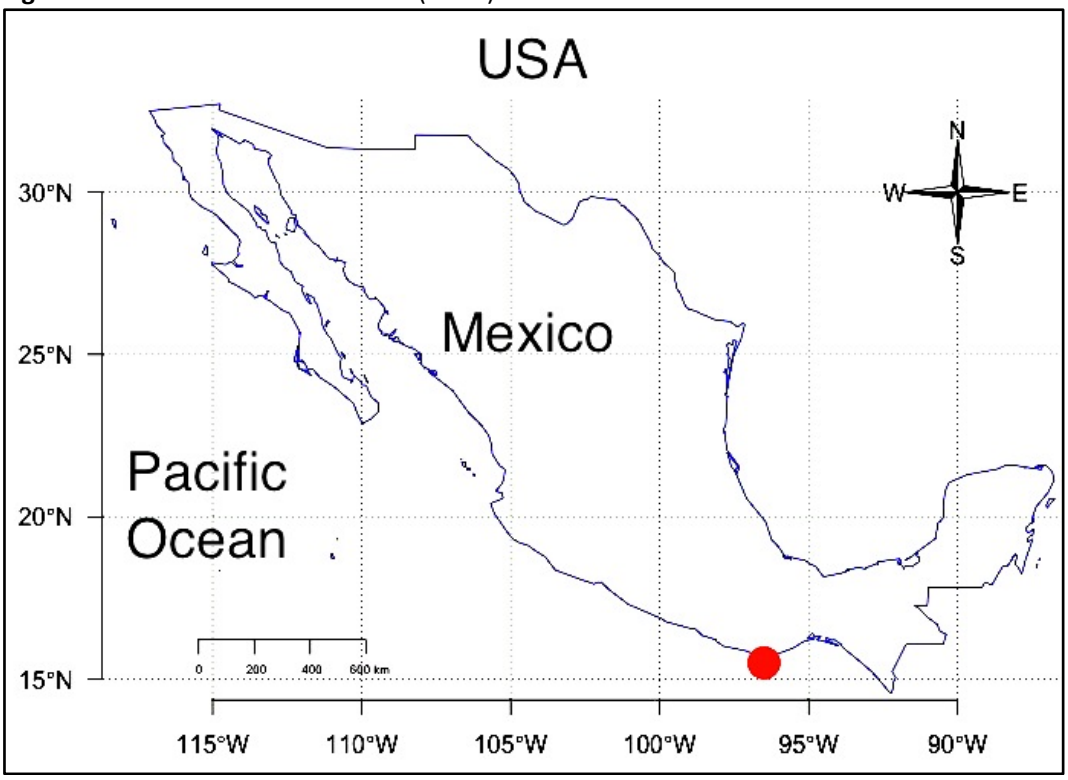
3.1.2.1 Sample collection

Samples (blood and tissues) were collected at EB, in the State of Oaxaca (Southeast Mexico, Eastern Pacific, 96°44'W and 15°47'N) (Figure 1). Beach monitoring was performed between August and September 2012 (nesting season, third arribada event). Turtles were measured and the curve carapace length (CCL) was used to determine age (Gardner et al., 2006). The CCL of each turtle was recorded, taken from the anterior nuchal notch to the furthest rear point of the carapace.

Blood from *L. olivacea* was sampled (Permit SAGARPA Number: SGPA/DGVS/10445/11) from 41 nesting females selected at random. Samples were taken from the dorsal cervical sinus, using 5 mL non-heparinized tubes and 21" needles. Before blood extraction, the neck region was carefully cleaned using ethanol and deionized water. Liver and kidney samples were taken from 13 dead turtles found along the beach in the same period (*n*= 13). Six of

these turtles died from head trauma possibly caused by boat propellers; the cause of death of the other 7 turtles is unknown. All samples were transferred into 1.5 mL microtubes and kept at -20^{o} until processed.

Figure 1. La Escobilla beach location (circle).



3.1.2.2 Metal analysis

Prior to the analysis, all the samples and laboratory materials used were thoroughly rinsed with deionized water (Milli-Q) to avoid contamination. It was then performed a pre-treatment of the samples: 0.5 g of each sample (blood, liver and kidney in wet weight) was taken for an acid digestion using 4 mL of HNO_3 (69%) and 1 mL of H_2O_2 (33%) mixed in special Teflon reaction tubes in a microwave digestion system (UltraClave-Microwave Milestone®) for 20 minutes at 220°C and finally diluted with 25 mL of double deionized water (Milli-Q).

Metal concentrations were determined using an inductively coupled plasma optical emission spectrophotometer (ICP-OES, ICAP 6500 Duo Thermo®). All concentrations are expressed in microgram per gram in wet weight. Detection limits for all elements were 0.01 μg g^{-1}. Two readings of each sample were made. To check for possible contamination, 1 blank sample for every 11 samples was also analysed.

Multielement calibration standards were prepared with different concentrations of inorganic elements, taking as reference the UNE-EN ISO 11885 for the determination of elements by inductively coupled plasma atomic emission spectroscopy. In addition, intermediate patterns of all elements were prepared. The equipment calibration was established by batch. Calibration was established with a minimum of three points for each lot. Each run began with the calibration standards and continued with samples and intermediate patterns, finishing the series with intermediate patterns (10% variation coefficient). The wavelengths were as follows:

Element	**As**	**Cd**	**Cu**	**Mn**	**Ni**	**Pb**	**Se**	**Zn**
λ(nm)	193.759	214.438	324.754 224.700	259.373 259.373R	231.604	220.353	196.090 203.985	206.200

The uncertainty percentages of the elements were as follows: As=5.56, Cd=4.56, Cu=4.12, Mn=6.15, Ni=4.83, Pb=6.14, Se=6.43, Zn=5.02.

3.1.2.3 Data analysis

Statistical analyses were performed using SPSS v.19.0. All reported statistics are arithmetic means, standard deviation (SD) and range (i.e., minimum and maximum concentrations) in micrograms per gram on a wet weight (ww) basis. Spearman's rank correlation coefficient test for non-parametric variables was applied to establish correlation coefficients between metal concentration for each tissue and turtle CCL as a relative indicator of age. This test

was also applied to observe correlations between metals for each tissue. In all cases, *p values* of less than 0.05 were considered statistically significant.

3.1.3 Results

Concentration of Pb, Cd, Cu, Zn, Mn, Se, Ni and As (μg g^{-1}, wet weight) in blood, liver and kidney are presented in Table 1. All of the samples analysed exhibited values above the instrumental limit of detection for all of the elements evaluated. Cadmium and zinc were the elements with highest concentrations in tissues and blood respectively, while lead and nickel were the elements with lowest concentration.

Table 1. Concentration of Pb, Cd, Cu, Mn, Zn, Se, Ni and As in blood, liver and kidney of Olive Ridley Turtles (*Lepidochelys olivacea*) from La Escobilla beach (Oaxaca, Mexico). Mean±SD (range in parenthesis) in μg g^{-1} wet weight.

Element	Blood	Liver	Kidney
Pb	0.02 ±0.01 (0.01-0.05)	0.11± 0.08 (0.03-0.22)	0.06± 0.03 (0.02-0.11)
Cd	0.17± 0.08 (0.06-0.41)	82.88± 36.65 (33.39-163.93)	150.88± 110.99 (0.31-318.26)
Cu	0.61± 0.11 (0.45-0.95)	16.37± 10.34 (3.29-39.19)	1.42± 0.69 (0.27-2.74)
Mn	0.59± 0.10 (0.39-0.90)	3.36± 1.34 (1.93-7.07)	2.71± 1.20 (0.83-5.86)
Zn	10.55± 3.68 (5.29-24.13)	46.65± 16.39 (24.58-80.39)	40.62± 22.62 (10.12-83.46)
Se	5.75± 2.48 (2.02-12.02)	8.25± 2.47 (3.33-12.07)	1.67± 0.78 (0.43-3.38)
Ni	0.04± 0.02 (ND-0.10)	0.08± 0.06 (0.01-0.21)	0.07± 0.04 (0.02-0.17)
As	1.16± 0.70 (0.27-4.21)	3.34± 1.51 (1.05-5.96)	1.38± 0.86 (0.35-3.51)

ND= not detected (Detection limit=0.01 μg g^{-1})

The percentage of blood samples with high concentrations (outliers) were 2.44 (As, one sample with 4.21 μg g^{-1}), 12.20 (Cd, 5 samples with more than 0.32 μg g^{-1}), 7.32 (Cu, 3 samples with more than 0.84 μg g^{-1}), 2.44 (Mn, one sample with 0.90 μg g^{-1}), 2.44 (Pb, one sample with 0.05 μg g^{-1}), 2.44 (Se, one sample with 12.02 μg g^{-1}) and 4.88 (Zn, two samples with more than 22.00 μg g^{-1}). Outliers were not found in the case of Ni. We did not find outlier in blood samples with low metal concentrations. In liver, we found only one outlier with high Mn concentrations (7.07 μg g^{-1}) and one outlier with low Se levels (3.33 μg g^{-1}). Finally, we found one outlier in kidney with high levels of As (3.51 μg g^{-1}), Mn (5.86 μg g^{-1})

and Se (3.38 µg g^{-1}), and several for Cd, with three low values (0.31, 2.11 and 4.67 µg g^{-1}) and four high concentrations (246.95, 257.82, 311.52 and 318.26 µg g^{-1}).

Table 2. Correlation between metals in Blood

	Pb	Cd	Cu	Mn	Zn	Se	Ni	As
Pb	1.00	0.43*	0.43*	0.47*	0.40*	0.61*		0.39
Cd		1.00	0.42*	0.36	0.63*	0.60*		0.61*
Cu			1.00	0.67*	0.78*	0.73*		0.43*
Mn				1.00	0.61*	0.52*		
Zn					1.00	0.82*		0.58*
Se						1.00		0.61*
Ni							1.00	
As								1.00

Table 3. Correlation between metals in Liver

	Pb	Cd	Cu	Mn	Zn	Se	Ni	As
Pb	1.00		-0.57					
Cd		1.00			0.65	0.58		0.60
Cu			1.00					
Mn				1.00			0.71*	
Zn					1.00			
Se						1.00	0.78*	0.58*
Ni							1.00	
As								1.00

Table 4. Correlation between metals in Kidney

	Pb	Cd	Cu	Mn	Zn	Se	Ni	As
Pb	1.00			0.68*	0.56		0.58	
Cd		1.00	0.82*		0.75*			
Cu			1.00		0.96*	0.58		
Mn				1.00				
Zn					1.00			
Se						1.00		
Ni							1.00	
As								1.00

Significant correlation at level 0.05
* Significant correlation at level 0.01

CCL was 65.50±2.39 cm (range 62.50-70.70 cm). The statistically significant relationships between CCL and metal concentration levels were a negative relationship for Mn (*p=0.027*) in liver and kidney, and a positive relationship for Cd (*p=0.050*) in kidney. Positive correlations were found when comparing metal levels in different tissue for the following: Zn and Cd in the three tissues (*p=0.000*); Cd and As in blood (*p=0.000*) and liver (*p=0.010*)

and with Se in blood (*p=0.000*). Also in blood, Pb had a positive relationship with Se (*p=0.000*) and Se with Cu (*p=0.000*); and finally, between Zn and Cu in blood (*p=0.000*) and kidney (*p=0.000*).

3.1.4 Discussion

Biomonitoring is used as an essential tool in assessing the quality of the environment, providing information on environmental degradation and the potential effects of pollutants (Zhou et al., 2008). On the coast of Mexico, a large number of sea turtles arrive to nest; this is due to their longevity and their fidelity to certain feeding areas making them good bio-indicator organisms. According to Páez-Osúna et al. (2010a), their use in biomonitoring marine pollution by metals can give valuable information about the quality of the water they inhabit, which is of great importance in the risk assessment of vulnerable or endangered species. Thus, several authors have shown that concentrations of inorganic elements vary in sea turtles due to their environment and their specific feeding habits, as there are herbivorous, omnivorous and carnivorous species (Camacho et al., 2013b; da Silva et al., 2014; Ley-Quiñónez et al., 2011; Páez-Osúna et al., 2010a; Torrent et al., 2004). With this in mind, and because there are not many papers published on *L. olivacea*, we compared our results with *Caretta caretta* and *Lepidochelys kempii* (Tables 5 to 7) as these species have some similarities in their eating habits with *L. olivacea*, sharing an analogous position in the food chain. We have also made comparisons with other species (*Dermochelys coriacea*, *Chelonia mydas* or *Natator depressus*) when the information was deemed relevant. To enable comparisons, all published results in dry weight have been estimated regarding wet weight using the humidity percentage content in blood (80%) reported by Guirlet et al. (2008) and obtained by García-Fernández et al. (2009) for liver and kidney (75 and 64% respectively). Finally, according to Walton (2001), our number of samples (41 blood and 13 tissues) suggest a probability of 99% (in blood) or 50% (in tissues) that the population will be fall between the lower and upper limits of the observed values.

3.1.4.1 Lead

The mean blood lead concentration was 0.02±0.01 μg g^{-1} ww, a value lower than those reported and estimated from other authors in different turtle species and locations (Table 5), except for Ley-Quiñónez (2009); Ley-Quiñónez et al. (2011) who did not detect the element in *L. olivacea* and *C. caretta* in Mexico and USA. Páez-Osúna et al. (2010a) reported a concentration about 8 times higher than ours, from the arribada of 2005 with a mean of 0.19±0.03 μg g^{-1} in blood (n=25). In a study on tissues from *C. caretta*, Storelli *et al*. (2005) deemed that Pb concentrations below 0.5 μg g^{-1} (ww) should be considered low. In the present study, the concentrations found in liver and kidney fell below these limits, with a mean of 0.11±0.08 and 0.06±0.03 μg g^{-1} ww, respectively. Compared with those found in other studies, the levels detected in liver and kidney are intermediate (Tables 6 and 7), although lower than those described for *L. olivacea* by Frias-Espericueta et al. (2006), who detected 3.32 μg g^{-1} in liver and 4.46 μg g^{-1} in kidney. In the same year and species, Gardner et al. (2006) did not detect lead in liver (detection limit 0.006 μg g^{-1}), and concentration in kidney was very low (Table 7). Nor did Talavera-Saenz et al. (2007) detect lead in liver of *C. mydas*, and the mean in kidney was low. This may indicate that the feeding zone could be responsible for this different lead accumulation (Gulf of Mexico *vs* Pacific Ocean). In this study, the low concentrations found in blood and tissues indicate a reduced exposure to lead, compared with turtles elsewhere in the world as well as a decreased concentration in turtles from the same nesting area compared to 2005 (Páez-Osúna et al., 2010a). According to Maffucci et al. (2005) and Páez-Osúna et al. (2010a), *L. olivacea* has a moderate organic regulation of this element through homeostatic processes. However, such low lead concentrations in tissues in *L. olivacea* and *C. mydas* could also be due to the gradual disuse of leaded gasoline in Mexico and Central America since the 1990s, as the feeding grounds of the turtles that nest in EB extends to the Central Pacific (Morreale et al., 2007) but not the Gulf of Mexico. This does not explain the decrease from the concentration reported by Páez-Osúna et al. (2010a)), indicated that lead detected was from natural sources, although, the number of samples used for lead isotopic analysis was small (*n*= 4). The highest mean

concentration of lead was found in liver, but no clear tropism was shown toward any tissue analysed (Table 6).

3.1.4.2. Cadmium

Mean concentration of cadmium in blood was 0.17±0.08 μg g^{-1} with a range of 0.06-0.41 μg g^{-1}, about 80% higher than those reported by Páez-Osúna et al. (2010b) in samples of *L. olivacea* collected in 2005 also in EB, and almost 10 times higher than those reported in *L. kempii* by Wang (2005) (0.01±0.01 μg g^{-1}) in the Gulf of Mexico. Nonetheless, these concentrations were lower than those reported in the Mexican Pacific North by Ley-Quiñónez (2009) (0.55 and 1.01 μg g^{-1} in *L. olivacea* and *C. caretta* respectively) and by Ley-Quiñónez et al. (2011) in *C. caretta* in Baja California Sur (1.8±0.63 μg g^{-1}). As we can see in Table 5, our results were higher than most of those obtained elsewhere in the world. In tissues analysed, Cd levels were also high (82.88±36.65 μg g^{-1} in liver and 150.88±110.99 μg g^{-1} in kidney), and as in the case of blood, higher than those described by other authors in the Mexican Pacific in *L. olivacea* (Frias-Espericueta et al., 2006; Gardner et al., 2006; Talavera-Saenz et al., 2007) in Baja California, northwest coast of Mexico and Magdalena Bay. To our knowledge, the mean renal cadmium levels found in our study is the highest ever reported for any sea turtle species.

In studies of *C. caretta* in other areas of the world, the highest levels of Cd in tissues have been described in samples from the Eastern Pacific (Japan) with 9.74±3.37 and 38.3±17.5 μg g^{-1} (ww) in liver and kidney respectively (Sakai et al., 2000). In the Mediterranean Sea, García-Fernández et al. (2009) found 5.85±13.42 μg g^{-1} (ww) in liver and 10.49±23.58 μg g^{-1} (ww) in kidney, while Maffucci et al. (2005) found 4.3±8.5 μg g^{-1} (ww) in liver and 19.4±11.8 μg g^{-1} (ww) in kidney. Meanwhile, Torrent et al. (2004) reported in the Canary Islands (Atlantic Ocean) mean of 2.53±0.45 and 5.01±1.02 μg g^{-1} (ww) in the same organs. If we eliminate the three extreme values found in our study, the mean renal Cd concentration (195.40 μg g^{-1}) exceeds all the others, becoming the highest value of Cd described in all species of sea turtles worldwide.

In marine turtles, as in mammals, Cd does not perform any known role in biological systems, and it is only poorly excreted, resulting in long-term storage, specially in kidney, organ to which Cd shows a clear tropism (Andreani et al., 2008b; D'Ilio et al., 2011; Frias-Espericueta et al., 2006; García-Fernández et al., 2009; Storelli et al., 2008; Storelli and Marcotrigiano, 2003). Usually, Cd accumulation in liver and kidney is mainly due to the binding of this metal by metallothionein (MT) (Andreani et al., 2008b), which is heavy metal regulatory and detoxifying intracellular proteins to both physiological (Zn, Cu, Se) and xenobiotic (Cd, Pb, As) elements (Andreani et al., 2008b; ATSDR, 2007a, b, 2012; Cherian et al., 2003). It might have a protective role as an acute-phase protein during toxicity (Sonne et al., 2009). Cd–MT complexes formed in the liver and intestine are preferentially transferred to the kidney where they accumulate (Sonne et al., 2009).This accumulation, associated to its limited degradation and excretion, could explain the high levels of Cd found in kidney.

High Cd blood and tissue concentrations (especially in kidney), higher than values recorded in 2005, indicate that the turtles nesting on EB are chronically exposed to this metal, since Cd levels are as a result of that most of the turtles showed an elevated concentration of this element in both tissues (data not shown). Cd is one of the most toxic elements for wildlife (Barbieri, 2009; Camacho et al., 2013b). Several authors suggest that Cd has a low metabolic regulation in sea turtles (Andreani et al., 2008b; García-Fernández et al., 2009) and it is known that this metal can cause many organic and physiological alterations in marine turtles and other vertebrate species. In this sense, Cd cause endocrine-disruption with effects in hormone systems (Rana, 2014), negative effects on hormone synthesis, disruption in the Ca balance (Boughammoura et al., 2013; Sonne et al., 2009) and negative correlations between red blood cells and Cd concentration (Camacho et al., 2013b). According to Storelli et al. (2005), concentrations of 3.36 μg g^{-1} (ww) in liver and 8.35 μg g^{-1} (ww) in kidney were high enough to damage threatened or endangered marine species health. In our study, all samples of liver and ten kidney samples had higher Cd concentration than reported by these authors as dangerous, so Cd levels found in these sea turtles could be related to their death.

Table 5. Metal concentrations (mean ± SD, µg g^{-1} wet weight) in blood of marine turtles from different locations

Source	Species	Locality	*n*	Pb	Cd	Mn	Cu	Zn	Se	Ni	As
Sakai et al 2000.	*C. caretta*	Japan	7	0.08 ±0.03	9.74±3.37	2.18±0.40	17.7±8.93	28.1±4.73	NA	<0.03	NA
García-Fernández et al., 2009	*C. caretta*	Spain	16	0.69±0.41	5.85±13.42	NA	5.40±2.01	26.8±20.6	NA	NA	NA
Jerez *et al.* 2010 *	*C. caretta*	Spain	13	0.05±0.02	0.20±0.12	NA	NA	7.55±3.05	0.77±0.36	NA	3.18±3.25
Storelli *et al.* 2005.	*C. caretta*	Italy	19	0.16±0.05	3.36±1.94	NA	7.69±4.63	29.3±7.71	3.54±1.49	NA	NA
Andreani *et al.*, 2008*	*C. caretta*	Italy	34	0.025±0.02	0.6±0.1	1.99±0.35	4.37±0.61	25.75±3.5	NA	NA	NA
	C. mydas	Costa Rica	11	0.018±0.003	2.65±0.27	2.24±0.23	25±2.75	20.62±1.22	NA	NA	NA
Torrent *et al.,* 2004	*C. caretta*	Spain (Canary Islands)	78	2.94±0.59	2.53±0.45	NA	15.02±2.07	13.48±1.70	NA	2.88±0.35	17.07±2.9
Gardner *et al.* 2006*	*L.olivacea*	Mexico	6	ND	4.47	0.025	9.18	11.78	NA	0.14	NA
Frías-Espericueta *et al.,* 2006	*L.olivácea*	Mexico	7	3.32	3.28	NA	8.20	NA	NA	NA	NA
Talavera-Saenz *et al.,* 2007	*C. mydas*	Mexico	8	0.00	4.23	0.06	19.13	22.73	NA	0.00	NA
Presente estudio	*L.olivacea*	Mexico	13	0.106±0.075	82.87±36.64	3.36±1.34	16.37±10.34	46.65±16.38	8.245±2.473	0.078±0.055	3.34±1.50

ND = Not detected
NA= Not analysed

Table 6. Metal concentrations (mean ± SD, μg g^{-1} wet weight) in liver of marine turtles from different locations

Source	Species	Locality	*n*	Pb	Cd	Mn	Cu	Zn	Se	Ni	As
Camacho et al., 2013	*C.caretta*	Cape Verde	201	0.06 ± 0.02	0.29 ± 0.25	0.03 ± 0.03	1.27 ± 8.46	4.97 ± 2.9	2.53 ± 2.21	1.41 ± 6.66	0.58 ± 0.95
Guirlet et al., 2008	*D.coriacea*	French Guiana	78	0.18±0.05	0.08±0.03	NA	1.34±0.28	11.10±0.28	9.98±0.05	NA	NA
Jerez et al., 2010*	*C. caretta*	Spain	5	0.062±0.062	0.024±0.042	NA	NA	1.41±0.57	0.56±0.26	NA	1.40±1.86
Ancora *et al.* 2012*	*C. caretta*	Italy	55	0.058±0.075	0.107±0.087	NA	NA	NA	NA	NA	NA
Wang 2005	*L. kempii*	USA	33	0.048±0.024	0.0149±0.0137	NA	0.40±0.09	22.70±12.60	NA	NA	NA
Ley-Quiñones, 2009	*L..olivacea*	México	17	ND	0.549	2.478	1.09	37.19	10.922	2.176	1.876
Ley-Quiñones *et al*., 2011	*C. caretta*	México	22	ND	1.8±0.63	0.61±0.55	2.83±0.62	44.81±17.53	6.14±3.58	1.159±2.42	4.09±2.56
Páez-Osuna *et al.*2010*	*L. olivacea*	México	25	0.19 ± 0.036	0.09 ± 0.04	NA	0.47 ± 0.08	11.68 ± 0.94	NA	0.56 ± 0.26	NA
Present study	*L. olivacea*	México	41	0.022±0.010	0.166±0.098	0.588±0.097	0.603±0.116	10.43±4.12	5.736±2.503	0.036±0.022	1.189±0.695

ND = Not detected
NA= Not analysed

Table7. Metal concentrations (mean ± SD, µg g^{-1} wet weight) in kidney of marine turtles from different locations

Source	Specie	Locality	*n*	Pb	Cd	Mn	Cu	Zn	Se	Ni	As
Sakai et al 2000.	*C. caretta*	Japan	7	0.16±0.05	38.3±17.5	1.50±0.51	1.30±0.216	25.4±4.39	NA	0.217±0.093	NA
García-Fernández et al., 2009	*C. caretta*	Spain	19	0.17±0.16	10.49±23.58	- NA	1.26±1.17	9.29±8.92	NA	NA	NA
Jerez *et al.* 2010*	*C. caretta*	Spain	7	0.41 ±0.29	2.33 ±2.12	- NA	NA -	12.46 ±4.14	1.90 ±1.96	NA	11.80±10.81
Storelli *et al.* 2005.	*C. caretta*	Italy	19	0.12±0.07	8.35±4.83	NA	1.21±0.54	23.1±4.53	NA	NA	NA
Andreani *et al.,* 2008*	*C. caretta*	Italy	9	0.003±0.023	1.97±0.47	2.38±0.44	1.89±0.32	40.46±2.72	NA	NA	NA
	C. mydas	Costa Rica	33	0.018±0.003	13.32±1.05	1.95±0.09	2.83±0.59	26.31±0.64	NA	NA	NA
Torrent *et al.,* 2004	*C. caretta*	Canary Islands	78	2.44±0.48	5.01±1.02	NA -	4.60±0.97	9.09±0.96	NA	5.81±1.10	13.80±2.40
Gardner *et al.* 2006*	*L. olivacea*	Mexico	6	0.01	20.41	1.80	1.65	2.32	NA	0.54	NA
Frías-Espericueta *et al.,* 2006	*L.olivacea*	Mexico	7	4.46	5.28	NA -	6.40	NA	NA	NA	NA
Talavera-Saenz *et al.,* 2007	*C. mydas*	Mexico	8	0.017	37.4	0.51	1.98	64.26	NA	1.08	NA
Presente estudio	*L.olivacea*	Mexico	13	0.057±0.030	150.87±110.99	2.70±1.19	1.41±0.68	40.61±22.61	1.66±0.77	0.071±0.044	1.38±0.85

ND = Not detected
NA= Not analysed

Several authors assert that sea turtles exposure to this and other elements is essentially due to their diet (Andreani et al., 2008b; García-Fernández et al., 2009; Gardner et al., 2006; Maffucci et al., 2005). However, other researchers suggest that diet may not be of great importance, and relate the high accumulation of these metals to the physiology and age of each species (Caurant et al., 1999; Ley-Quiñónez et al., 2011; Saeki et al., 2000; Storelli et al., 2008). Adult olive ridley turtles are facultative carnivorous and generalist predators, and their diet includes fish, crustaceans, molluscs, cephalopods and occasionally algae, among others, occupying a high level on the food chain (CONANP, 2011a; Maffucci et al., 2005; Márquez, 1990; Marquez et al., 2005). Furthermore, cephalopods are reported to be an important vector of Cd to top marine predators, which at the same time, feed with other cephalopods, crustaceans, bivalves and polychetes (Maffucci et al., 2005). Consequently, diet composition and biomagnification in the trophic web could be the main source of Cd for these turtles. On the other hand, the carapace length is used to estimate the age of sea turtles (Gardner et al., 2006) and in our study we found a significant correlation between the CCL and the concentration of Cd (*p=0.050*), so the turtle longevity, along with the MT induction, could be an important factor on the bioaccumulation of Cd in tissues.

3.1.4.3 Manganese, copper, zinc, selenium, nickel and arsenic

Other trace elements in blood of marine turtles are poorly documented (Table 5). In *L. olivacea* there are only two studies where the levels of Cu, Zn and Ni are determined (Ley-Quiñónez, 2009; Páez-Osúna et al., 2010b) and only one work which studies Mn, Se and As (Ley-Quiñónez, 2009). In this species, Gardner et al. (2006) analysed Mn, Cu, Zn and Ni in liver and kidney, while Frias-Espericueta et al. (2006) analysed only Cu.

In our study, the mean blood concentration of Zn (10.55±3.68 µg g^{-1} ww) was similar to that described by Páez-Osúna et al. (2010b) in *L. olivacea* from the same beach (11.68 µg g^{-1} ww) and to that described by Guirlet et al. (2008) on *D. coriacea* in French Guiana (11.10 µg g^{-1} ww), but much lower than those described by Ley-Quiñónez (2009) in *L. olivacea* (37.19 µg g^{-1} ww) in Northern Mexico. Other studies highlight the 44.81 µg g^{-1} (ww) found in *C. caretta*

by Ley-Quiñónez *et al.* (2011) in Mexico, compared to 1.41 μg g^{-1} (ww) found in the same species by Jerez et al. (2010b) in Spain, or 0.151 μg g^{-1} (ww) found in *Natatus depresus* (Ikonomopoulou et al., 2011) in Australia. In the liver, the mean concentration of Zn (46.65±16.39 μg g^{-1} ww) was higher than reported in *L. olivacea* and other species (Table 6). Furthermore, kidney concentration of Zn (40.62±22.62 μg g^{-1} ww) was similar to that found in Italy by Andreani et al. (2008b) in *C. caretta* and superior to the *L. olivacea* from Mexico (Gardner et al., 2006) and other species (Table 7). According to Mas and Azcue (1993), the largest proportions of Zn present in marine habitat precipitates in the form of carbonates deposited in sediments, so the benthonic habits of the adult turtles may lead to a higher degree of exposure in the medium term, with age being a factor which increases the potential accumulation of heavy metals and essential elements (Jerez et al., 2010b; Lutcavage et al., 1997).

Moreover, blood concentrations of Cu were intermediate in *L. Olivacea* from Mexico and other sea turtles (Table 5). In liver, Cu concentration was higher than that reported by Gardner et al. (2006) and Frias-Espericueta et al. (2006) in the same species from Mexico, but with an intermediate value compared to other species and locations (Table 6). In kidney, our results were intermediate compared to all previous works (Table 7). We therefore cannot establish a relationship between these elements and the species or area of origin due to the current lack of information on the subject.

Se and As were also detected in blood at concentrations above 1 μg g^{-1} (5.75±2.48 and 1.16±0.70 μg g^{-1}). Ley-Quiñónez (2009) found in *L. olivacea* Se and As concentrations higher than those detected in our study in blood (Table 5), while we have found no study which evaluates these elements in tissues for this species. Storelli et al. (2005) reported that the accumulation of Se in some marine mammals is always elevated, and that in aquatic reptiles it is difficult to establish whether concentrations of Se can be toxic or merely background level. A previous publication described that higher Se levels were identified in the pelagic phase of the species, when turtles are carnivorous (Aguirre et al., 1994b).

Marine animals such as molluscs and crustaceans, which are a part of the Olive Ridley turtle sea turtle's diet, may retain concentrations of As, but only a small percentage in toxic form (Márquez, 1990; Mas and Azcue, 1993; Montenegro and Bernal, 1982; Ozretić et al., 1990). If we compare this with other species of sea turtles (Tables 5 to 7), blood and tissue concentrations of Se and As were intermediate (Se and As in blood and As in liver), highest (Se in liver), and lowest (Se and As in kidney), so those concentrations were not connected with species or area of origin, and we cannot specify a reference standard in the concentrations of these elements.

Finally, comparing the concentrations of Mn and Ni with other studies in *L. olivacea* (Tables 5 to 7), we found that concentrations in blood (0.59±0.10 and 0.04±0.02 μg g^{-1} ww respectively) were lower than those described by Ley-Quiñónez (2009) and Páez-Osúna et al. (2010b), while the concentration of Mn in liver and kidney was higher than that described by Gardner et al. (2006). However, Ni in liver and kidney was far below that seen by the same authors. For other sea turtles, Mn and Ni concentrations in blood and tissue have only been measured in *C. caretta* (Andreani et al., 2008b; Camacho et al., 2013b; Ley-Quiñónez et al., 2011; Sakai et al., 2000; Torrent et al., 2004) and *C. mydas* tissues (Andreani et al., 2008b; Talavera-Saenz et al., 2007). In these works, concentrations of Mn in both species were lower than in our study (except that reported by Ley-Quiñónez *et al.* in 2011 in blood of *C. caretta* from Mexico), and concentrations of Ni were higher, except that reported by Sakai et al. (2000) and Talavera-Saenz et al. (2007) in liver of *C. caretta* from Japan and *C. mydas* from Mexico.

3.1.4.4 Element correlations

Several authors have reported correlations between concentrations of metals in tissues and blood of different species of sea turtles (Camacho et al., 2013b; García-Fernández et al., 2009; Gardner et al., 2006; Ikonomopoulou et al., 2011; Jerez et al., 2010b; Ley-Quiñónez et al., 2011; Maffucci et al., 2005; Torrent et al., 2004). In *L. olivacea*, we have only found

one work with metal correlations in tissues (Gardner et al., 2006), and our results do not concur with them. However, some of the correlations observed in the present study have been previously found in other turtle species, such as the blood correlations reported in *C. caretta* by Jerez et al. (2010b), Ley-Quiñónez et al. (2011) and Camacho et al. (2013b), or in *Natator depressus* by Ikonomopoulou et al. (2011). In our study, some kidney correlations were also similar to those reported in *C. caretta* (García-Fernández et al., 2009; Gardner et al., 2006; Maffucci et al., 2005; Torrent et al., 2004) and *C. mydas* (Gardner et al., 2006); and some liver correlations were similar to those reported in *C. caretta* (Maffucci et al., 2005; Torrent et al., 2004) and *C. mydas* (Gardner et al., 2006).

A positive correlation between Zn and Cd in blood ($p<0.01$), liver ($p<0.05$) and kidney ($p<0.01$) was also found (Tables 2 to 4). In our study, Cd and Zn concentration was higher, particularly in kidney (150.88±110.99 and 40.62±22.62 μg g^{-1} respectively), and other studies on sea turtles show high concentrations of Zn when the mean concentrations of Cd were also high (Tables 5 to 7). In many species, a certain Cd quantity accumulates linked to MT (Andreani et al., 2008b). Zn is also considered an inductor of MT in terrestrial and marine animals (García-Fernández et al., 2009). Therefore, according to several authors (ATSDR, 2012; Maffucci et al., 2005), the significant positive correlation between Cd and Zn in kidneys could suggest the implication of these MTs in the transport of these metals and in the prevention of the toxic effects of cadmium in *L. olivacea*. On the other hand, a positive correlation between Pb, Cu, Mn, Se and As with Zn was also found in blood (Table 2), and a similar relationship of Pb and Cu with Zn was described in kidney (Table 4). Therefore, as occurs in loggerhead, green and hawksbill turtles with Cd, Zn and Cu (Anan et al., 2002; García-Fernández et al., 2009; Maffucci et al., 2005; Storelli et al., 2005), olive ridley turtles could be able to regulate the concentrations of these metals by metabolic processes linked also to the MT.

The concentrations of Se were positively associated in blood with Pb, Cd, Cu, Zn, Mn and As (Table 2), in liver with Cd, Ni and As (Table 3), and in kidney with Cu (Table 5). Essential

elements like Cu, Mn, Se, and Zn play vital roles in tissue metabolism and growth, but the negative effects of some of these elements on sea turtles are not well known (Camacho et al., 2013b). According to Maffucci et al. (2005), it appears that loggerhead turtles can regulate Se concentrations through homeostatic processes in a balance between metabolic requirements and prevention against toxic effects. Also, the presence of Se in tissues reduces Cd toxicity by storing and reallocating these toxic metals and thus, diminishing its potential adverse effect (Siscar et al., 2014). Heavy metals or metalloids, including Pb, As and Cd, have an important physiological role in reactive oxygen species (ROS)-generating reactions (Konigsberg Fainstein, 2008), and elements such as Se, Zn, Cu or Mn have a protective role in preventing metals toxicity in aquatic organisms due to their association with antioxidant enzymes (Livingstone, 2001), such as glutathione peroxidase and superoxide dismutase. However, knowledge about the antioxidant responses and oxidative stress in wildlife is scarce (McGraw et al., 2010; Nussey et al., 2009) and in sea turtles, almost non-existent (Labrada-Martagon et al., 2011a).Therefore, and because data relating to elements like Se, As or Mn in sea turtles is scarce, further studies are warranted to explain correlations of these elements in blood and tissue of Olive Ridley turtles.

Since no method for age determination in marine turtles has been established, the CCL was used to determine size as a relative indicator of age (Gardner et al., 2006). As we have already mentioned, the statistically significant correlations between the CCL and the trace elements were a negative correlation with Mn (*p=0.027*), in liver and kidney, and the positive correlation with Cd (*p=0.050*) above mentioned. Correlation with Mn may be caused by the diet of the animals, as certain types of algae are an important source of inorganic elements (Sierra-Vélez and Álvarez-León, 2009), such as Mn, so this correlation could indicate that algae represents a greater percentage of the diet of younger turtles than older turtles, which tend to be more carnivorous (Marquez et al., 1996).

3.1.5 Conclusions

This study establishes that turtles of the species *L. olivacea* that nest on the beach "La Escobilla" (one of the most important beaches in the world for the reproduction of this species) contain one of the highest concentrations of Cd reported worldwide, a heavy metal capable to produce serious pathologies in sea turtles, in the reproductive, skeletal or immune system, inter alia. Nonetheless, Pb levels have declined in recent years in this population. Due to the lack of information on trace elements, it is difficult to establish whether the levels found are physiological or not. This research provides information needed to better understand possible variations in sea turtle natural environment, but it is necessary to closely monitor the levels of all these elements in blood and tissues in order to better know the risk they may pose to this population.

References

Aguirre, A.A., Balazs, G., Zimmerman, B., Spraker, T.R., 1994a. Evaluation of Hawaiian green turtles (*Chelonia mydas*) for potential pathogens associated with fibropapillomas. J. Wildl. Dis. 30, 8-15.

Aguirre, A.A., Balazs, G.H., Zimmerman, B., Galey, F.D., 1994b. Organic contaminants and trace metals in the tissues of green turtles (*Chelonia mydas*) afflicted with fibropapillomas in the Hawaiian Islands. Marine Pollution Bulletin 28, 109-114.

Alava, J.J., Keller, J.M., Kucklick, J.R., Wyneken, J., Crowder, L., Scott, G.I., 2006. Loggerhead sea turtle (*Caretta caretta*) egg yolk concentrations of persistent organic pollutants and lipid increase during the last stage of embryonic development. Science of the Total Environment 367, 170-181.

Anan, Y., Kunito, T., Sakai, H., Tanabe, S., 2002. Subcellular distribution of trace elements in the liver of sea turtles. Marine Pollution Bulletin 45, 224-229.

Andreani, G., Santoro, M., Cottignoli, S., Fabbri, M., Carpene, E., Isani, G., 2008. Metal distribution and metallothionein in Loggerhead (*Caretta caretta*) and Green (*Chelonia mydas)* sea turtles. Science of the Total Environment 390, 287-294.

ATSDR, 2007a. Toxicological profil for arsenic, Agency for Toxic Substances and Disease Registry. U.S. Departament of Health and Human Services. Public Health Service. Agency for Toxic Substances and Disease Registry, Atlanta, Georgia, p. 500.

ATSDR, 2007b. Toxicological profile for lead. U.S. Departament of Health and Human Services. Public Health Service. Agency for Toxic Substances and Disease Registry, Atlanta, Georgia.

ATSDR, 2012. Toxicological profile for cadmium. U.S. Departament of Health and Human Services. Agency for Toxic Substances and Disease Registry, Georgia, U.S.

Barbieri, E., 2009. Concentration of heavy metals in tissues of Green turtles (*Chelonia mydas*) sampled in the Cananéia Estuary, Brazil. Brazilian Journal of Oceanography 57, 243-248.
Boughammoura, S., Kessabi, K., Chouchene, L., Messaoudi, I., 2013. Effects of cadmium and high temperature on some parameters of calcium metabolism in the killifish (*Aphanius fasciatus*). Biological Trace Element Research 154, 73-80.
Camacho, M., Oros, J., Boada, L.D., Zaccaroni, A., Silvi, M., Formigaro, C., Lopez, P., Zumbado, M., Luzardo, O.P., 2013. Potential adverse effects of inorganic pollutants on clinical parameters of Loggerhead sea turtles (Caretta caretta): Results from a nesting colony from Cape Verde, West Africa. Marine Environmental Research 92, 15-22.
Caurant, F., Bustamante, P., Bordes, M., Miramand, P., 1999. Bioaccumulation of cadmium, copper and zinc in some tissues of three species of marine turtles stranded along the French Atlantic coasts. Marine Pollution Bulletin 38, 1085-1091.
Cherian, M.G., Jayasurya, A., Bay, B.-H., 2003. Metallothioneins in human tumors and potential roles in carcinogenesis. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 533, 201-209.
CONANP, 2011. Ficha de la tortuga golfina, in: Marinas, P.N.p.l.C.d.l.T. (Ed.), Dirección para Especies Prioritarias para la Conservación. Comisión Nacional de Áreas Naturales Protegidas, México, D.F., p. 7.
D'Ilio, S., Mattei, D., Blasi, M.F., Alimonti, A., Bogialli, S., 2011. The occurrence of chemical elements and POPs in loggerhead turtles (*Caretta caretta*): an overview. Marine Pollution Bulletin 62, 1606-1615.
da Silva, C.C., Varela, A.S., Jr., Barcarolli, I.F., Bianchini, A., 2014. Concentrations and distributions of metals in tissues of stranded green sea turtles (Chelonia mydas) from the southern Atlantic coast of Brazil. Science of the Total Environment 466-467, 109-118.
Franzellitti, S., Locatelli, C., Gerosa, G., Vallini, C., Fabbri, E., 2004. Heavy metals in tissues of loggerhead turtles (*Caretta caretta*) from the northwestern Adriatic Sea. Comp Biochem Physiol C Toxicol Pharmacol 138, 187-194.
Frias-Espericueta, M.G., Osuna-Lopez, J.I., Ruiz-Telles, A., Quintero-Alvarez, J.M., Lopez-Lopez, G., Izaguirre-Fierro, G., Voltolina, D., 2006. Heavy metals in the tissues of the sea turtle *Lepidochelys olivacea* from a nesting site of the northwest coast of Mexico. Bulletin of Environmental Contamination and Toxicology 77, 179-185.
Fuentes-Mascorro, G., López-Rojas, F., Domínguez-Martínez, M., 2007. XXV Aniversario de conservación e investigación en tortuga marina. Universidad Autónoma Benito Juárez, Oaxaca, México.
García-Fernández, A.J., Gómez-Ramírez, P., Martínez-López, E., Hernández-García, A., María-Mojica, P., Romero, D., Jiménez, P., Castillo, J.J., Bellido, J.J., 2009. Heavy metals in tissues from loggerhead turtles (*Caretta caretta*) from the southwestern Mediterranean (Spain). Ecotoxicology and Environmental Safety 72, 557-563.
Gardner, S.C., Fitzgerald, S.L., Vargas, B.A., Rodriguez, L.M., 2006. Heavy metal accumulation in four species of sea turtles from the Baja California peninsula, Mexico. Biometals 19, 91-99.
Godley, B.J., Gaywood, M.J., Law, R.J., McCarthy, C.J., McKenzie, C., Patterson, I.A.P., Penrose, R.S., Reid, R.J., Ross, H.M., 1998. Patterns of marine turtle mortality in British

waters (1992- 1996) with reference to tissue contaminant levels. Journal of the Marine Biological Association of the United Kingdom 78, 973-984.
Godley, B.J., Thompson, D.R., Furness, R.W., 1999. Do heavy metal concentrations pose a threat to marine turtles from the Mediterranean sea? Marine Pollution Bulletin 38, 497-502.
Gordon, A.N., Pople, A.R., Ng, J., 1998. Trace metal concentrations in livers and kidneys of sea turtles from south-eastern Queensland, Australia. Mar. Freshwater Res. 49, 409-414.
Guirlet, E., Das, K., Girondot, M., 2008. Maternal transfer of trace elements in leatherback turtles (*Dermochelys coriacea)* of French Guiana. Aquatic Toxicology 88, 267-276.
Homer, B., Foley, A., Fick, K., Lores, M., Redlow, A., Jacobson, E., 2000. Lesions, pathogens and toxins identified in 13 stranded marine turtles in Florida, Proceedings of the 18th International Symposium on Sea Turtle Biology and Conservation, NOAA Technical Memorandum, NMFS-SEFSC-436, Mazatlan, Sinaloa, Mexico, pp. 117-118.
Ikonomopoulou, M.P., Olszowy, H., Limpus, C., Francis, R., Whittier, J., 2011. Trace element concentrations in nesting flatback turtles (*Natator depressus*) from Curtis Island, Queensland, Australia. Marine Environmental Research 71, 10-16.
IUCN, 2012. Red List of Threatened Species, Version 2012.1. International Union for Conservation of Nature, http://www.iucnredlist.org/.
Jerez, S., Motas, M., Canovas, R.A., Talavera, J., Almela, R.M., Bayón del Rio, A., 2010. Accumulation and tissue distribution of heavy metals and essential elements in loggerhead turtles (*Caretta caretta*) from Spanish Mediterranean coastline of Murcia. Chemosphere 78, 256-264.
Kaska, Y., Çelik, A., Bağ, H.s., Aureggi, M., Özel, K., Elçi, A., Kaska, A., Elçi, L., 2004. Heavy metal monitoring in stranded sea turtles along the Mediterranean coast of Turkey. Fresenius Environmental Bulletin 13, 769-776.
Konigsberg Fainstein, M.A.-M., 2008. Radicales libres y estrés oxidativo: aplicaciones médicas.
Labrada-Martagon, V., Rodriguez, P.A., Mendez-Rodriguez, L.C., Zenteno-Savin, T., 2011. Oxidative stress indicators and chemical contaminants in East Pacific green turtles (*Chelonia mydas*) inhabiting two foraging coastal lagoons in the Baja California peninsula. Comparative Biochemistry and Physiology Part C 154, 65-75.
Ley-Quiñónez, C., 2009. Determinación de Metales Pesados en tortugas del noreste de México, Departamento de Medio Ambiente. INSTITUTO POLITÉCNICO NACIONAL, p. 76.
Ley-Quiñónez, C., Zavala-Norzagaray, A.A., Espinosa-Carreon, T.L., Peckham, H., Marquez-Herrera, C., Campos-Villegas, L., Aguirre, A.A., 2011. Baseline heavy metals and metalloid values in blood of loggerhead turtles (*Caretta caretta*) from Baja California Sur, Mexico. Marine Pollution Bulletin 62, 1979-1983.
Livingstone, D., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Marine pollution bulletin 42, 656-666.
Lutcavage, M.E., Plotkin, P., Witherington, B., Lutz, P.L., 1997. Human impacts on sea turtle survival. The biology of sea turtles 1, 387-409.

Maffucci, F., Caurant, F., Bustamante, P., Bentivegna, F., 2005. Trace element (Cd, Cu, Hg, Se, Zn) accumulation and tissue distribution in loggerhead turtles (*Caretta caretta*) from the Western Mediterranean Sea (Southern Italy). Chemosphere 58, 535-542.
Márquez, M.R., 1990. Sea Turt[es of the World. An annotated and ilustrated catalogue of sea turtles species known to date, FAO Species Catalogue. Food and Agriculture Organization of the United Nations, Rome, Italia, p. 81.
Marquez, M.R., Peñaflores, C., Vasconcelos, J.C., 1996. Olive ridley turtles (*Lepidochelys olivacea*) show signs of recovery at La Escobilla, Oaxaca. Marine Turtle Newsletter 73, 5-7.
Marquez, R., Burchfield, P., Diaz, J., Sanchez, M., Carrasco, M., Jimenez, C., Leo, A., Bravo, R., Pena, J., 2005. Status of the Kemp's ridley sea turtle, Lepidochelys kempii. Chelonian Conservation and Biology 4, 761-766.
Mas, A., Azcue, J.M., 1993. Metales en Sistemas Biológicos. . PPU, Barcelona,, p. 324.
McGraw, K.J., Cohen, A.A., Costantini, D., Hõrak, P., 2010. The ecological significance of antioxidants and oxidative stress: a marriage between mechanistic and functional perspectives. Functional Ecology 24, 947-949.
Montenegro, S.B., Bernal, G., 1982. Análisis del contenido estomacal de *Lepidochelys olivacea*. Universidad Nacional Autónoma de México, México.
Morreale, S.J., Plotkin, P.T., Shaver, D.J., Kalb, H.J., 2007. Adult migration and habitat utilization. Biology and conservation of Ridley sea turtles. Johns Hopkins University Press, Baltimore, Maryland, 213-229.
Nussey, D.H., Pemberton, J.M., Pilkington, J.G., Blount, J.D., 2009. Life history correlates of oxidative damage in a free - living mammal population. Functional Ecology 23, 809-817.
Ozretić, B., Krajnović-Ozretić, M., Santin, J., Medjugorac, B., Kraš, M., 1990. As, Cd, Pb, and Hg in benthic animals from the Kvarner-Rijeka Bay region, Yugoslavia. Marine pollution bulletin 21, 595-598.
Páez-Osúna, F., Calderon-Campuzano, M.F., Soto-Jiménez, M.F., Ruelas-Inzunza, J., 2011. Mercury in blood and eggs of the sea turtle *Lepidochelys olivacea* from a nesting colony in Oaxaca, Mexico. Mar Pollut Bull 62, 1320-1323.
Páez-Osúna, F., Calderón-Campuzano, M.F., Soto-Jiménez, M.F., Ruelas-Inzunza, J.R., 2010a. Lead in blood and eggs of the sea turtle, *Lepidochelys olivacea*, from the Eastern Pacific: concentration, isotopic composition and maternal transfer. Marine Pollution Bulletin 60, 433-439.
Páez-Osúna, F., Calderón-Campuzano, M.F., Soto-Jiménez, M.F., Ruelas-Inzunza, J.R., 2010b. Trace metals (Cd, Cu, Ni, and Zn) in blood and eggs of the sea turtle *Lepidochelys olivacea* from a nesting colony of Oaxaca, Mexico. Archives of Environmental Contamination and Toxicology 59, 632-641.
Perrault, J., Wyneken, J., Thompson, L.J., Johnson, C., Miller, D.L., 2011. Why are hatching and emergence success low? Mercury and selenium concentrations in nesting leatherback sea turtles (Dermochelys coriacea) and their young in Florida. Marine Pollution Bulletin 62, 1671-1682.
Rana, S.V., 2014. Perspectives in endocrine toxicity of heavy metals-a review. Biological Trace Element Research 160, 1-14.

Saeki, K., Sakakibaea, H., Sakai, H., Kunito, T., Tanabe, S., 2000. Arsenic accumulation in three species of sea turtles. Biometals 13, 241-250.
Sakai, H., Saeki, K., Ichihashi, H., Suganuma, H., Tanabe, S., Tatsukawa, R., 2000. Species-specific distribution of heavy metals in tissues and organs of Loggerhead turtle (*Caretta caretta*) and Green turtle (*Chelonia mydas*) from Japanese Coastal Waters. Marine Pollution Bulletin 40, 701-709.
Sierra-Vélez, L., Álvarez-León, R., 2009. Comparación Bromatológica de las algas nativas (Gracilariopsis tenuifrons, Sargassum filipendula) y exóticas (Kappaphycus Alvarezii) del caribe colombiano. BOLETÍN CIENTÍFICO CENTRO DE MUSEOS MUSEO DE HISTORIA NATURAL Vol. 13 No. 2, 17.
Siscar, R., Koenig, S., Torreblanca, A., Sole, M., 2014. The role of metallothionein and selenium in metal detoxification in the liver of deep-sea fish from the NW Mediterranean Sea. Sci Total Environ 466-467, 898-905.
Sonne, C., Aspholm, O., Dietz, R., Andersen, S., Berntssen, M.H., Hylland, K., 2009. A study of metal concentrations and metallothionein binding capacity in liver, kidney and brain tissues of three Arctic seal species. Science of the Total Environment 407, 6166-6172.
Storelli, M.M., Barone, G., Storelli, A., Marcotrigiano, G.O., 2008. Total and subcellular distribution of trace elements (Cd, Cu and Zn) in the liver and kidney of green turtles (*Chelonia mydas*) from the Mediterranean Sea. Chemosphere 70, 908-913.
Storelli, M.M., Ceci, E., Marcotrigiano, G.O., 1998. Comparison of Total Mercury, Methylmercury, and Selenium in Muscle Tissues and in the Liver of Stenella coeruleoalba (Meyen) and Caretta caretta (Linnaeus). Bull. Environ. Contam. Toxicol. 61, 541-547.
Storelli, M.M., Marcotrigiano, G.O., 2003. Heavy metal residues in tissues of marine turtles. Marine Pollution Bulletin 46, 397-400.
Storelli, M.M., Storelli, A., D'Addabbo, R., Marano, C., Bruno, R., Marcotrigiano, G.O., 2005. Trace elements in loggerhead turtles (*Caretta caretta*) from the eastern Mediterranean Sea: overview and evaluation. Environmental Pollution 135, 163-170.
Talavera-Saenz, A., Gardner, S.C., Riosmena Rodriquez, R., Acosta Vargas, B., 2007. Metal profiles used as environmental markers of green turtle (Chelonia mydas) foraging resources. Sci Total Environ 373, 94-102.
Torrent, A., Gonzalez-Diaz, O.M., Monagas, P., Oros, J., 2004. Tissue distribution of metals in loggerhead turtles (*Caretta caretta*) stranded in the Canary Islands, Spain. Marine Pollution Bulletin 49, 854-860.
Walton, R.M., 2001. Establishing reference intervals: health as a relative concept, Seminars in Avian and Exotic Pet Medicine. Elsevier, pp. 66-71.
Wang, H.-C., 2005. Trace metal uptake and accumulation pathways in Kemp's Ridley. Texas A&M University, p. 275.
Zhou, Q., Zhang, J., Fu, J., Shi, J., Jiang, G., 2008. Biomonitoring: an appealing tool for assessment of metal pollution in the aquatic ecosystem. Anal Chim Acta 606, 135-150.

3.2 Chapter III. Three years of inorganic elements monitoring

3.2.1 Introduction

Long-live marine species as cetaceans and sea turtles are becoming an important tool in ecotoxicology, because of their sensitivity to marine environmental change, especially regarding pollution. Indeed, they usually occupy on high trophic levels in the marine food web, therefore they accumulate different pollutants during their life time (Aguirre and Lutz, 2004; Caceres-Saez et al., 2013; Camacho et al., 2013b). In marine turtles inorganic element concentration shown to vary depending on several factors, such as diet, age, location and migration habits (Andreani et al., 2008a; Guirlet et al., 2008; Reijnders, 2003). Inorganic elements found in the marine environment are natural components of the Earth's crust but they have also been introduced to the ecosystem by anthropogenic sources. Being fundamental elements, they cannot be degraded; rather they can be toxic. Some of them, such as metals and metalloids, tend to bioaccumulate and this can alter the natural biological equilibrium (Camacho et al., 2013b; Haynes and Johnson, 2000). As a consequence of the aforementioned circumstances several authors concluded (Camacho et al., 2014a; Ley-Quiñónez et al., 2011; Páez-Osúna et al., 2011) that databases of inorganic elements contamination must be established for species and population areas. Baseline measurements will allow comparisons with further studies into the health status of the populations and it will increase our knowledge about the pollution levels of the areas where these studied turtles live.

Most of the data available in literature have been focus on three metals (Pb, Cd and Hg) and few trace elements (Zn, Cu, Mn, Se commonly and Ni and As sometimes). As far as we know, except for Ca, Mg and K baseline level for other inorganic elements have been little or never establish for marine turtle populations (these last usually in haematology studies), neither as some other heavy metals that, although being less distributed in the environment, can be toxic to both humans and animals, such Sb, Ti or Tl (Bannon, 2015; Burford et al., 2011; Chen et al., 2014).

Blood is thought to be a good tool for the simultaneous monitoring of environmental contaminants and clinical parameters (Camacho et al., 2013b), however is well know that, in blood, only the elements of a recent exposition can be found (acute exposure). In order to better know the chronic exposure and accumulation of these elements, it is necessary to use their accumulation target organs. For necropsies, these studies involve specimens derived usually from bycatch or stranding events (Lahaye et al., 2006). Nevertheless, significant difficulties to have access to carcasses existed, and many times they are undergone post-mortem autolysis organs, or animals died from some pathological process, which may alter results. Because, in most cases, these kind of samples are the only one available for research, it represent an opportunistically but basic source of information (Storelli et al., 1998). Thus, it is important that methods for measuring elemental contents in carcasses are developed to obtain as much information as possible, such as the animals' health, nutritional status and their relation with the marine environment (Agusa et al., 2011; Caceres-Saez et al., 2013; Lavery et al., 2008; Lavery et al., 2009).

Seven species of marine turtles exist in the world; all of them being listed in Appendix I of the Convention of International Trade in Endangered Species (CITES) and in the Red List of the IUCN (International Union for Conservation of Nature) (IUCN, 2012). In Mexico, six of the seven species can be found nesting (*Lepidochelys olivacea, Lepidochelys kempii, Caretta caretta, Dermochelys coriacea, Chelonia mydas,* and *Eretmochelys imbricata*). Also, all sea turtles are considered as endangered species within the regulation norm NOM-ECOL-059-2010 (SEMARNAT, 2010), and they have been made a priority species for conservation. The Olive Ridley (*Lepidochelys olivacea*) is the most abundant and recovered turtles in the country, and some authors affirm that on Mexicans beaches one quarter of the nests world's population are deposited (Abreu-Grobois *et al*., 2007), most of them in the State of Oaxaca, especially on the Escobilla Beach (EB), which is one of the most important beaches in the world for Ridleys "arribadas". All this makes La Escobilla a beach of great importance to 1) the study of the health of Mexican population, and 2) as a bioindicator of pollutants

from the Eastern Pacific region and how this changes on an annual basis (Páez-Osúna et al., 2010a, b).

During the arribadas, up to 400,000 turtles in a week period can nest (Ocana, 2010). This high concentration of turtles on the same point is often a cause of accidents, both from human causes (propellers of boats that are close to this beach), or a vast carapace/head trauma caused by waves or crashing two turtles among themselves while trying to get to the sand. In this context, recently dead turtles or even turtles with major injuries agonizing can be found along the beach during the arribadas. All these events make an opportunity of have access to apparently healthy alive turtles as well as dead turtles without a clear cause of death (possible cause for diseases, migration extenuation, age related, *inter alia*).

The aims of this work was to assess concentrations of inorganic essential elements (Al, As, Cu, Cr, Fe, Mn, Ni, Se and Zn) and non-essential (Pb, Cd, Li, Sr, Ti and Tl) in blood of live animals; and in blood, liver, kidney muscle, bone, brain, fat and eggs from dead turtles. To make it possible, 241 nesting marine turtles and 58 dead turtles were sampled during 3 consecutive years (2012-2014) within 8 different arribadas on La Escobilla beach. Using these data we will: 1) show the variation in metal concentration and accumulation during the 3 monitoring years; 2) establish baseline levels of selected essential and non-essential elements in blood and tissues of this species from this area of the Eastern Pacific waters; 3) find differences between alive and death turtles blood metal concentrations; and finally, 4) establish the utility of using only blood samples in biomonitoring programs and its relation with the accumulation organs in this sea turtle.

3.2.2. Material and methods

3.2.2.1 Sample collection

Samples (blood and tissues) were collected at La Escobilla beach, in the State of Oaxaca (Southeast Mexico, Eastern Pacific, 96°44'W and 15°47'N) (Figure 1). Beach monitoring was performed between August and September 2012 (nesting season, third arribada event),

August to October 2013 (third and fourth arribadas) and August to October 2014 (fourth to sixth arribadas). curved carapace length (CCL), Curve carapace wide (CCW) and mass of the turtles was recorded.

Two hundred and forty-one apparently healthy nesting turtles were selected at random for blood samples. Blood was taken from the dorsal cervical sinus, using 5 mL non-heparinized tubes and 21" needles. Before blood extraction, the neck region was carefully cleaned using ethanol and deionized water. Blood, liver, kidney, muscle, bone, fat and brain samples were taken from 58 dead turtles found along the beach in the same periods. Twenty-five of these turtles died from head trauma possibly caused by boat propellers; the cause of death of the other 33 turtles is unknown. CCL and CCW were also recorded, additionally we give a value for the cause of dead of the individuals. The decomposition of the carcasses was also recorded, giving a value from 1 (fresh carcasses) to 5 (very rotten carcasses), to explore if the carcasses condition could alter the concentration of the studied elements. All samples were transferred into 1.5 mL microtubes and kept at -20^{o} until processed (Permit SAGARPA Numbers: SGPA/DGVS/10445/11, SGPA/DGVS/00905/13 and SGPA/DGVS/07774/14).

3.2.2.2 Element Analysis

Prior to the analysis, all the samples and laboratory materials used were thoroughly rinsed with deionized water (Milli-Q) to avoid contamination. It was then performed a pre-treatment of the samples: 0.5 g of each sample (blood, liver and kidney in wet weight) was taken for an acid digestion using 4 mL of HNO_3 (69%) and 1 mL of H_2O_2 (33%) mixed in special Teflon reaction tubes in a microwave digestion system (UltraClave-Microwave Milestone®) for 20 minutes at 220°C and finally diluted with 25 mL of double deionized water (Milli-Q).

Metal concentrations were determined using an inductively coupled plasma optical emission spectrophotometer (ICP-OES, ICAP 6500 Duo Thermo®). All concentrations are expressed in microgram per gram in wet weight. Detection limits for all elements were

0.01 µg g^{-1}. Two readings of each sample were made. To check for possible contamination, 1 blank sample for every 11 samples was also analysed.

Multielement calibration standards were prepared with different concentrations of inorganic elements, taking as reference the UNE-EN ISO 11885 for the determination of elements by inductively coupled plasma atomic emission spectroscopy. In addition, intermediate patterns of all elements were prepared. The equipment calibration was established by batch. Calibration was established with a minimum of three points for each lot within the range of observed concentrations. Each run began with the calibration standards and continued with samples and intermediate patterns, finishing the series with intermediate patterns (10% variation coefficient).

3.2.2.3 Statistics

Data for this paper was separated into live and dead individuals. Histograms and Shapiro Wilk tests were used to assessed the normality of the data. Since the distribution was not normal different approaches were used: 1) Spearman's rank correlation coefficient test for non-parametric variables were applied to stablish correlations coefficients between elements and biometrics in live turtles, and between elements, biometrics, cause of dead and decomposition level in every tissue in dead turtles for the 3 monitored years; 2) In order to explore the differences in all the elements concentrations and the three sampled years, we used log() function to normalized the data before tested for the analysis of the variance with one-way ANOVA test (years *vs* every element in each tissue), in both live and dead turtles; 3) In a similar way, one-way ANOVAs were used to assess the relationships among element concentrations in blood and in every tissue of dead turtles. In all cases *p* values of less than 0.05 were included as significant results. All statistical analysis were performed using R 3.4.0 (R Core Team, 2017).

3.2.3. Results and Discussion

As it has been shown in Chapter I (The Art State), inorganic elements are worldwide distributed for the 7 species of marine turtles. However, the absorption and accumulation of these elements seem to be related to 1) the bioavailability of the element in the environment, 2) the diet of the different species and 3) the physiology of each species. In a previous work (Chapter II), it has been shown high concentrations of certain of these elements, specially Cd, with the highest report worldwide. Regarding non-essential elements (Cd and Pb), it was impossible to establish if these concentrations were from one-time exposition or if the concentration of these elements is constant all the time in the feeding and nesting areas of these turtles. On the other hand, the concentration of essential inorganic elements was also difficult to assess if they were normal for this population or they were influenced for some external sources of pollution.

This was a middle-term program regarding inorganic elements surveillance in *L. olivacea* turtles during three consecutive years and 8 different arribada events. Thus, a database for some essential elements (Al, As, Cr, Cu, Fe, Mn, Ni, Se and Zn) as well as the concentration of some non-essential elements (Cd, Li, Pb, Sr, Ti and Tl) has been created. With this information, we will know 1) the level of pollution for the measured elements in this population environment and their variations through the time; and 2) the elements with high/alarming concentrations that could affect the health of theses turtles and focus more research studies on them.

3.2.3.1 Live turtles

The use of blood to assess the level of exposure to elements is an attractive option for studies on wildlife health because blood can be non-lethally collected and allows the determination of many environmental pollutants with a small blood sample (1 mL). This present study represents the largest research study that has investigated the concentrations in blood of inorganic pollutants and essential elements of sea turtles (*n*=241). CCL and CCW are given in Table 1.

Table 1. Curved carapace length (CCL) and width (CCW) of alive Olive Ridleys from La Escobilla.

	CCL	CCW
MIN	55	60.5
1st Qu	64	67.4
Median	65.5	70
Mean	65.64	69.5
3rd Qu	67.62	71
Max	76	79
***n*=**	179	65

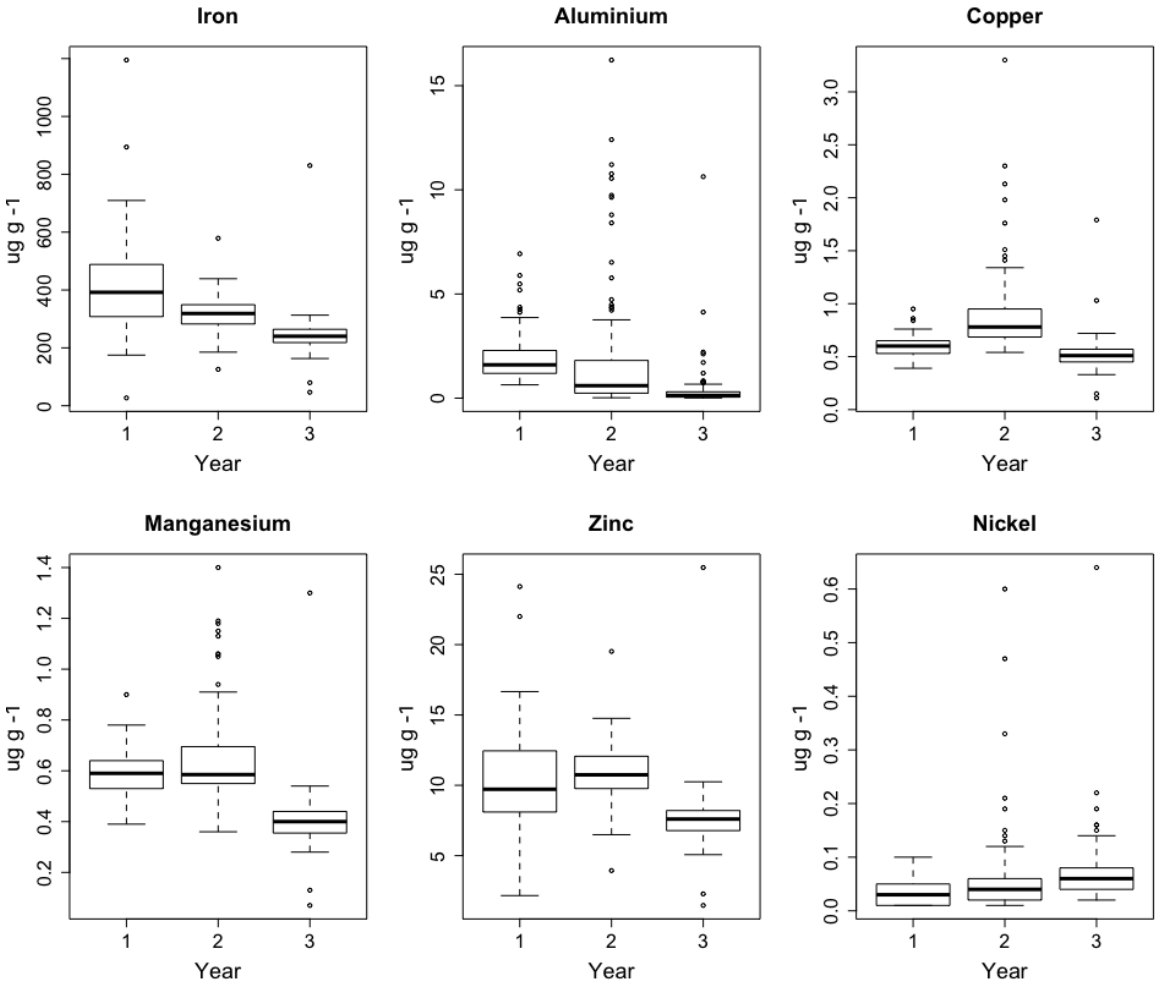
3.2.3.1.1 Essential elements

Concentrations of trace essential inorganic elements are given in Table 2. For the eight assessed elements (Al, As, Cr, Cu, Fe, Mn, Ni, Se and Zn), concentrations were very variable, with some individuals reaching very high concentrations (except for Cr, Mn and Ni). Iron has been consecutively decreasing year after year (Figure 1), during this monitoring program (2012-2014). In sharp contrast to the other elements, most of the Fe in vertebrates is concentrated in the blood (65%), attached to red blood cell hemoglobin and transporting oxygen to cells for oxidative phosphorylation and other purposes (Linder, 2013). Then, it is not surprising that this element was present with the highest concentrations compared to the other essential elements (mean 305 μg g^{-1} ww). However, as happened with most of the included essential elements in this work, both cellular iron deficiency and excess have adverse consequences. The cumulative deficit of Fe resulting from the difference between hemoglobin synthesis and hemoglobin loss through erythrocyte turnover is manifested as anemia of inflammation; and free Fe promotes generation of oxygen radicals and then, oxidative stress (Ganz and Nemeth, 2006). Regarding marine turtles, there are not many studies determining Fe. Thus, it is difficult to establish if Fe concentrations found are normal for this species. In this sense, Villa et al. (2017) reported concentrations from 100 to 440 μg g^{-1} ww in Green Turtles (*n*=131) from 4 different populations in Australia. These authors results seem much more homogenous than our population (range 26-1194 μg g^{-1} ww). Finally, Fe has positive significant relationships with both, CCL and CCW (Table 3), meaning that the size (and possible age) of the individuals also influence Fe concentration in these turtles.

Table 2. Concentrations of essential elements in blood of alive Olive Ridleys. Results are in µg g^{-1} wet weight.

	Al	As	Cr	Cu	Fe	Mn	Ni	Se	Zn
Min	0.01	0.05	0.01	0.11	26	0.07	0.01	1.01	1.46
1st Qu	0.16	0.67	0.15	0.52	238	0.41	0.03	5.08	7.51
Median	0.54	1.03	0.17	0.61	284	0.51	0.04	6.16	8.93
Mean	1.69	1.13	0.19	0.84	305	0.53	0.06	6.42	9.43
3rd Qu	1.62	1.43	0.21	0.76	335	0.60	0.07	7.37	11.1
Max	16.2	7.59	0.85	3.30	1194	1.40	0.64	27.7	25.4
BDL	30	1	3	0	0	0	13	0	0

Figure 1. Blood essential elements with significant correlations among the 3 years (2012,2013 and 2014).



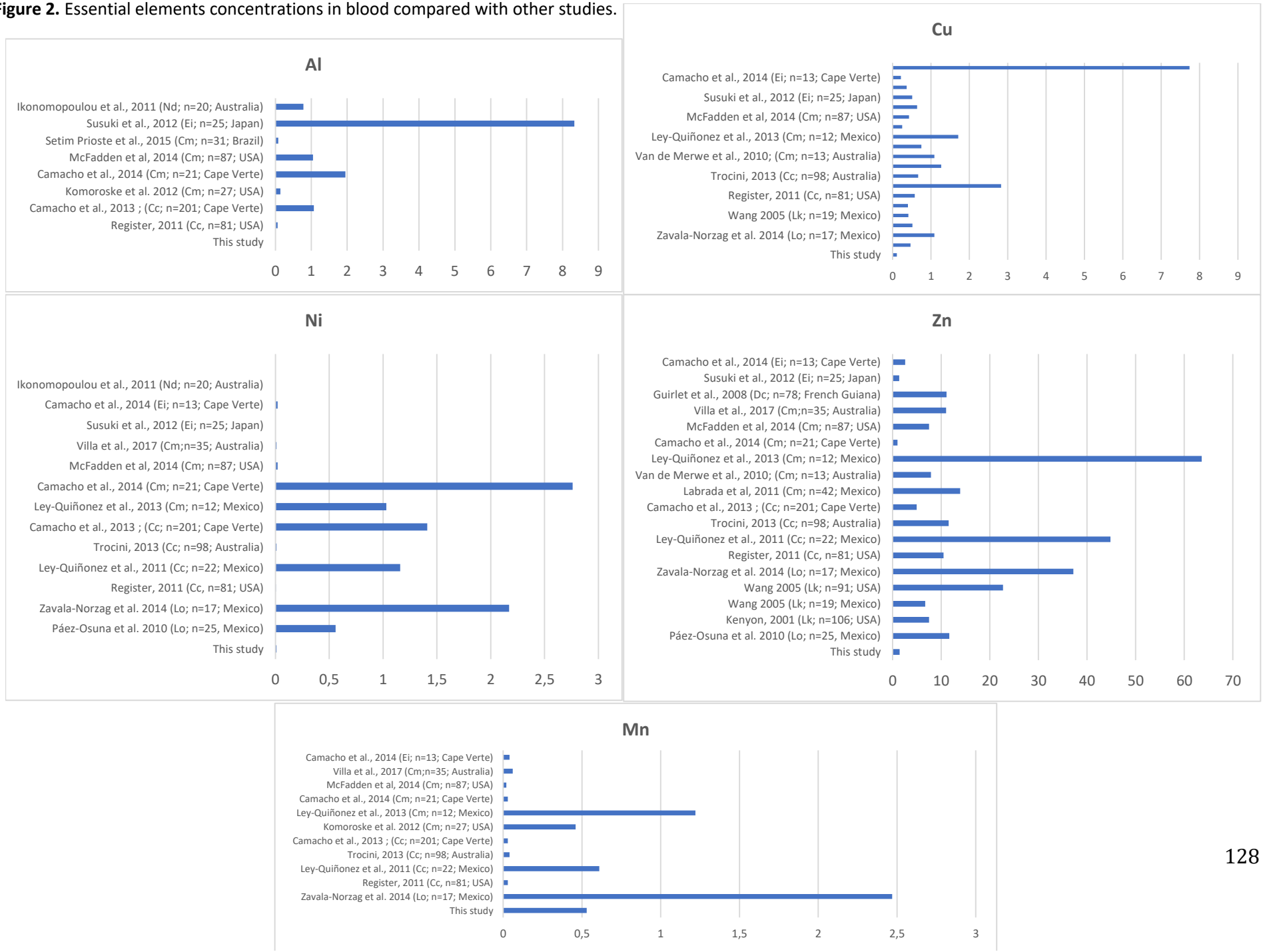
Al, Cu, Mn and Zn have also significantly decreased (Table 3) during the three years of monitoring (Figure 1). All these elements are essential for many organisms in low levels, including plants. Thus, one of its main sources in this region is from fertilizers fields, with

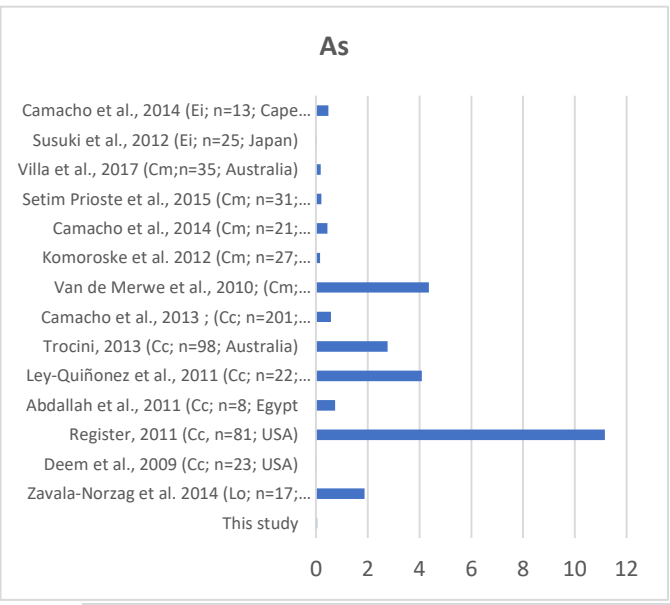
the rain they can easily reach rivers and the sea. There are many fields of cultivation with an important use of fertilizers all around La Escobilla beach and along the coast of Oaxaca (personal observation). Before an *arribada* starts, turtles can spend from few days to several weeks waiting just in front of the beach (Ocana, 2010). Moreover, an important river flows out on this beach, sending all the waste in its path to the ocean from many kilometers inland, especially after strong rains (personal observation). The most important *arribadas* occur during raining season (Jun to November); then, precipitations along the cost may play an important role for the availability of these elements every year. Al and Mn also presented positive correlations with CCL and CCW (Table 3). Mn was present in all the individuals above the detection limit (>0.01 μg g^{-1}), but Al was not found in 30 individuals, this could be just due to the detection limit and the low concentrations usually found of this element in blood (Figure 2-Al). Some individuals shown very high concentrations (Figure 1), compared with the majority of the individuals and to other populations, especially in the second year. There are not many studies about Al toxicity in reptiles, but in experimental rats the DL50 has been calculated around 250 μg g^{-1} (ATSDR, 2008); even if turtles were more sensitive to this metal, the highest concentrations found in this study still far lower from the lethal levels in other species.

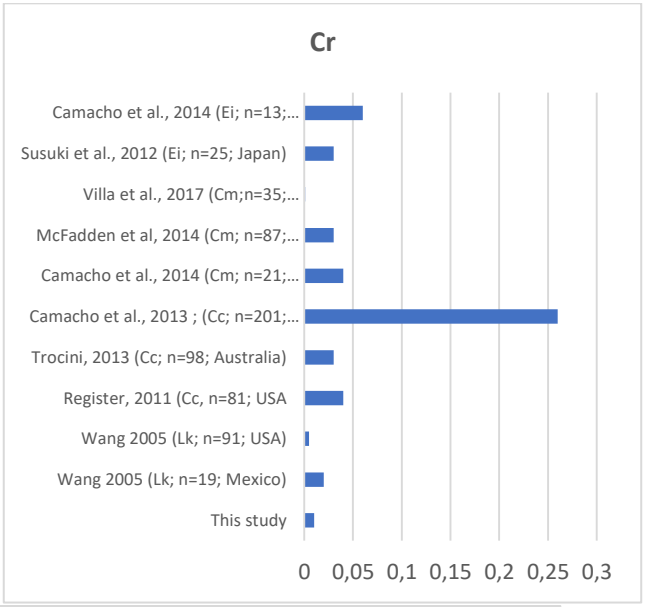
Table 3. Spearman significant correlations among biometrics and essential elements (3 years) in blood of *L. olivacea*. Only *r* and *p* values from significant results are included.

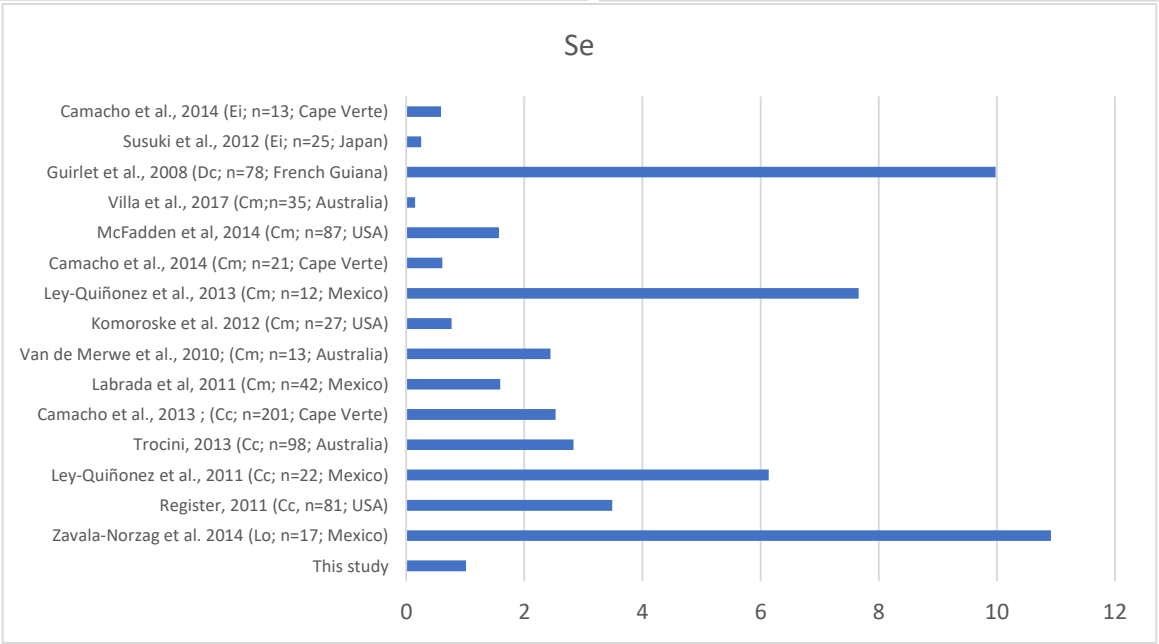
	CCL	CCW	Al	As	Cr	Cu	Fe	Mn	Ni	Se	Zn
CCL	1.000		0.167				0.191	0.215		0.165	
CCW	0.000	1.000					0.158	0.151			
Al	0.040		1.000		0.261	0.300	0.376	0.603		-0.1826	0.2945
As				1.000	0.232		0.237			0.472	0.213
Cr			0.000	0.000	1.000	0.516	0.548	0.423	0.385	0.396	0.590
Cu			0.000		1.000	1.000	0.621			0.282	0.772
Fe	0.010	0.035	0.000	0.000	0.000	0.000	1.000		-0.192	0.377	0.874
Mn	0.004	0.044	0.000		0.000	0.000	0.000	1.000	-0.192	0.171	0.733
Ni					0.000		0.003	0.0032	1.000		-0.119
Se	0.028		0.007	0.000	0.000	0.000	0.000	0.0076		1.000	0.450
Zn			0.000	0.000	0.000	0.000	0.000	0.000	0.072	0.000	1.000

Figure 2. Essential elements concentrations in blood compared with other studies.









Nickel was the only element with a significant increase during the three years (Figure 1). However, Ni concentration still among the lowest concentrations for these essential elements compared with other species and populations (Figure 2-Ni), with a range of 0.01 to 0.64 µg g^{-1}. This element plays a regulatory role in mineral metabolism at low concentrations (Rana, 2014). Then, compared with other populations and species (Figure 2-Ni), usually Ni does not reach more than 2 µg g^{-1} ww, except for the work of Labrada-Martagon et al. (2011a), reporting more than 70 µg g^{-1} ww in Green turtles from the

Mexican Pacific. This extremely high concentration of Ni was suggested mostly due to natural causes, such as currents, upwelling, volcanism and tectonic activity (this study was not included in the figure and be able to see the difference between the rest of the works).

Table 3 also shows all the significant correlations among all the elements. There were many significant correlations, most of them positive. Only Ni showed negative correlations (Fe, Mn and Zn) and only one positive with Cr. All the other elements had only positive relationships; Cr, Fe and Zn present significant correlations with all the elements; Se with all except Ni; Cu and Al with all but Ni and As; Mn and As have 5 and 4 significant correlations respectively. All these correlations must be related to the essentiality and physiological interaction of all these elements in the organism. Then, Fe, Zn, Se and Cr demonstrated their essentiality to the turtles organism, and their participation in many physiological pathways, as happened in many other species (Al-Jawad et al., 2016; Budis et al., 2013; da Silva et al., 2014; Kunito et al., 2008; Nardini et al., 2013; Santamaria, 2008; Siscar et al., 2014; Stefanidou et al., 2006; Sun et al., 2014). Al and Ni were the elements with more BDL individuals (30 and 13 respectively), but still seem important and essential elements for the turtles. Negative correlations of Ni, Al, Mn and As could be related just to different physiological processes compared with the rest of the elements.

Blood is not a storage tissue for these elements (except for Fe); then, anomalous or very high concentrations in blood must be duo recent/acute expositions. Their possible accumulation or chronic exposition is only possible when their target organs are analyzed. Figure 2 compares all these essential elements with other works in the same and different species and populations around the world. Concentrations in blood for turtles in this study are within the populations with low or medium concentrations. Any of these elements show any outstanding level to suspect about any source of very high contamination with these elements around this beach (once again, excluding Fe for insufficient data to compared it). These values then, can be used as a database for normal values for Olive Ridley turtles in Oaxaca.

3.2.3.1.2 Non-essential elements

Concentrations of 6 non-essential elements (Pb, Cd, Li, Sr, Ti and Tl) are given in Table 4. Pb and Cd were the only two previously assessed elements in the first approach (Chapter II). Compared to those results, Pb seems a slightly increased (from 0.01-0.05 to 0.01-0.6 μg g^{-1} ww) but still in low concentrations and with any significant correlation between the 3 years of monitoring. Cd seems very similar (from 0.06-0.41 to 0.02-0.52 μg g^{-1} ww), and only one individual was BDL (<0.01). All the elements were detected in most of the samples, except Tl, which was detected in just 37% of the individuals sampled.

Table 4. Concentrations of non-essential elements for the 3 years in blood of *L. olivacea*. Results are in μg g^{-1} wet weight.

	Pb	Cd	Li	Sr	Ti	Tl
Min	0.01	0.02	BDL	0.18	0.01	0.01
1st Qu	0.02	0.1	0.02	0.81	0.02	0.01
Median	0.03	0.12	0.04	0.99	0.03	0.15
Mean	0.04	0.14	0.04	1.06	0.31	0.33
3rd Qu	0.05	0.17	0.06	1.26	0.11	0.37
Max	0.6	0.52	0.3	3.13	7.73	11.4
BDL	21	1	42	0	9	152

All these non-essential elements, except Li, showed significant differences between the 3 monitoring years (Figure 4). Blood Cd seems to be decreasing annually, this may indicated lower concentrations of this element in the waters around the nesting beach (since Cd accumulates in kidney and liver principally). Sr was higher the first year to slightly decreased the following 2 years. Pb, Ti and Tl had a peak in their availability during the second year, but concentrations decrease again the 3rd year. Sr is the element with the highest mean concentration (1.06 μg g^{-1} ww), but Ti and Tl presented some individuals with concentrations as high as 7.73 and 11.4 μg g^{-1} ww, which are very high concentrations in blood for these potentially toxic elements. Most of these elements are of high concern, in very low doses (Pb, Cd and Tl), and with not many information available (Sr and Ti) (Bannon, 2015; Budis et al., 2013; Matovic et al., 2015; McPherson et al., 2014; Nunes et al., 2014; Pemmer et al., 2013; Rana, 2014; Shi et al., 2013). For instance, Tl is considered highly toxic

to humans, domestic and wild organisms; it can cross the placental, hematoencephalic, and gonadal barriers; it's more severe effects are reported in the nervous system (Rodriguez-Mercado and Altamirano-Lozano, 2013). Then, it is an important element to consider in monitoring programs.

Figure 4. Non-essential inorganic elements in blood of *L. olivacea* with significant relationships between the 3 years.

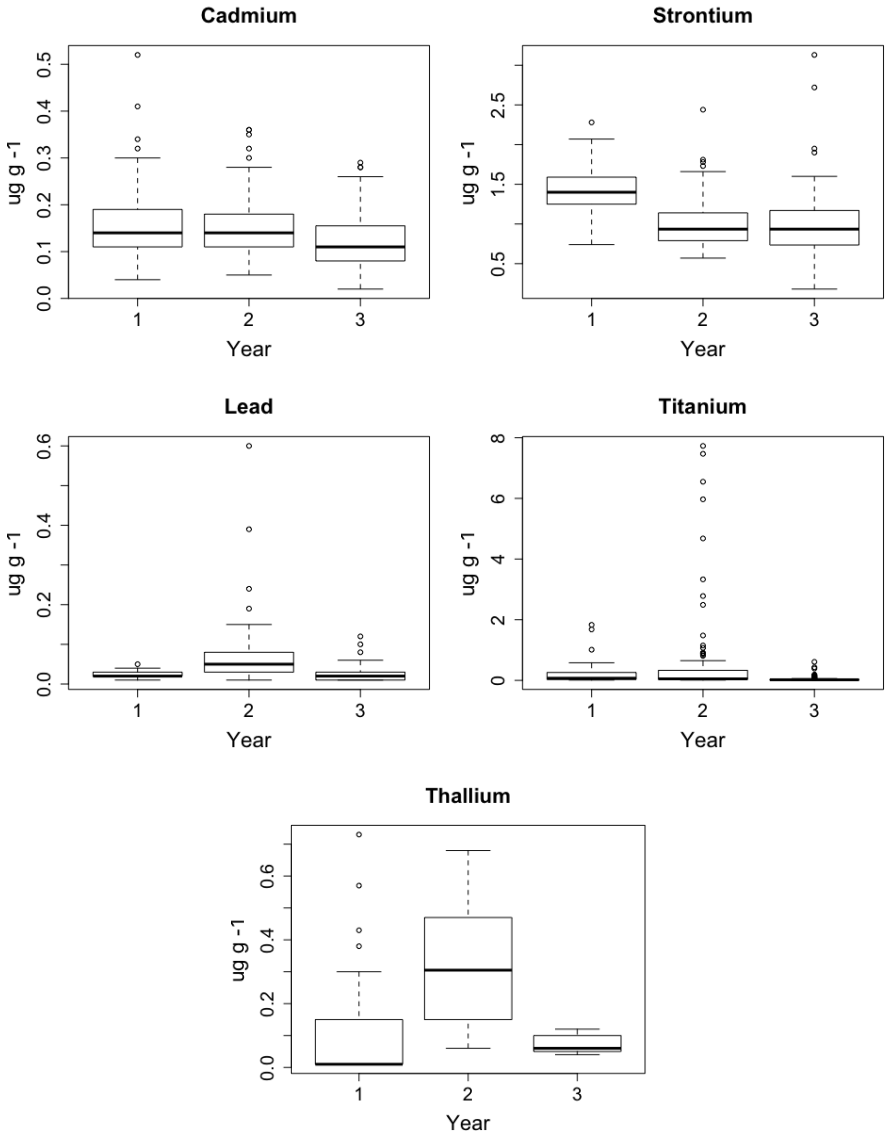


Table 5 shows all significant correlations found among all the elements, and between the elements and the size of the individuals. CCL had two significant positive correlations with Pb and Ti, while CCW had one with Tl. This information, along with the CCL and CCW

correlations with essential elements, show us that these two measures can give different information about elements accumulation related with the age of the individuals. All the elements also showed several significant correlations among them; there were only three negative correlations between all of them, two of Tl (with Li and Sr) and one of Pb (with Li). All the other correlations were positive. This, make us think about a common source for most of these elements. For example, Tl is obtained during the refining and smelting processes for Cd, Pb, Fe and Zn ores (Rodriguez-Mercado and Altamirano-Lozano, 2013). Moreover, in the essential element section, Fe and Zn also had a similar decreasing tendency for the 3 monitoring years. These essential elements also had positive significant correlations with most of the non-essential ones: Zn with all of them (Li and Sr p >0.01; Cd, Pb, Ti, Tl and Fe p>0.000), and Fe with Cd, Pb, Sr and Ti (p>0.000).

Table 5. Significant correlations among biometrics and essential elements in blood of *L. olivacea*.

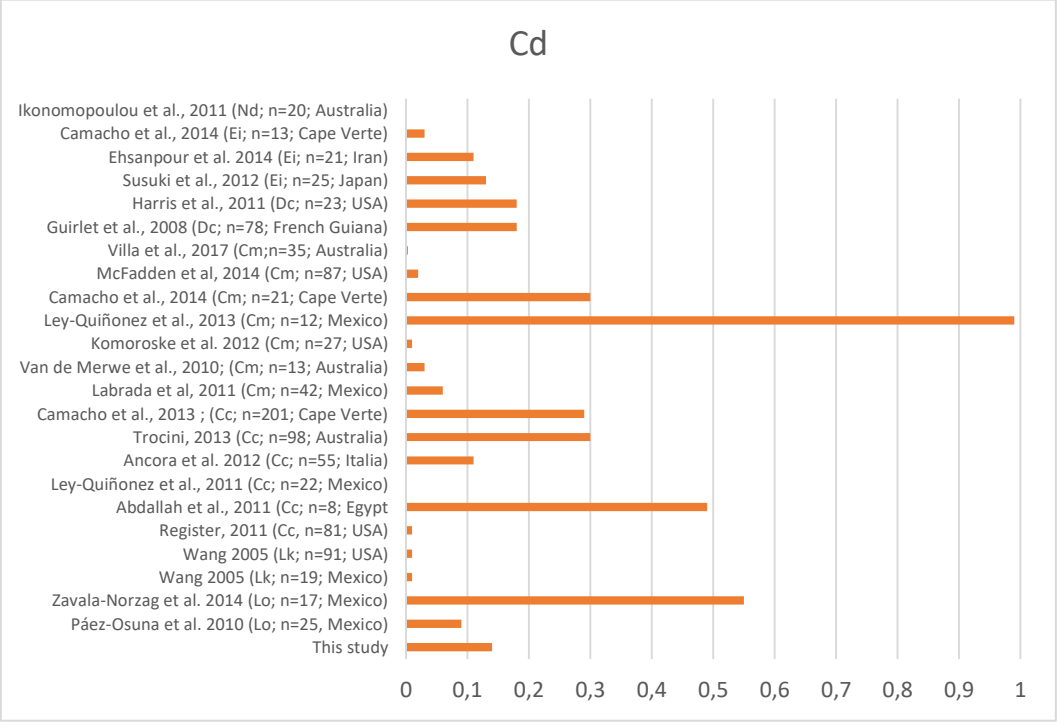
	CCL	CCW	Cd	Li	Pb	Sr	Ti	Tl
CCL	1.000				0.187		0.168	
CCW	0.000	1.000						0.351
Cd			1.000		0.219		0.158	0.264
Li				1.000	-0.345	0.359		-0.499
Pb	0.01		0.001	0.000	1.000		0.461	0.462
Sr				0.000		1.000		-0.279
Ti	0.02		0.01		0.000		1.000	
Tl		0.01	0.01	0.000	0.000	0.007		1.000

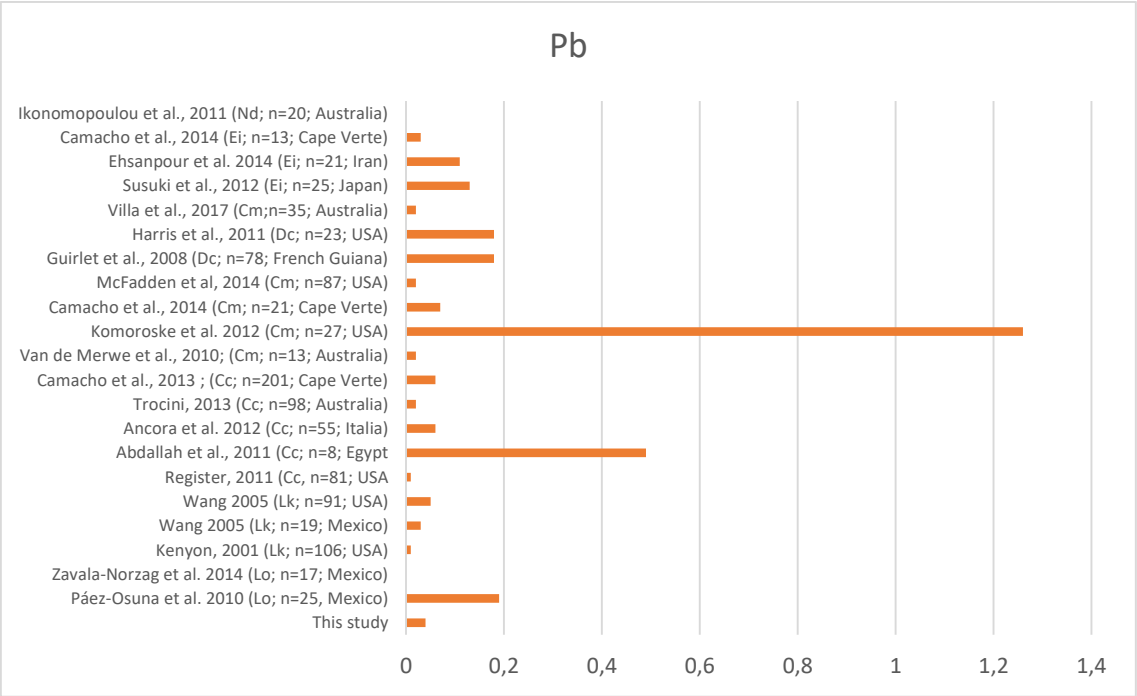
Comparing with the existing literature worldwide with our results, only Cd and Pb were found in several studies in the 7 sea turtle species (Figure 5). Only three works in *L. olivacea* for those two metals were found (Páez-Osúna et al., 2010a, b; Zavala-Norzagaray et al., 2014), both of them from the Mexican Pacific (one North and other South), and one of them was undertaken in the same beach seven years before this monitoring study. Blood Cd concentrations looks very similar to the results of Páez-Osúna et al. (2010b), but almost 5 times lower compared to the work from the North of Mexico (Zavala-Norzagaray et al., 2014). Compared with other species our results are low but, in all cases, above de detection limit. Pb concentrations in the other hand, was lower than the results from 7 years earlier and it was also among the lowest concentrations compared with other species. As well as

Cd, Pb concentrations were also above the detection limit in most cases (in 220 from 241). These results denote that Cd and Pb are in low concentrations, but constantly present, around this nesting beach. The North of Mexico on the other hand, presented the highest Cd concentrations in blood in *C. mydas*, this differences could be due to the species or de different availability of this element in the feeding areas.

There is almost nothing published about the rest of the non-essential elements for marine turtles to compared with. We only found one work giving Ti and Tl concentrations in blood of Green turtles from Australia (Villa et al., 2017). Regarding Tl, our concentrations were three times higher than those from Villa et al. (2017) (mean 0.33 vs 0.11 µg g^{-1} ww); our range were even higher (0.01-7.73 vs 0.05-0.4 µg g^{-1} ww). Ti on the contrary, presented considerable higher concentrations in the Green turtles from Australia (mean 26, range 14-41 µg g^{-1} ww) than in Ridleys from Mexico (mean 0.31, range 0.01-7.73 µg g^{-1} ww). Here the diet and availability of these elements in each population must be the reason of these differences. This results also demonstrated the usefulness of turtles to indicate the regions with more concentrations of specific pollutants.

Figure 5. Non-essential element (Cd and Pb) studies worldwide compared with the present work.





3.2.3.2 Dead turtles

Tissues are the place where pollutants accumulate, and depending on their proprieties, inorganic elements have different target organs. For instance, the highest Cu and Zn are usually found in liver, Cd in kidney, Pb in bone and As and Mn and muscle (see Chapter I). Some of these elements are able to accumulate for more than 20 years (e.g. Pb in bone) (Vighi et al., 2016). Other elements have more efficient detoxification pathways, and can be excreted faster (e.g. Se and As) (Dyc et al., 2016; Rana, 2014). Either way, target tissues are useful to examine long-term trends in metal pollution (Vighi et al., 2016).

3.2.3.2.1 Essential elements

Concentration of the nine essential elements (Al, As, Cr, Cu, Fe, Mn, Ni, Se and Zn) in the six tissues (liver, kidney, muscle, brain, bone, blood and fat) analyzed are given in Table 6. As a macronutrient, Fe is the element with the higher concentrations in all the tissues; the highest concentration was found in liver, follow for blood > bone > brain > kidney > muscle and finally fat. As we previously mention in section 3.2.3.1.1, Fe is attached to red blood cell hemoglobin to transport oxygen to cells and other purposes. In the face of uncertainties in

Fe bioavailability, many organism have evolved a complex system to retain and store iron not immediately in use, and to make it available when and where it is needed. Then, Fe is stored innocuously in the large hollow protein, ferritin, particularly in cells of the liver and bone marrow (Linder, 2013). Thus, explains very well the distribution of Fe found in the different tissues in this study. However, compared with other species and populations (see Chapter I), liver presents the highest concentrations (all the other studies range from 3.12 to 760 and this study range from 291 to 12647 μg g^{-1} ww); bone and brain look high as well, but there were only 1 study to compared (Sakai et al., 2000).

The second most abundant element in all tissues was Zn. It was present in all samples, and the concentrations by tissue decreased in the follow order bone > liver > kidney > muscle > blood > brain and fat. Zn is an essential trace element required for normal cell growth, development, and differentiation (Tapiero and Tew, 2003). Zn in certain concentration is desirable for the growth of animals, but its over accumulation is hazardous to exposed organisms (Senthil Murugan et al., 2008). Most of Zn is present in bone for most species, for this reason Zn is consider an essential component of the calcified matrix (Pemmer et al., 2013). Some other studies in fish has reported liver, kidney and muscle as accumulation tissues, and this accumulation has been directly related with Zn consumption through water or food (Gündoğdu et al., 2009). Although mortality for high Zn doses is rarely observed in experiments, concentrations of 70 μg g^{-1} ww in liver and 16 μg g^{-1} (ww) in muscle have been linked with increased lethargy and loss of equilibrium in Rainbow trout (Gündoğdu et al., 2009). These researchers suggest a physiological acclimation or genetic adaptation, or a combination of both to Zn in humans and fish (Gündoğdu et al., 2009; Pemmer et al., 2013; Senthil Murugan et al., 2008). Compared with other populations and species (see Chapter I), Zn in this study is the highest in most tissues (bone 124, liver 48 and muscle 26 μg g^{-1} ww), except in fat (4.9 μg g^{-1} ww).

Aluminium was the third element with the highest concentrations and it was above the detection limit in most of the samples (Table 7). Bone and brain were the elements with the

highest concentrations (94 and 69 9 μg g^{-1} ww), follow for kidney > liver > fat > blood and finally muscle (with concentrations from 5 to 17 μg g^{-1} ww). The high concentration of Al in bone has been explained in mammals due to the absorption of Al through the calcium channels (Kumar and Gill, 2009). However, the same study mentions that most of the soft tissues have a concentration of about 0.3–0.8 mg kg^{-1} ww, which are concentrations far lower than concentrations found in our study. Other authors also reported that brain has been found to accumulate lower concentrations of Al than many other tissues in humand and experimental rats (Yokel, 2006; Yokel and McNamara, 2001), the opposite than our turtles have. It has been suggested that there is a relationship between high levels of Al and increased risk of a number of pathogenic disorders, such as osteomalacia and microcytic anaemia (González Muñoz and Meseguer Soler, 2009). Kumar and Gill (2009) also mention that, in humans, Al is a potent neurotoxicant and may induce cognitive deficiency and dementia when it enters the brain. We only found 3 studies regarding Al in marine turtles to compare: Camacho et al. (2013b) in blood of Loggerhead reported 1.3 μg g^{-1} ww; Aguirre et al. (1994b) analyzed liver and kidney of Green turtles with 2.3 and 1.3 μg g^{-1} ww respectively; and finally Torrent et al. (2004) analyzed liver, kidney, muscle and blood of Loggerheads with 2.2, 0.7, 1.5 and 30 μg g^{-1} ww respectively.

Selenium was the follow element in decreasing concentration order. It was higher in liver, then blood > brain > kidney >muscle > fat and finally bone (Table 6). Aguirre et al. (1994b) mention that most terrestrial animal livers present <1.5 μg g^{-1} of Se and that concentrations found in the turtles populations they studied were very high (2.5 and 3.4 μg g^{-1} ww). Once again, Ridleys from La Escobilla are above these concentrations with 10 μg g^{-1} ww in liver. Compared with other turtles population and species, concentrations for most of the tissues are among the highest (see Chapter I). Nevertheless, Dyc et al. (2016) conducted an experiment in *Trachemys scripta* exposed to high levels of Se; Se reach until 17 μg g^{-1} dw (4.3 μg g^{-1} ww) in liver, 45 μg g^{-1} dw (9 μg g^{-1} ww) in kidney, 35 μg g^{-1} dw (7 μg g^{-1} ww) in muscle, and 5.9 μg g^{-1} dw in blood (1.1 μg g^{-1} ww). According to this author, despite the high exposition, individuals showed grown normally in size and mass and their feeding behavior

seemed not affected. Thus, lead us to think that either reptiles use more Se than other species, and turtles seems to adapted well to high concentrations of this element.

Other essential elements (As, Cr, Cu, Mn and Ni) showed low or medium concentrations compared to other populations and species in most of the tissues (see tables Chapter I). Arsenic for instance, had the highest concentration in liver and muscle (4.3 and 4.2 μg g^{-1} ww respectively), but these concentrations are average on individual compares with other species. However, As is well known for its high toxicity, especially as a reactive oxygen species (even in low concentration) and then, oxidative stress inducer in many species, as most metals do (Sun et al., 2014).

Table 6. Concentrations of essential elements in all tissues. Results are in μg g^{-1} wet weight

		Al	As	Cr	Cu	Fe	Mn	Ni	Se	Zn
Liver	*mean±SD*	10±14	4.3±2.6	1.0±0.7	12±8	2474±2064	2.7±1	0.1±0.05	10±4	48±20
***n*=56**	*max-min*	BDL-56	0.7-11	0.2-3.8	1.0-39	291-12647	0.8-7	BDL-0.2	3.3-24	17-151
	BDL	1	0	0	0	0	0	3	0	0
Kidney	*mean±SD*	17±29	1.2±0.7	0.1±0.1	1.4±0.6	82±102	1.6±0.9	0.04±0.03	2±1	38±18
***n*=56**	*max-min*	BDL-141	0.2-3.5	0.03-0.8	0.2-3	10-618	0.2-6	BDL-0.17	0.4-7.3	5.4-83
	BDL	2	0	1	0	0	0	3	0	0
Muscle	*mean±SD*	5.1±6	4.2±2.1	0.14±0.1	0.8±0.2	48±46	1.4±0.4	0.06±0.1	1.9±0.4	26±10
***n*=44**	*max-min*	BDL-27	0.8-11	0.01-0.57	0.4-1.5	17-244	0.9-2.7	BDL-0.7	1.2-2.9	13-62
	BDL	4	0	0	0	0	0	4	0	0
Brain	*mean±SD*	69±97	0.3±0.4	0.3±0.3	0.8±0.4	138±178	2.1±1.9	0.1±0.2	2.3±1.4	5.8±3
***n*=37**	*max-min*	BDL-382	0.01-2	0.01-1.4	0.09-2.2	0.01-690	0.01-7.9	BDL-0.8	0.01-5.5	0.01-15
	BDL	3	1	0	1	0	0	6	0	0
Bone	*mean±SD*	94±21	0.6±0.5	1.5±0.6	1±0.4	167±265	19±3	0.4±2	1.1±3.5	121±8
***n*=44**	*max-min*	1-651	0.01-2.2	0.2-9.4	0.2-16	15-680	7-40	0.03-7	0.6-2.5	13-326
	BDL	0	0	0	0	0	0	0	0	0
Fat	*mean±SD*	9.9±2.6	1.5±0.8	0.1±0.08	0.3±0.5	41±67	0.6±0.8	0.05±0.03	1.2±0.4	4.9±4.7
***n*=23**	*max-min*	0.01-100	0.2-4.2	0.01-0.42	0.07-2	5-290	0.12-3.6	0.01-0.17	0.6-2.7	1.2-22
	BDL	0	0	0	0	0	0	0	0	0

Blood	*mean±SD*	9.1±21	1.0±0.5	0.3±0.6	0.9±1	274±265	1.2±3.1	0.08±0.1	5.5±3.5	11±8
***n*=38**	*max-min*	BDL-103	0.07-2.3	0.01-3.3	0.06-5.7	5-1493	0.06-20	BDL-0.9	0.1±17	0.3-36
	BDL	5	0	0	0	0	0	6	0	0

All the elements with significant relationships among the sampled years (since fat was sampled only one year it was excluded) are shown in Table 7. Se in liver and kidney and As in brain were the only 3 positive relationships found, meaning that those elements had increased over the 3 years. However, there were several negative relationships for most of the elements (except Ni, which had any), and different tissues showing a decreasing tendency for the most of the elements concentration over the monitoring years. Specially Al had negative significant correlations with all the tissues analyzed ($p<0.000$). Followed for Mn with 5 significant correlations (in all tissues except liver). As we previously mention, these elements are natural components of the earth crust, but they are also introduce by anthropogenic sources like pollution, then substantial variation could be due to natural alterations (such as earthquakes and volcanic activity) or intensive pollution. But natural sources should be detected as sporadic and isolate events, while pollution shows more constant alterations.

Table 7. Significant relationships between years and essential elements by tissue. All correlations found were negative except those marked with (*).

	Al	**As**	**Cr**	**Cu**	**Fe**	**Mn**	**Ni**	**Se**	**Zn**
Liver	0.000	0.003						0.003*	
Kidney	0.000		0.006		0.000	0.001		0.015*	
Muscle	0.000		0.019	0.000	0.000	0.000			0.000
Brain	0.000	0.024*	0.003		0.000	0.001			
Bone	0.000			0.009		0.000			0.002
Blood	0.000		0.000	0.01	0.009	0.019			0.000

3.2.3.2.2 Non-essential elements

Unlike essential elements, as the name implies, non-essential elements are not essential for most organisms, even more, they can be toxic in very low levels (Register, 2011). In the Table 8 are given the concentrations of these elements by tissue.

Cadmium was the element with the highest concentration compared, not only to the other non-essential elements, but also compared to other turtle populations and species worldwide, as reported in the first approach (Chapter II). These very high concentrations for Cd were especially in kidney and liver (84 and 145 µg g^{-1} ww respectively). From all the studies described in Chapter I, Cd in liver has a range from 0.23 µg g^{-1} ww (Barbieri, 2009) in *C. mydas* from Brazil to 9.74 µg g^{-1} ww (Sakai et al., 2000) in *C. caretta* from Japan; and in kidney from 0.89 µg g^{-1} ww (Lam et al., 2004) in *C. mydas* from China to 43 µg g^{-1} ww (Gardner et al., 2006) in *C. mydas*. Kidney is the organ where Cd accumulate the most; then, it is not surprising to find here its highest concentrations. However, the great difference existing between Ridleys from this study and all the other populations is surprising.

Table 8. Concentrations of non-essential elements in all tissues. Results are in µg g^{-1} wet weight. BLD= below the detection limit (0.01).

		Cd	Li	Pb	Sr	Ti	Tl
Liver	*mean±SD*	84±49	0.01±0.09	0.1±0.1	1.7±1.9	1.1±1.8	1.9±1.2
***n*=56**	*max-min*	1-183	BDL-0.6	0.01±0.6	0.2-9.8	0.01-9.2	BDL-6.1
	BDL	0	8	0	0	0	0
Kidney	*mean±SD*	145±105	0.05±0.02	0.04±0.03	1.3±0.9	2.5±4.4	0.5±0.3
***n*=56**	*max-min*	0.3-348	BDL-0.13	BDL-0.13	0.01-0.13	0.01-23	BDL-1.3
	BDL	0	22	3	0	0	18
Muscle	*mean±SD*	0.8±0.4	0.07±0.1	0.04±0.03	0.6±0.5	1.3±2.3	0.4±0.2
***n*=44**	*max-min*	0.2-2.6	BDL-0.49	BDL-0.15	0.01-3	BDL-13	BDL-1-2
	BDL	0	27	11	0	1	10
Brain	*mean±SD*	0.7±0.5	0.08±0.07	0.09±0.1	2.5±3.4	6.1±9.4	0.7±0.6
***n*=37**	*max-min*	0.05-6.5	BDL-0.27	BDL-0.5	0.01-21	0.01-42	BDL-2.2
	BDL	1	17	4	0	0	24
Bone	*mean±SD*	0.6±0.2	3.0±1.6	0.7±0.7	885±367	8.7±9.9	17±7
***n*=44**	*max-min*	0.3-1.2	0.1-6.9	BDL-2.7	4-1562	0.01-45	6.4-35
	BDL	0	1	3	0	0	1

Fat	*mean±SD*	2.2±4.5	0.09±0.1	0.03±0.02	1.2±1.1	1.0±2.3	0.7±0.4
***n*=23**	*max-min*	0.4-22	BDL-0.38	BDL-0.07	0.01-4.2	0.04-8.5	BDL-1.3
	BDL	0	6	5	0	0	16
Blood	*mean±SD*	4.26±13.2	0.05±0.08	0.06±0.1	1.82±2	3.3±15	0.54±0.97
***n*=38**	*max-min*	0.01-81	BDL-0.46	0.01-0.6	0.09-9.2	0.01-94	BDL-3.7
	BDL	0	5	0	0	0	25

Concentrations of Cd in all tissues were above the detection limit; only 8 individuals had concentrations below 8 μg g^{-1} ww in kidney. Cd is regarded as one of the most toxic inorganic elements in the environment (ATSDR, 2012). At the biochemical level, Cd can negatively affect DNA, RNA, and ribosome synthesis (Klaassen et al., 2009; Rana, 2014), as well as inhibate several enzyme systems (Rana, 2014), and it is an important precursor of the production of reactive oxygen species (Matovic et al., 2015). At the organism level, Cd is known to be teratogenic and embryotoxic (Storelli et al., 2005). In addition chronic Cd exposure results in lower fecundity and decreased overall reproductive success in marine species (Burger, 2008; Caurant and Amiard-Triquet, 1995). Cd concentrations on this species are high compared to other turtle species but also to mammals and other terrestrial animals. In experiments with freshwater turtles (*Chrysemys picta*), Rie et al. (2001) described the effect of metallothioneins (MT), meta-binding proteins, as an adaptive response of these turtles to Cd exposure. Then, it would be interesting to analyze MT levels in this species, to compared with metal concentrations and explore if MT are increasing accordingly with metal concentrations as an adaptation of the organism, or if this species is just able to accumulate and highly tolerant to Cd.

Strontium was the second element with some remarkable concentrations, especially in bone (885±367, range from 4 to 1562 μg g^{-1} ww). Concentrations in other organs was considerably lower with brain > blood > liver > kidney > fat and muscle, with concentrations ranging from 0.6 to 2.4 μg g^{-1} ww. Sr is chemically very similar to calcium (Ca), and can replace it, but still little is known about the role of Sr in normal bone metabolism as well as in bone disorders (Pemmer et al., 2013). We only found one work reporting Sr concentrations in marine turtles (Anan et al., 2001) in liver, kidney and muscle from Green

(0.35, 1.13 and 0.81 μg g^{-1} ww) and Hawksbill turtles (0.76, 3.04 and 0.83 μg g^{-1} ww respectively). Only kidney concentration from Hawksbills seems dash higher than Greens or Ridleys. We were not able to find any work reporting Sr in bone to compare the high concentrations found.

For all these non-essential elements, except for Cd, bone was the tissue with the highest concentrations, tissue known for accumulate metals (Pemmer et al., 2013; Vighi et al., 2016). Then, Tl was the element, after Sr, with the highest concentrations in this tissue (17±7 μg g^{-1} ww). Once again, we could not find any publication about Tl in bone in turtles, but we did find one work in blood (Villa et al., 2017), with a lower concentration compared with our results (0.0001 *vs* 0.54 μg g^{-1} ww). In liver, kidney and muscle Anan et al. (2001) reported 0.005, 0.003 and 0.006 μg g^{-1} ww in Green turtles and 0.002, 0.002 and 0.001 μg g^{-1} ww respectively for Hawksbill turtles. These concentrations are around 20 to 80 times lower than those for Ridleys in this study. Despite concentrations look relatively low, Tl is more acutely toxic than most metals and the principal sources of Tl in water pollution, are various mining and refining processes (Bannon, 2015). Laboratory experiments have also demonstrated that Tl induce decrease in sperm motility in chronic low doses exposures (Bannon, 2015).

Titanium was found in a concentration of 8.7±9.9 μg g^{-1} ww in bone. Traditionally, Ti nanoparticles have been considered as poorly soluble, low toxicity particles; however, in recent years it has been widely increase used in many industrial and consumer products (Shi et al., 2013). In laboratory experiments Ti has been found to produce reactive oxygen species, especially in brain, found being the most sensitive organ for Ti toxicity (Long et al., 2006; Ramsden et al., 2009; Shimizu et al., 2009). Concentrations in brain in this study were 6.1±9.4 μg g^{-1} ww, the second highest tissue, with all samples above the detection limit. The unique work reporting Ti in turtles was Villa et al. (2017) in blood, with 0.03 4 μg g^{-1} ww, 20 times lower than in this study (3.3±15 μg g^{-1} ww).

On the other hand, Pb and Li were found in very low concentrations in all tissues (Table 9), and many times individuals were below the detection limit. Once again, their higher concentrations and more individual above the detection limit were found in bone. As the other metals, this tissue is where this element can accumulates for several years (da Silva et al., 2014). These low concentrations may indicate that this population is very low exposed and/or good excreting these metals in the last years. Compared to other populations (see Chapter I), Pb concentrations are among the lowest, we did not find any publication regarding Li in marine turtles.

Non-essential elements showed decreasing concentrations tendencies thought the monitoring years, at least in one tissue for each element (Table 9). Pb and Ti show negative relationships for 5 tissues (Pb all except liver and Ti all except bone); Sr in 4 (except in liver and brain); the rest of the elements showed one negative correlation: Cd and Tl in bone, and Li in kidney.

Table 9. Significant relationships between year and non-essential elements by tissue. All regressions found with ANOVA were negative except those marked with (*).

	Cd	Li	Pb	Sr	Ti	Tl
Liver					0.000	
Kidney		0.008	0.000	0.021	0.000	
Muscle			0.000	0.003	0.000	
Brain			0.000		0.000	
Bone	0.048		0.001	0.041		0.011
Blood		0.041*	0.000	0.007	0.000	

Li in blood was the single element with one positive relationship with the 3 monitoring years (meaning that Li concentration significantly increased during the years). Despite this significant increased, Li presents the lowest concertation in most of the tissues compared with the rest of the elements, with always less than 0.1 μg g^{-1} ww. Except in bone, the tissue where this metal accumulates (Pigatto et al., 2016), here it was present in all the samples with a mean of 3.3 μg g^{-1} ww.

3.2.3.2.3 Significant correlations

In the Table 10, the CCL and CCW for the individuals included in this study are shown. Unlike to other marine turtle species, Olive Ridleys tend to be wider than longer in curve carapace measures. Then, we used both curve measures (CCL and CCW) to compared in the correlation analysis. Since CCL and CCW are two biometric measures related with the size of the individuals, they had significant positive correlations in all tissues as expected. However, they were not correlated in like manner to the elements analyzed, even more, most of the times they were differently correlated among these elements. This mean that, even if both measures are closely correlated, they do not give the same information related to the concentration of the inorganic elements and the size (and maybe age) of the individuals. CCL was the measure with more significant correlations.

Liver was the tissue with more significant correlations between the size of the individuals and most of the elements (Table 11). CCL presented six positive significant correlations: four with essential elements, As (p=0.01), Cr (p=0.001), Fe (p=0.001) and Se (p=0.003); and two with non-essential elements, Cd (p= 0.000) and Tl (p=0.04). Liver also present one negative correlation with Ti (p= 0.03). For the other tissues, CCL had only two other significant correlations: one positive in kidney with Cd (p= 0.02) and one negative in muscle with Al (p=0.03). On the other hand, CCW presented only two significant correlations: one positive in fat with Tl (p= 0.04), and one negative in bone with As (p=0.03). All these correlations are presented in Figures 6 (positives) and 7 (negatives).

Table 10. Curve carapace length (CCL) and width (CCW) from dead turtles.

	CCL	**CCW**
Min.	62.80	67.0
1st Qu.	65.50	70.0
Median	66.50	71.0
Mean	66.97	71.5
3rd Qu	68.00	73.0
Max.	73.50	79.0

This important difference of correlations in liver compared with the other tissues could be explained for the fact that liver in the first filter organ after ingestion, where all the elements pass through before, either be excreted or accumulated in this or other organs. Since all these elements arrive to the liver after ingestion, the quantity of ingested pollutants may be proportional to the quantity of food ingested. Then, especially for the elements that are not accumulated in liver, these correlations should be more related to: bigger the turtles are, more food is ingested and more inorganic elements (in this case) enter in the liver. On the other hand Cd is an element that accumulates in liver and kidney with very high concentrations, and it had positive correlations in both organs and CCL; this could be more related to real bioaccumulation through the years, and to a poor excretion of Cd in these turtles. Fe is another element with very high concentrations in liver (Table 8). The positive correlation with CCL lead us propose that this element is poorly excreted. To talk about bioaccumulation through the years, significant relationships should be expected in the target accumulation tissues. However, it is clear that for certain elements the size of the individual influence the concentrations of the elements ingested.

Figure 6. Positive significant relationships between CCL and CCW and elements by tissues.

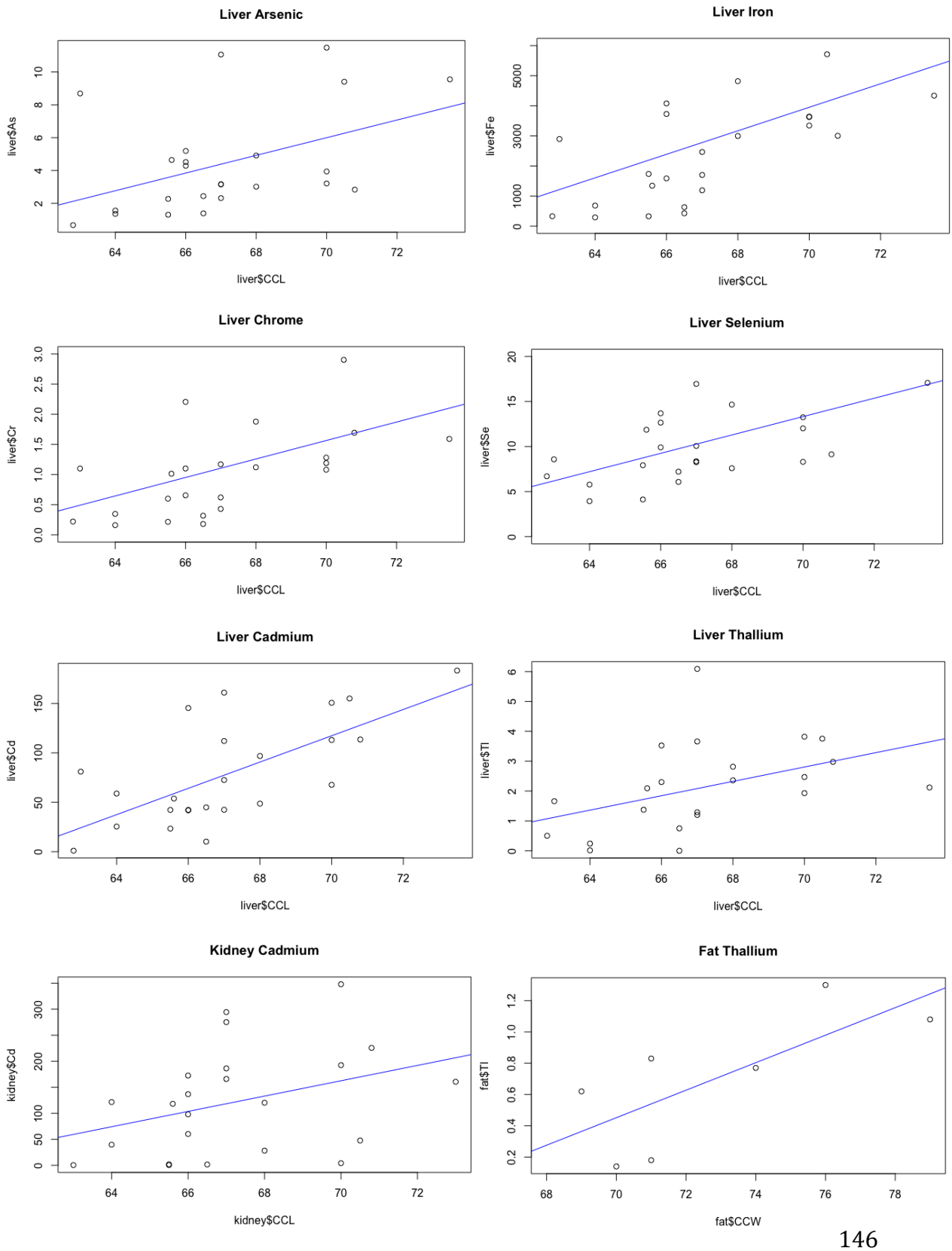
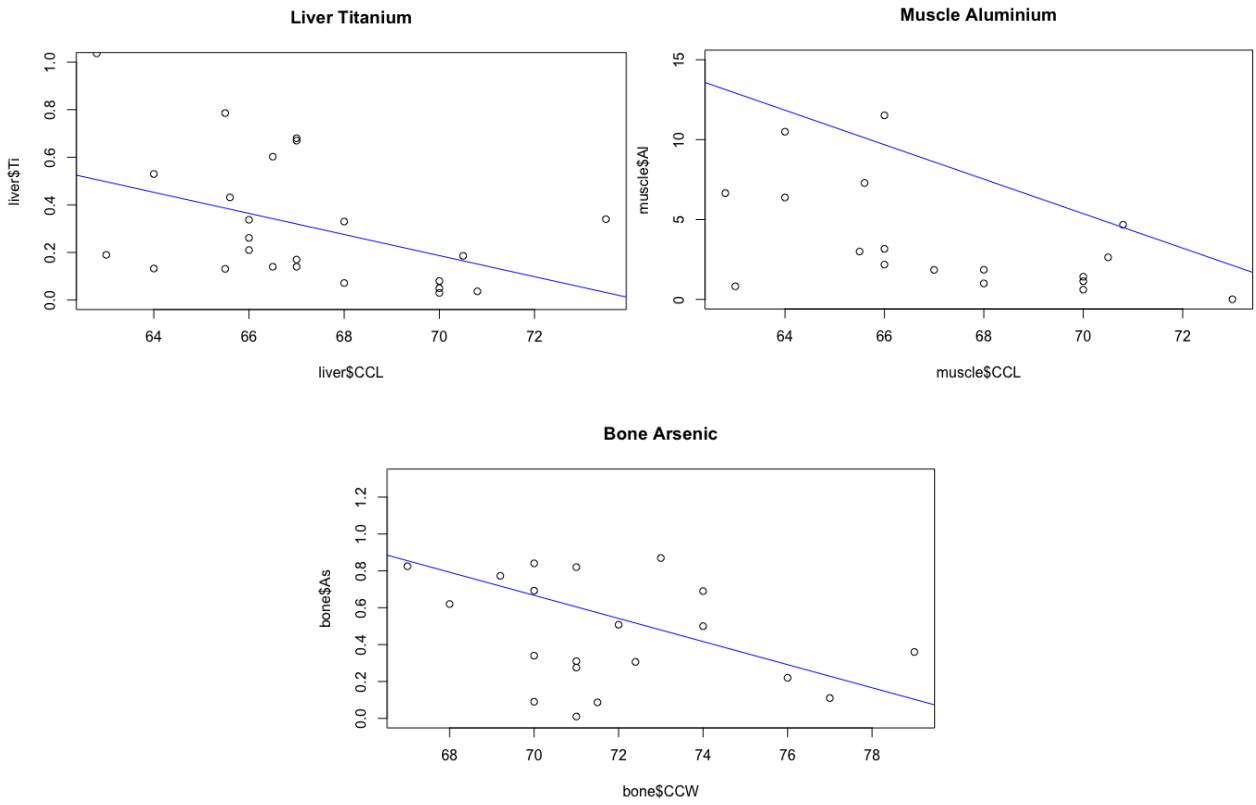


Figure 7. Negative significant relationships between CCL and CCW and elements by tissues.



The state of decomposition of the carcasses was also included for the correlation test. Aluminium was the element with more significant relationships: 3 positives in liver, kidney and muscle (Tables 11, 12 and 13). During the decomposition processes, a concerted release of certain ions, including Al^{+}, take place during early stages (Forbes et al., 2017). This could explain the apparently rise of Al with the rise of the decomposition stage. On the other hand, muscle and fat were the tissue with more positive correlations among decomposition and elements (Cr, Fe, Mg, Cd, Li, Pb, Sr and Ti for muscle and Cu, Fe, Mn, Zn, Cd and Li for fat). Even if these are not the first tissues showing liquefaction during the decomposition process, they seems to be the first suffering alterations in those elements concentrations. Brain is one of the first tissues presenting liquefaction signs, however, this event does not appear to alter its inorganic elements concentrations since it did not present any significant correlation. Bone was another tissue with not significant correlations with decomposition

and elements concentrations. This information is important to be taken into account when inorganic elements are study in dead animals.

The cause of the dead (traumatism *vs* unknown cause) was significant related only with Se in bone and blood, Zn in bone and Ti in blood (higher for unknown causes). Here it does not appear that these studied elements presented any strong influence among these two dead causes. However, more detailed necropsies, with macroscopic and microscopic pathologies stablish, are necessary to really understand if any of this inorganic elements can have a real link with the cause of the dead.

Tables 11 to 17 are all the significant correlations by tissue for both, essential and non-essential elements. Surprisingly, almost all correlations were positive: kidney, brain, fat and blood presented only positive correlations for all the elements. Liver presented only two negative correlations, both of Sr (with Cd and Cu); muscle had also only two negative correlations: Sr-As and Li-Tl; and finally bone, also only two negative correlations, both of As (with Ni and Ti). For essential elements these positive correlations and the fact that almost all the samples were above the detection limit must be related to the essentiality and physiological interaction of all these elements in the organism. Regarding all positive correlations between non-essential elements, as well as in blood for live turtles, reinforce the theory of a common source for most of these elements for this turtle population. Another important observation is that, with all these positive correlations, does not seems negative interactions between non-essential and essential inorganic elements.

Table 11. Significant Spearman correlations in **liver**. Variables without significant correlations were excluded.

	CCL	CCW	DESC	Al	As	Cr	Cu	Fe	Mn	Ni	Se	Zn	Cd	Li	Pb	Sr	Ti	Tl
CCL	NA	0.677			0.476	0.644		0.647			0.575		0.672				-0.446	0.584
CCW	0.001	NA																
DESC			NA	0.474											0.378		0.625	
Al			0.007	NA					0.364						0.355	0.622	0.858	0.316
As	0.019				NA	0.559		0.639		0.356	0.788	0.336	0.582					0.432
Cr	0.001				0.000	NA				0.530	0.620	0.332	0.000		0.666			
Cu							NA		0.396			0.274				-0.364		
Fe	0.001				0.000	0.000		NA		0.493	0.587		0.000		0.567			0.826
Mn				0.006			0.003		NA				0.010					
Ni					0.008	0.000		0.000		NA	0.421		0.010	0.001	0.405			0.539
Se	0.003				0.000	0.000		0.000		0.002	NA	0.377	0.000					0.491
Zn					0.011	0.012	0.041		0.001		0.004	NA	0.000					
Cd	0.000				0.000	0.658		0.658	0.319	0.324	0.621	0.638	NA			-0.366		0.456
Li										0.482		0.421		NA			0.450	
Pb			0.033	0.008		0.000		0.000		0.003					NA			0.671
Sr				0.000			0.006						0.005			NA	0.478	0.304
Ti	0.029		0.000	0.000					0.001							0.000	NA	
Tl	0.004			0.021	0.001			0.000		0.000	0.000		0.000		0.000	0.025		NA

Table 12. Significant Spearman correlations in **kidney**. Variables without significant correlations were excluded.

	CCL	DESC	Al	Cr	Cu	Fe	Mn	Ni	Se	Zn	Cd	Li	Pb	Sr	Ti	Tl
CCL	NA										0.470					
DESC		NA	0.477												0.507	
Al		0.008	NA	0.584		0.606	0.393	0.799				0.567		0.467	0.926	
Cr			0.000	NA		0.738	0.564	0.627	0.184			0.577	0.425	0.691	0.511	0.437
Cu					NA		0.462			0.777	0					
Fe			0.000	0.000		NA	0.527	0.596	0.383			0.581	0.503	0.756	0.596	0.436
Mn			0.000	0.000	0.000	0.000	NA	0.531		0.377	0.001	0.000	0.427	0.619	0.540	0.361
Ni			0.005	0.000		0.000	0.000	NA				0.000		0.545	0.365	
Se				0.187		0.004			NA					0.344		0.330
Zn					0.000		0.005			NA	0.000					
Cd	0.024				0.855		0.426			0.763	NA					
Li			0.000	0.001		0.000	0.757	0.609				NA	0.579	0.624	0.571	
Pb			0.000	0.002		0.000	0.002					0.001	NA	0.337	0.751	
Sr			0.000	0.000		0.000	0.000	0.000	0.011			0.000	0.016	NA	0.441	0.620
Ti		0.004	0.000	0.000		0.000	0.000	0.008				0.001	0.000	0.001	NA	
Tl				0.009		0.008	0.031		0.049					0.000		NA

Table 13. Significant Spearman correlations in **muscle**. Variables without significant correlations were excluded.

	CCL	CCW	DESC	Al	As	Cr	Cu	Fe	Mn	Se	Zn	Cd	Li	Pb	Sr	Ti	Tl
CCL	NA	0.679		-0.464													
CCW	0.001	NA		-0.464													
DESC			NA	0.505		0.382		0.473	0.540			0.541	0.695	0.499	0.420	0.397	
Al	0.034	0.046	0.004	NA		0.482	0.629	0.828	0.840		0.683			0.876			
As					NA										-0.384		
Cr			0.028	0.002		NA		0.432	0.353		0.335			0.391	0.491	0.413	
Cu				0.000			NA		0.715		0.875	0.017	0.517	0.593	0.361	0.482	
Fe			0.005	0.000		0.003	0.000	NA	0.810		0.716	0.013	0.500	0.882	0.499	0.758	
Mn			0.001	0.000		0.019	0.000	0.000	NA		0.655	0.009	0.001	0.908	0.497	0.735	
Se										NA			0.056				
Zn				0.000		0.026	0.000	0.000	0.000		NA			0.000	0.002	0.001	
Cd			0.001				0.360	0.371	0.392			NA	0.585	0.345	0.314		
Li			0.026				0.034	0.041	0.715	0.472		0.014	NA		0.607		-0.487
Pb			0.011	0.000		0.024	0.000	0.000	0.000		0.579	0.050		NA	0.593	0.876	
Sr			0.015	0.001	0.010	0.001	0.016	0.001	0.001		0.454	0.038	0.010	0.000	NA	0.395	
Ti			0.022	0.000		0.006	0.001	0.000	0.000					0.000	0.009	NA	
Tl											0.505		0.047				NA

Table 14. Significant Spearman correlations in **brain**. Variables without significant correlations were excluded.

	Year	Al	As	Cr	Cu	Fe	Mn	Ni	Se	Zn	Cd	Li	Pb	Sr	Ti	Tl
Year	NA	-0.571	0.377	-0.480		-0.634	-0.511						-0.733		-0.557	
Al	0.000	NA		0.826		0.889	0.890	0.463				0.639	0.791	0.708	0.962	
As	0.024		NA						0.415	0.345						
Cr	0.003	0.000		NA		0.781	0.837	0.648		0.365		0.526	0.709	0.604	0.785	
Cu					NA		0.339	0.417	0.664	0.711				0.339		
Fe	0.000	0.000		0.000		NA	0.821	0.464					0.786	0.485	0.829	
Mn	0.001	0.000		0.000	0.043	0.000	NA	0.596		0.325		0.004	0.804	0.736	0.890	
Ni		0.011		0.000	0.020	0.009	0.000	NA				0.001	0.580	0.369	0.461	
Se			0.012		0.000				NA	0.758	0.008					
Zn			0.039	0.026	0.000		0.050		0.000	NA	0.001			0.002		0.002
Cd									0.438	0.536	NA	-0.453				0.593
Li		0.004		0.017			0.621	0.695			0.045	NA	0.505	0.440	0.611	
Pb	0.000	0.000		0.000		0.000	0.000	0.001				0.032	NA	0.565	0.777	
Sr		0.000		0.000	0.043	0.002	0.000	0.041		0.494		0.052	0.001	NA	0.687	0.764
Ti	0.000	0.000		0.000		0.000	0.000	0.009				0.004	0.000	0.000	NA	
Tl										0.769	0.033		1.000	0.002		NA

Table 15. Significant Spearman correlations in **bone**. Variables without significant correlations were excluded.

	Year	CCW	DC	Al	As	Cr	Cu	Fe	Mn	Ni	Se	Zn	Cd	Li	Pb	Sr	Ti	Tl
Year	NA			-0.536			-0.389		-0.575			-0.446	-0.299		-0.499	-0.310		-0.383
CCW		NA			-0.449													
DC			NA								0.435	0.457						
Al	0.000			NA				0.393	0.431	0.404					0.483		0.464	0.416
As		0.036			NA					-0.350			0.562				-0.34	
Cr						NA	0.441	0.612	0.466	0.680						0.345		0.329
Cu	0.009					0.003	NA	0.709	0.361	0.497	0.322		0.023				0.438	
Fe				0.008		0.000	0.000	NA		0.610							0.473	
Mn	0.000			0.003		0.001	0.016		NA	0.374		0.806		0.001	0.743	0.789		0.896
Ni				0.007	0.020	0.000	0.001	0.000	0.012	NA							0.315	0.333
Se			0.011				0.033				NA	0.306	0.032		0.010			
Zn	0.002		0.007						0.000		0.044	NA		0.003	0.000	0.000		0.000
Cd	0.048				0.000		0.342				0.324		NA					
Li					0.009				0.504			0.448		NA	0.331	0.675		0.703
Pb	0.001			0.001					0.000		0.400	0.776		0.037	NA	0.626		0.683
Sr	0.041					0.022			0.000			0.809		0.000	0.000	NA		0.776
Ti				0.002	0.022		0.003	0.001		0.037							NA	
Tl	0.011			0.006		0.031			0.000	0.029		0.713		0.000	0.000	0.000		NA

Table 16. Significant Spearman correlations in **blood**. Variables without significant correlations were excluded.

	Year	DC	DESC	Al	As	Cr	Cu	Fe	Mn	Ni	Se	Zn	Cd	Li	Pb	Sr	Ti	Tl
Year	NA	-0.384		-0.631		-0.538	-0.413	-0.417	-0.379			-0.572		0.358	-0.636	0.432	-0.570	
DC	0.048	NA									0.401						0.389	
DESC			NA				0.616					0.396	0.515		0.429			0.618
Al	0.000			NA		0.788	0.625	0.614	0.727			0.754	0.457		0.747		0.805	0.720
As					NA	0.457	0.463	0.540	0.498		0.519	0.501	0.365			0.324		
Cr	0.000			0.000	0.004	NA	0.852	0.816	0.864		0.644	0.875	0.000		0.725		0.745	0.702
Cu	0.010		0.001	0.000	0.003	0.000	NA	0.809	0.813		0.503	0.860	0.000		0.652		0.601	0.648
Fe	0.009			0.000	0.000	0.000	0.000	NA	0.641		0.827	0.879	0.002		0.655		0.603	0.786
Mn	0.019			0.000	0.001	0.000	0.000	0.000	NA	0.433	0.446	0.788	0.000		0.635		0.758	0.511
Ni									0.013	NA				0.000				
Se		0.038			0.001	0.000	0.001	0.000	0.005		NA	0.675			0.009		0.465	
Zn	0.000		0.041	0.000	0.001	0.000	0.000	0.000	0.000		0.000	NA	0.000		0.000		0.000	0.049
Cd			0.006	0.007	0.024	0.604	0.775	0.477	0.695			0.720	NA		0.480		0.470	
Li	0.041									0.667				NA		0.552		
Pb	0.000		0.025	0.000		0.000	0.000	0.000	0.000		0.416	0.681	0.002		NA		0.635	0.688
Sr	0.007				0.047				0.079					0.001		NA		
Ti	0.000	0.045		0.000		0.000	0.000	0.000	0.000		0.003	0.709	0.003		0.000		NA	
Tl			0.043	0.006		0.007	0.017	0.001				0.555			0.009			NA

Table 17. Significant Spearman correlations in **fat**. Variables without significant correlations were excluded.

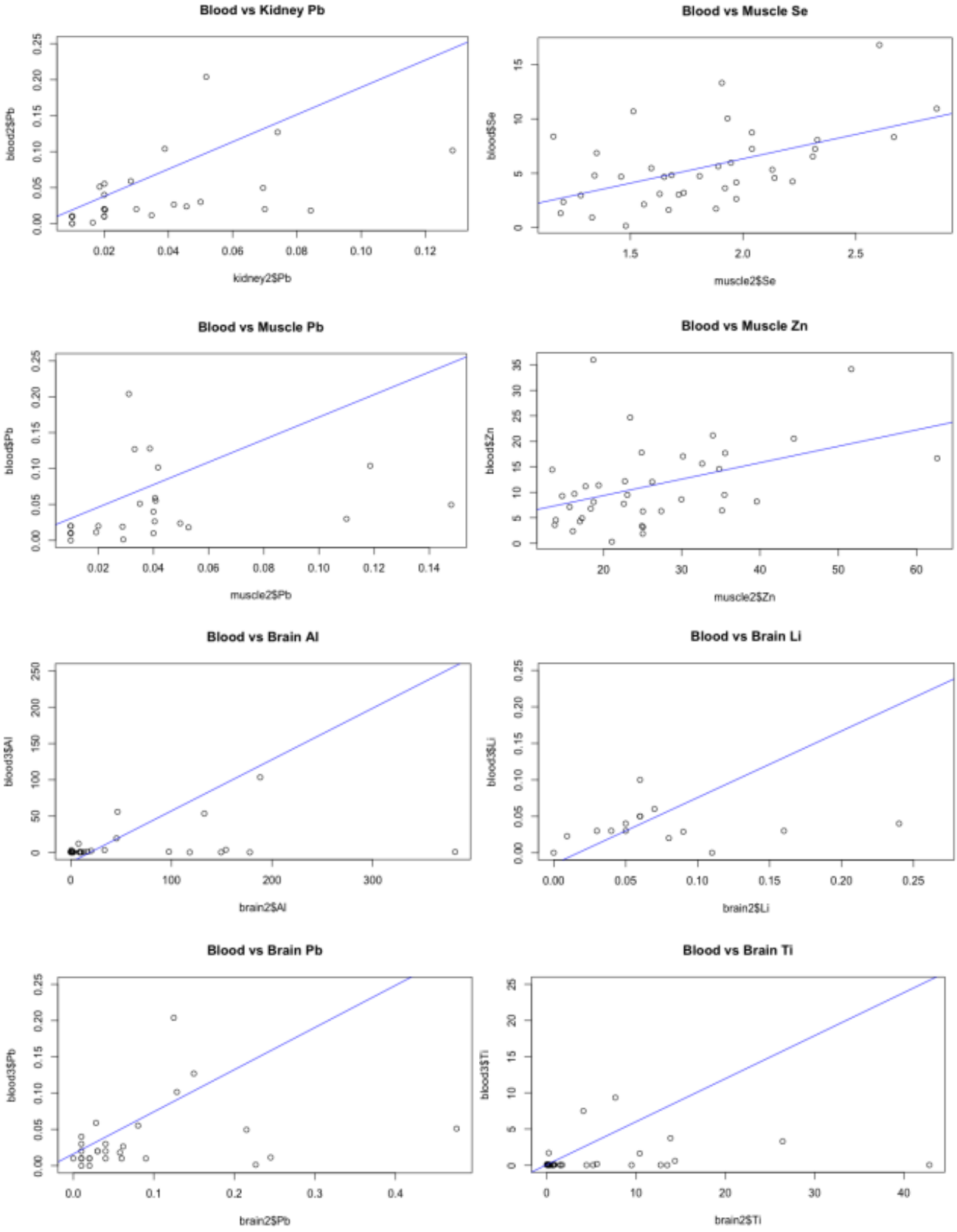
	CCW	DESC	Al	As	Cr	Cu	Fe	Mn	Ni	Se	Zn	Cd	Li	Sr	Ti	Tl
CCW	NA															0.775
DESC		NA				0.720	0.886	0.733			0.733	0.916	0.629			
Al			NA					0.471	0.422						0.563	
As				NA	0.533	0.455			0.415			0.429				
Cr				0.009	NA				0.567							
Cu		0.008		0.029		NA		0.801		0.685	0.754	0.000	0.546	0.509		
Fe		0.000					NA	0.782		0.752	0.489	0.000	0.627	0.557	0.476	
Mn		0.007	0.023			0.000	0.000	NA		0.565	0.663	0.000	0.006	0.685		
Ni			0.045	0.049	0.005				NA							
Se						0.000	0.000	0.005		NA	0.533	0.003		0.458	0.441	
Zn		0.007				0.000	0.018	0.001		0.009	NA	0.008		0.011		
Cd		0.000		0.041		0.770	0.803	0.792		0.588	0.542	NA	0.511	0.710		
Li		0.038				0.023	0.007	0.638				0.036	NA			
Sr						0.013	0.006	0.000		0.028	0.518	0.000		NA		
Ti			0.005				0.022			0.035					NA	
Tl	0.041															NA

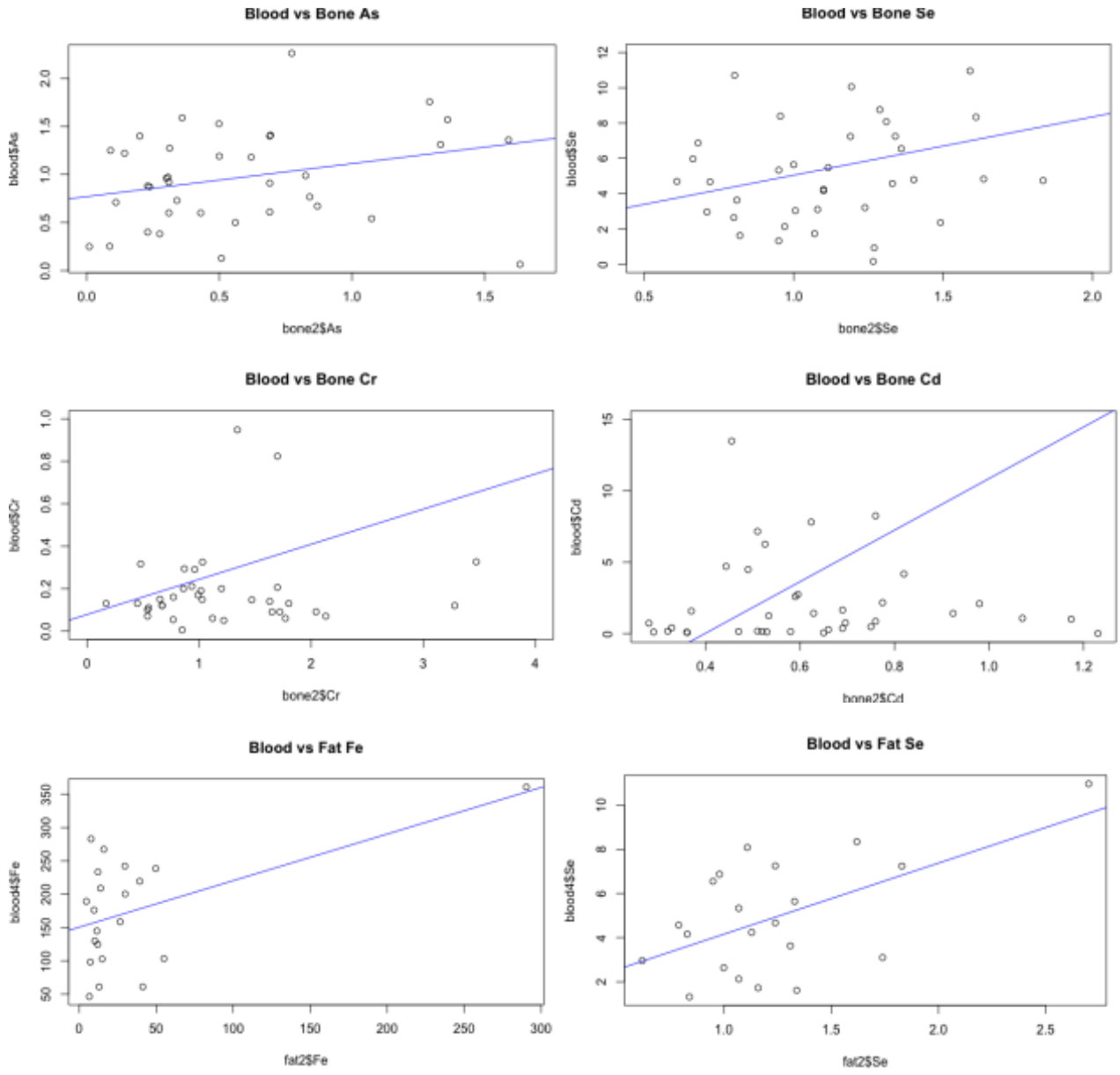
3.2.4. Blood versus tissues concentrations

Finally, we wanted to validated the usefulness of blood in monitoring inorganic elements accumulation in live turtles. In the Figure 8 are represented all the significant relationships found, all of them were positive. Brain and blood were the tissues with more relationships, 4 each one; muscle has 3, fat 2 and kidney one. Despite all these correlations found, most of them were found comparing blood to tissues that are not the accumulation tissues where the highest concentration of the element tested were found. This means that these correlations, even of some of them are very strong, are not useful to indicate the accumulation of those elements through only blood analysis. The only elements with positive relationships between blood and tissues with high concentrations were Al and Ti in brain and Zn in muscle.

Here is important to mention that concentrations of some elements, specifically Cd, Al and Ti, were visibly higher in dead than in live turtles. Cd in blood of live turtles presented a mean of 0.14 with a range of 0.02 to 0.52 μg g^{-1} ww, and dead turtles had 4.26 range of 0.01 to 81 μg g^{-1} ww. Al pass from 1.69 μg g^{-1} ww (range 0.01 to 16) in live turtles to 9.1 μg g^{-1} ww (range of 0.01 to 103) in dead ones; and Ti pass from0.04 μg g^{-1} ww (range of 0.01 to 7.7) to 6.1 μg g^{-1} ww (range of 0.01-42). There are only two explanations for these vast differences: 1) the technique taking the samples (live turtles from the cervical sinuous and dead turtles from the heart), or 2) many dead turtles were recently exposed to very high concentrations of these elements.

Figure 8. Significant relationships between blood and elements in all the tissues.





3.2.5 Conclusions

In both, live and dead turtles, most of the inorganic elements analyzed showed a decreasing tendency over the three years of monitoring (2012-2014). This is a very important result, especially regarding those elements found in high or very high concentrations compared with other populations (Fe in blood and liver, Al and Ti in brain, Sr and Tl in bone). Al was the element showing significant decreasing for all the tissues analyzed, Pb Ti and Sr also had significant decreasing in most tissues. Cd on the other hand, is the most alarming non-

essential element with the highest concentrations worldwide for any sea turtle species, it does not appear to be decreasing during this monitoring program. On the contrary Cd showed a slight increase compared with the first approach program. Bone was the tissues were, except for Cd, all the non-essential elements showed their highest concentrations. Then, bone is a excellent accumulation indicator to many metals. Liver was the tissue with more positive significant correlations between essential and non-essential elements and CCL. Then, this could be the ideal tissue to study the relationships among the size or the age and inorganic elements. Regarding the carcasses decomposition, Al was the element with more alterations related to the decomposition; and muscle and fat look to be the tissues with more alterations in the inorganic elements concentrations depending on the decomposition processes. This is important to take into account in monitoring programs. Finally, try to correlate the concentrations in blood to predict the accumulation of these elements in tissues, does not appear to be accurate; at least our results suggest that it can be useful only for Al and Ti accumulated in brain and Zn in muscle. Blood still a very useful tool regarding acute expositions; though, in order to assess the accumulation and chronic exposition to inorganic elements, tissues still crucial.

References

Aguirre, A.A., Balazs, G.H., Zimmerman, B., Galey, F.D., 1994. Organic contaminants and trace metals in the tissues of green turtles (*Chelonia mydas*) afflicted with fibropapillomas in the Hawaiian Islands. Marine Pollution Bulletin 28, 109-114.

Aguirre, A.A., Lutz, P.L., 2004. Marine turtles as sentinels of ecosystem health: Is fibropapillomatosis an indicator? EcoHealth 1, 275-283.

Agusa, T., Yasugi, S.Y., Iida, A., Ikemoto, T., Anan, Y., Kuiken, T., Osterhaus, A.D., Tanabe, S., Iwata, H., 2011. Accumulation features of trace elements in mass-stranded harbor seals (Phoca vitulina) in the North Sea coast in 2002: the body distribution and association with growth and nutrition status. Mar Pollut Bull 62, 963-975.

Al-Jawad, F.H., Sharquie, K.E., Abu Raghif, A., Nashtar, S.B., 2016. Hepatoprotective effects of zinc sulphate and silymarin against thallium-induced poisoning in rats.

Anan, Y., Kunito, T., Watanabe, I., Sakai, H., Tanabe, S., 2001. Trace element accumulation in Hawksbill turtles (*Eretmochelys imbricata*) and Green Turtles (*Chelonia mydas*) from Yaeyama Islands, Japan. Environmental Toxicology and Chemistry 20, 2802-2814.

Andreani, G., Santoro, M., Cottignoli, S., Fabbri, M., Carpene, E., Isani, G., 2008. Metal distribution and metallothionein in loggerhead (Caretta caretta) and green (Chelonia mydas) sea turtles. Sci Total Environ 390, 287-294.

ATSDR, 2008. Toxicological Profile for aluminium. Agency for Toxic Substances and Disease Registry. US Deparment, Atlanta, Georgia.
ATSDR, 2012. Toxicological profile for cadmium. U.S. Departament of Health and Human Services. Agency for Toxic Substances and Disease Registry, Georgia, U.S.
Bannon, D.I., 2015. Chapter 20. Wildlife toxicity assessment for thallium, Wildlife Toxicity Assessments for Chemicals of Military Concern. Elsevier Science.
Barbieri, E., 2009. Concentration of heavy metals in tissues of Green turtles (*Chelonia mydas*) sampled in the Cananéia Estuary, Brazil. Brazilian Journal of Oceanography 57, 243-248.
Budis, H., Kalisinska, E., Lanocha, N., Kosik-Bogacka, D.I., 2013. The concentration of manganese, iron and strontium in bone of red fox *Vulpes vulpes* (L. 1758). Biological Trace Element Research 155, 361-369.
Burford, N., Carpenter, Y.-y., Conrad, E., Saunders, C.D.L., 2011. The Chemistry of Arsenic, Antimony and Bismuth, in: Sun, H. (Ed.), Biological Chemistry of Arsenic, Antimony and Bismuth. John Wiley & Sons Ltd, United Kingdom.
Burger, J., 2008. Assessment and management of risk to wildlife from cadmium. Science of the Total Environment 389, 37-45.
Caceres-Saez, I., Ribeiro-Guevara, S., Dellabianca, N., Goodall, N., Cappozzo, L., 2013. Heavy metals and essential elements in Commerson's dolphins (*Cephalorhynchus c. commersonii*) from the southwestern South Atlantic Ocean. Environmental Monitoring and Assessment 185, 5375-5386.
Camacho, M., Boada, L.D., Oros, J., Lopez, P., Zumbado, M., Almeida-Gonzalez, M., Luzardo, O.P., 2014. Monitoring organic and inorganic pollutants in juvenile live sea turtles: Results from a study of *Chelonia mydas* and *Eretmochelys imbricata* in Cape Verde. Science of the Total Environment 481, 303-310.
Camacho, M., Oros, J., Boada, L.D., Zaccaroni, A., Silvi, M., Formigaro, C., Lopez, P., Zumbado, M., Luzardo, O.P., 2013. Potential adverse effects of inorganic pollutants on clinical parameters of Loggerhead sea turtles (*Caretta caretta*): Results from a nesting colony from Cape Verde, West Africa. Marine Environmental Research 92, 15-22.
Caurant, F., Amiard-Triquet, C., 1995. Cadmium contamination in pilot whales *Globicephala melas* - Source and potential hazard to the species. Marine Pollution Bulletin 30, 207-210.
Chen, T., Yan, J., Li, Y., 2014. Genotoxicity of titanium dioxide nanoparticles. Journal of Food and Drug Analysis 22, 95-104.
da Silva, C.C., Varela, A.S., Jr., Barcarolli, I.F., Bianchini, A., 2014. Concentrations and distributions of metals in tissues of stranded green sea turtles (*Chelonia mydas*) from the southern Atlantic coast of Brazil. Science of the Total Environment 466-467, 109-118.
Dyc, C., Far, J., Gandar, F., Poulipoulis, A., Greco, A., Eppe, G., Das, K., 2016. Toxicokinetics of selenium in the slider turtle, Trachemys scripta. Ecotoxicology 25, 727-744.
Forbes, S.L., Perrault, K.A., Comstock, J.L., 2017. Microscopic Post-Mortem Changes: the Chemistry of Decomposition. Taphonomy of Human Remains: Forensic Analysis of the Dead and the Depositional Environment, 26.
Ganz, T., Nemeth, E., 2006. Regulation of iron acquisition and iron distribution in mammals. Biochim Biophys Acta 1763, 690-699.

Gardner, S.C., Fitzgerald, S.L., Vargas, B.A., Rodriguez, L.M., 2006. Heavy metal accumulation in four species of sea turtles from the Baja California peninsula, Mexico. Biometals 19, 91-99.
González Muñoz, M.J., Meseguer Soler, I., 2009. Elementos ultratrazas ¿Nutrientes o tóxicos? Toxicología 26, 93-103.
Guirlet, E., Das, K., Girondot, M., 2008. Maternal transfer of trace elements in leatherback turtles (*Dermochelys coriacea)* of French Guiana. Aquatic Toxicology 88, 267-276.
Gündoğdu, A.e., Yardım, O.z., Bat, L., Çulha, S.T.r., 2009. Accumulation of zinc in liver and muscle tissues of rainbow trout (*Onchorhyncus mykiss* Walbaum 1792). Fresenius Environmental Bulletin 18, 40-44.
Haynes, D., Johnson, J.E., 2000. Organochlorine, heavy metal and polyaromatic hydrocarbon pollutant concentrations in the Great Barrier Reef (Australia): a review. Marine Pollution Bulletin 41, 267-278.
IUCN, 2012. Red List of Threatened Species, Version 2012.1. International Union for Conservation of Nature, http://www.iucnredlist.org/.
Klaassen, C.D., Liu, J., Diwan, B.A., 2009. Metallothionein protection of cadmium toxicity. Toxicology and Applied Pharmacology 238, 215-220.
Kumar, V., Gill, K.D., 2009. Aluminium neurotoxicity: neurobehavioural and oxidative aspects. Arch Toxicol 83, 965-978.
Kunito, T., Kubota, R., Fujihara, J., Agusa, T., Tanabe, S., 2008. Arsenic in marine mammals, seabirds, and sea turtles. Reviews of Environmental Contamination and Toxicology, 31-69.
Labrada-Martagon, V., Rodriguez, P.A., Mendez-Rodriguez, L.C., Zenteno-Savin, T., 2011. Oxidative stress indicators and chemical contaminants in East Pacific green turtles (*Chelonia mydas*) inhabiting two foraging coastal lagoons in the Baja California peninsula. Comparative Biochemistry and Physiology Part C 154, 65-75.
Lahaye, V., Bustamante, P., Dabin, W., Van Canneyt, O., Dhermain, F., Cesarini, C., Pierce, G.J., Caurant, F., 2006. New insights from age determination on toxic element accumulation in striped and bottlenose dolphins from Atlantic and Mediterranean waters. Mar Pollut Bull 52, 1219-1230.
Lam, J.C., Tanabe, S., Chan, S.K., Yuen, E.K., Lam, M.H., Lam, P.K., 2004. Trace element residues in tissues of green turtles (*Chelonia mydas*) from South China waters. Marine Pollution Bulletin 48, 174-182.
Lavery, T.J., Butterfield, N., Kemper, C.M., Reid, R.J., Sanderson, K., 2008. Metals and selenium in the liver and bone of three dolphin species from South Australia, 1988-2004. Sci Total Environ 390, 77-85.
Lavery, T.J., Kemper, C.M., Sanderson, K., Schultz, C.G., Coyle, P., Mitchell, J.G., Seuront, L., 2009. Heavy metal toxicity of kidney and bone tissues in South Australian adult bottlenose dolphins (*Tursiops aduncus*). Marine Environmental Research 67, 1-7.
Ley-Quiñónez, C., Zavala-Norzagaray, A.A., Espinosa-Carreon, T.L., Peckham, H., Marquez-Herrera, C., Campos-Villegas, L., Aguirre, A.A., 2011. Baseline heavy metals and metalloid values in blood of loggerhead turtles (*Caretta caretta*) from Baja California Sur, Mexico. Marine Pollution Bulletin 62, 1979-1983.
Linder, M.C., 2013. Mobilization of stored iron in mammals: a review. Nutrients 5, 4022-4050.

Long, T.C., Saleh, N., Tilton, R.D., Lowry, G.V., Veronesi, B., 2006. Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity. Environmental Science & Technology 40, 4346-4352.
Matovic, V., Buha, A., Ethukic-Cosic, D., Bulat, Z., 2015. Insight into the oxidative stress induced by lead and/or cadmium in blood, liver and kidneys. Food Chem Toxicol 78, 130-140.
McPherson, C.A., Lawrence, G.S., Elphick, J.R., Chapman, P.M., 2014. Development of a strontium chronic effects benchmark for aquatic life in freshwater. Environmental Toxicology and Chemistry 33, 2472-2478.
Nardini, G., Leopardi, S., Bielli, M., 2013. Clinical hematology in reptilian species. Vet Clin North Am Exot Anim Pract 16, 1-30.
Nunes, B., Capela, R.C., Sergio, T., Caldeira, C., Goncalves, F., Correia, A.T., 2014. Effects of chronic exposure to lead, copper, zinc, and cadmium on biomarkers of the European eel, *Anguilla anguilla*. Environmental Science and Pollution Research 21, 5689-5700.
Ocana, M.A.S., 2010. Arribada nesting of olive ridley sea turtles (Lepidochelys olivacea) at La Escobilla, Mexico. Oregon State University, p. 89.
Páez-Osúna, F., Calderon-Campuzano, M.F., Soto-Jiménez, M.F., Ruelas-Inzunza, J., 2011. Mercury in blood and eggs of the sea turtle *Lepidochelys olivacea* from a nesting colony in Oaxaca, Mexico. Marine Pollution Bulletin 62, 1320-1323.
Páez-Osúna, F., Calderón-Campuzano, M.F., Soto-Jiménez, M.F., Ruelas-Inzunza, J.R., 2010a. Lead in blood and eggs of the sea turtle, *Lepidochelys olivacea*, from the Eastern Pacific: concentration, isotopic composition and maternal transfer. Marine Pollution Bulletin 60, 433-439.
Páez-Osúna, F., Calderón-Campuzano, M.F., Soto-Jiménez, M.F., Ruelas-Inzunza, J.R., 2010b. Trace metals (Cd, Cu, Ni, and Zn) in blood and eggs of the sea turtle *Lepidochelys olivacea* from a nesting colony of Oaxaca, Mexico. Archives of Environmental Contamination and Toxicology 59, 632-641.
Pemmer, B., Roschger, A., Wastl, A., Hofstaetter, J.G., Wobrauschek, P., Simon, R., Thaler, H.W., Roschger, P., Klaushofer, K., Streli, C., 2013. Spatial distribution of the trace elements zinc, strontium and lead in human bone tissue. Bone 57, 184-193.
Pigatto, P.D., Dell'Osso, B., Guzzi, G., 2016. Lithium overdosage and related tests. Int J Bipolar Disord 4, 1.
R Core Team, 2017. R: A language and environment for statistical computing, 3.4.0 ed. R Foundation for Statistical Computing, Vienna, Austria.
Ramsden, C.S., Smith, T.J., Shaw, B.J., Handy, R.D., 2009. Dietary exposure to titanium dioxide nanoparticles in rainbow trout,(Oncorhynchus mykiss): no effect on growth, but subtle biochemical disturbances in the brain. Ecotoxicology 18, 939-951.
Rana, S.V., 2014. Perspectives in endocrine toxicity of heavy metals-a review. Biological Trace Element Research 160, 1-14.
Register, A.L., 2011. Effects of Heavy Metal Pollution on the Loggerhead Sea Turtle, School of Science and Technology. Loma Linda University, Electronic Theses and Dissertations, p. 117.
Reijnders, P.J.H., 2003. Reproductive and developmental effects of environmental organochlorines on marine mammals. Taylor and Francis, London.

Rie, M., Lendas, K., Callard, I., 2001. Cadmium: tissue distribution and binding protein induction in the painted turtle, Chrysemys picta. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 130, 41-51.
Rodriguez-Mercado, J.J., Altamirano-Lozano, M.A., 2013. Genetic toxicology of thallium: a review. Drug and Chemical Toxicology 36, 369-383.
Sakai, H., Saeki, K., Ichihashi, H., Suganuma, H., Tanabe, S., Tatsukawa, R., 2000. Species-specific distribution of heavy metals in tissues and organs of Loggerhead turtle (*Caretta caretta*) and Green turtle (*Chelonia mydas*) from Japanese Coastal Waters. Marine Pollution Bulletin 40, 701-709.
Santamaria, A.B., 2008. Manganese exposure, essentiality & toxicity. Indian J Med Res 128, 484-500.
SEMARNAT, 2010. Norma Oficila Mexicana 059, in: Naturales, S.d.M.A.y.R. (Ed.).
Senthil Murugan, S., Karuppasamy, R., Poongodi, K., Puvaneswari, S., 2008. Bioaccumulation pattern of zinc in freshwater fish *Channa punctatus (*Bloch.) after chronic exposure. Turkish Journal of Fisheries and Aquatic Sciences 8, 55-59.
Shi, H., Magaye, R., Castranova, V., Zhao, J., 2013. Titanium dioxide nanoparticles: a review of current toxicological data. Particle and Fibre Toxicology, 10:15.
Shimizu, M., Tainaka, H., Oba, T., Mizuo, K., Umezawa, M., Takeda, K., 2009. Maternal exposure to nanoparticulate titanium dioxide during the prenatal period alters gene expression related to brain development in the mouse. Particle and Fibre Toxicology 6, 20.
Siscar, R., Koenig, S., Torreblanca, A., Sole, M., 2014. The role of metallothionein and selenium in metal detoxification in the liver of deep-sea fish from the NW Mediterranean Sea. Sci Total Environ 466-467, 898-905.
Stefanidou, M., Maravelias, C., Dona, A., Spiliopoulou, C., 2006. Zinc: a multipurpose trace element. Archives of Toxicology 80, 1-9.
Storelli, M.M., Ceci, E., Marcotrigiano, G.O., 1998. Comparison of Total Mercury, Methylmercury, and Selenium in Muscle Tissues and in the Liver of Stenella coeruleoalba (Meyen) and Caretta caretta (Linnaeus). Bull. Environ. Contam. Toxicol. 61, 541-547.
Storelli, M.M., Storelli, A., D'Addabbo, R., Marano, C., Bruno, R., Marcotrigiano, G.O., 2005. Trace elements in loggerhead turtles (*Caretta caretta*) from the eastern Mediterranean Sea: overview and evaluation. Environmental Pollution 135, 163-170.
Sun, H.J., Rathinasabapathi, B., Wu, B., Luo, J., Pu, L.P., Ma, L.Q., 2014. Arsenic and selenium toxicity and their interactive effects in humans. Environment International 69, 148-158.
Tapiero, H., Tew, K.D., 2003. Trace elements in human physiology and pathology: zinc and metallothioneins. Biomedicine & Pharmacotherapy 57, 399-411.
Torrent, A., Gonzalez-Diaz, O.M., Monagas, P., Oros, J., 2004. Tissue distribution of metals in loggerhead turtles (*Caretta caretta*) stranded in the Canary Islands, Spain. Marine Pollution Bulletin 49, 854-860.
Vighi, M., Borrell, A., Aguilar, A., 2016. Bone as a surrogate tissue to monitor metals in baleen whales. Chemosphere 171, 81-88.
Villa, C.A., Flint, M., Bell, I., Hof, C., Limpus, C.J., Gaus, C., 2017. Trace element reference intervals in the blood of healthy green sea turtles to evaluate exposure of coastal populations. Environmental Pollution 220, 1465-1476.

Yokel, R.A., 2006. Blood-brain barrier flux of aluminum, manganese, iron and other metals suspected to contribute to metal-induced neurodegeneration. Journal of Alzheimer's Disease 10, 223-253.
Yokel, R.A., McNamara, P.J., 2001. Aluminium Toxicokinetics: An Updated MiniReview. Pharmacology & Toxicology 88, 159-167.
Zavala-Norzagaray, A.A., Ley-Quiñónez, C.P., Espinosa-Carreon, T.L., Canizalez-Roman, A., Hart, C.E., Aguirre, A.A., 2014. Trace elements in blood of sea turtles *Lepidochelys olivacea* in the Gulf of California, Mexico. Bulletin of Environmental Contamination and Toxicology 93, 536-541.

SECOND PART. BIOMARKERS

3.3 Oxidative Stress

CHAPTER IV. Molecular oxidative stress markers in Olive Ridley turtles (*Lepidochelys olivacea*) and its relation to metal concentrations in wild populations

3.3.1 Introduction

Despite the fact that only seven species exist, sea turtles are globally distributed in all oceans and placed in important ecological niches; among them, Olive Ridley (*Lepidochelys olivacea*) constitutes the biggest population (Wallace et al., 2011). Considering how numerous this species has been historically at ecological level they have a great value as a prey for crabs, birds, fishes, small carnivores and sharks, and as a predator of fishes, jellyfish or sea grass (CONANP, 2011b). This ecological impact is especially meaningful during the *arribada* events, when thousands of turtles come out to nest to the same beach at the same time. They also have an active role in the transport of nutrients from the ocean to the beach, through the eggs (Silman et al., 2002), and in the transport of carbon from pelagic areas to abyssal zones (Buitrago, 2009). Olive Ridley turtles are classified as "vulnerable" by the International Union for Conservation of Nature-Red List (IUCN, 2012). In Mexico, all marine turtles are considered endangered species within the regulation norm NOM-ECOL-059-2010, and have been made a priority for conservation (CONANP, 2011b). In general, the main threats that have lead turtles to this point are bycatch, climate change, consumption of egg and meat in certain regions, nesting beaches development, pathogens and pollution (IUCN, 2012; Wallace et al., 2011).

Due to their longevity and extensive migration areas, marine turtles are able to accumulate diverse contaminants throughout many years; consequently, they represent interesting biomarker species for marine ecosystem's pollution (Andreani et al., 2008b; Godley et al., 1999a; Sakai et al., 2000). There are many different kinds of pollutants being inorganic elements, such as metals, one of the most studied due to their toxicity (REVIEW IF POSSIBLE). Bioaccumulation of these elements varies depending on several factors including geographic location, exposure to diverse pollutants, diet, species and tissues analyzed (Alava et al., 2006; Guirlet et al., 2008). The presence of some of these metals has been previously reported in Olive Ridley turtles from *La Escobilla* beach (Mexico), especially Cd, which has been found in very high concentrations compared to any other turtle species (Cortés-Gómez et al., 2014).

Oxidative stress is an unavoidable aspect of aerobic life. It is the result of an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defenses in the organisms. Therefore, this oxidative stress is produced by the cellular accumulation of ROS, molecules with strong oxidative properties (Valdivia et al., 2007), and this could occur by many factors. Among them, expose to a variety of environmental stressors, such as pollutants, produces increased production of ROS and reduced antioxidant potential leading to oxidative stress (Labrada-Martagon et al., 2011a). It has been demonstrated, in *in vivo* and *in vitro* works, that ROS have the potential for damaging proteins, lipids and nucleic acids, causing tissue damage, metabolic dysfunction and apoptosis cell death (Labrada-Martagon et al., 2011a; Morcillo et al., 2016; Valdivia et al., 2007).

Due to the relationship between molecular indicators of oxidative stress and environmental xenobiotics (e.g. heavy metals), superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) are considered indicators of the cell defense mechanisms against ROS, and therefore an important tool as biomarkers of pollution exposure (Morcillo et al., 2016). On the other hand, metallothioneins (MT) are among the most important proteins in the protection of cells against heavy metal toxicity (especially Cd, Cu and Zn) as clearly demonstrated using mouse models under acute and chronic Cd toxicity (Klaassen et al., 2009). In addition, MTs also have an important role in protecting cells from ROS-mediated injury and reducing oxidative stress (Andreani et al., 2008b; Ruttkay-Nedecky et al., 2013). Most of the information relies on determinations at protein or enzyme activity level of MTs and antioxidant enzymes and less attention has been paid to their regulation at gene expression (Matovic et al., 2015; Misra and Niyogi, 2009; Regoli and Giuliani, 2014; Venancio et al., 2013). Even less works on this subject have been driven on wildlife populations, since most of the works are developed in laboratory-controlled experiments. In the case of marine turtles, literature about oxidative stress is recent and very limited. Regarding antioxidant enzymatic activities, Labrada-Martagon et al. (2011a), with live animals and Valdivia et al. (2007) with incidental death turtles, did extensive works in three

foraging costal populations of Green turtles (*Chelonia mydas*), relating the antioxidant status with organic and inorganic pollutants in blood, liver and kidney tissues. Other works have also correlated the blood antioxidant enzymatic activities of Hawksbill sea turtles (*Eretmochelys imbricata*) and organochlorines (Tremblay et al. (2016) as well as of Kemp's Ridley turtles (*Lepidochelys kempii*) and brevetoxine (Perrault et al. (2014). About cellular protection and oxidative stress we find only one work in two different marine turtle populations (*C. caretta* and *C. mydas*) relating MT transcripts and metals (Andreani et al., 2008b). To our knowledge, there is no information about molecular indicators and enzyme activity (*sod, cat, gr* or *mt*) in Olive Ridley turtles.

For all above exposed, we wanted to establish if oxidative stress and MT are useful biomarkers in Olive Ridley turtles as in other species. Due to the lack of information existing about *sod, cat, gr* and *mt* in *L. olivacea* and their relation to metals, the aims of this work were: (1) partially sequence the genes of *sod, cat, gr* and *mt* from *Lepidochelys olivacea*, (2) assess gene expression of these biomarkers in blood, liver and kidney by real-time PCR, (3) measure SOD, CAT and GR enzyme activity in the same tissues, (4) asses heavy metal concentrations, and (5) make relationship analysis among gene expression, enzyme activity and heavy metal concentrations.

3.3.2. Material and methods

3.3.2.1 Sample collection

Olive Ridley turtles, 20 alive and 20 dead individuals, were sampled at *La Escobilla* beach, in the State of Oaxaca, Mexico (Southeast Mexico, Eastern Pacific, 96°440W and 15°470N) during four different *arribadas* in 2013 and 2014 (2 *per* year, in both cases during August and September). Curve carapace length (CCL) and straight carapace length (SCL) from all the individuals were recorded and used to determine size as a relative indicator of age (Balazs et al., 1987). Regarding live turtles, blood was taken from the dorsal cervical sinus, using a 5-mL heparinized syringe with 21G needle, divided in three samples and stored frozen at -

20°C till used: 1) whole blood; 2) plasma isolated by centrifugation; and 3) whole blood in Trizol® Reagent (Life Technologies).

Twenty recently dead turtles were also sampled (7 from 2013 and 13 from 2014). The cause of dead of 50% of the turtles was injuries caused by boat propel, and for the rest we could not establish the cause of dead. Liver and kidney samples were taken directly from the carcasses after the plastron was removed. Around 0.5 g of each tissue was stored into 1.5 mL microtubes with 1 mL of Trizol® Reagent, and another 1 g of tissue was stored alone in microtubes. All samples were finally frozen until processed. Permissions for this study to collect the samples were SGPA/DGVS/00905/13 and SGPA/DGVS/00774/14 and CITES for exportation were MX 67725 and MX 72799.

3.3.2.2 Metal analysis

Metal analysis was performed on blood samples from live turtles and in liver and kidney tissues from dead turtles. To do this, an acidic digestion was performed using 0.5 g of the sample into 4 mL of HNO_3 (69%) and 1 mL of H_2O_2 (33%) mixed in special Teflon reaction tubes in a microwave digestion system (UltraClave-Microwave Milestone) for 20 min at 220°C and finally diluted with 25 mL of double deionized water (Milli-Q). Metal concentrations were determined in whole blood, liver and kidney, using an Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES, ICAP 6500 Duo Thermo) following descriptions from Cortés-Gómez et al. (2014). All concentrations are expressed in microgram per gram of wet tissue weight. Detection limits for all elements were 0.01 $\mu g\ g^{-1}$.

3.3.2.3 Gene expression

Total RNA was extracted from whole blood, liver and kidney using Trizol® Reagent according to the manufacturer's instructions. The concentration and quality of RNA in the samples was assessed using NanoDrop 2000® Spectrophotometer resulting the 260/280 nm absorption ratio of 1.8-2.0 in all samples. The RNA was then treated with DNase I (Promega) to remove any genomic DNA contamination. Complementary DNA (cDNA) was synthesized

from 1 μg of total RNA using the SuperScript III reverse transcriptase (Life Technologies) with an oligo-dT18 primer.

First, we failed to find the sequences for *Lepidochelys olivacea sod, cat, gr* or *mt* genes in the NCIB gene databases. Therefore, to sequence them, liver cDNA was used in a first PCR amplification with degenerated primers (Table 1) designed against the known turtle respective sequences. PCR reactions were carried out using Taq polymerase (Life Technologies) and the amplification performed in a MasterCycler Gradient PCR: 95°C for 5 min; 35 cycles of 95°C for 45 s, 55C for 45 s, 72°C for 45 s; and followed by 72°C for 10 min. PCR products were separated on a 1% agarose gel containing 0.5 μg ml^{-1} ethidium bromide and visualized under UV light. Bands of the expected size were cut and the DNA purified from the gel and sequenced using an ABI PRISM 377 sequencer. Sequences were analyzed for similarity with other known sequences using the BLAST program (Altschul et al., 1990) within the ExPASy Molecular Biology server (http://us.expasy.org). Identified partial Olive Ridley turtles *sod*, *cat*, *gr* and *mt* sequences were now used to design specific primers (Table 1).

Table 1. Oligonucleotide primers used in the study.

Gene name	Gene abbreviation	Acc. number	Primer sequence (5′ → 3′)	Application
Cu/Zn Superoxide dismutase	*sod*	--	Fw1: TTTGACTGAAGGAAAACATGGCTTCC R1: TCCAAYAACACCACAAGCCAGACG	Cloning
		KY777501	F: TGGATGTACCAGTGCAGGTG R: CAATCACATTGCCAAGATCG	qPCR
Catalase	*cat*	--	Fw1: GTTTTCACTGATGAGATGGCTCATTT Fw2: AACAGGAAYCCTGTCAATTATTTTGC R1: AGACGACCCTGTAACATTTTATCAGG R2: CAGGTTAGATYKTTCCTTTGMAGACA	Cloning
		KY777503	F: GATCAGCCCAATTTGAAGGA R: TCACACAGTCTTTGGCGTTC	qPCR
Glutathione reductase	*gr*	--	Fw1: TTGGTGCTGGATACATTGCTGTGG R1: CATTTTGACTGCTACAGCGAAACC	Cloning
		KY777502	F: TGCACTCAGGAACTGGAGAA R: CCTCCAACAATCCAAGAGGA	qPCR
Metallothionein	*mt*	--	Fw1: GGTGGCTCSTGTACCTGTGCTG Fw2: RAACTGCAGSTGCACCTCTTGC R1: TTGCAGACACAGCCCTTGGCACA	Cloning
		KY777504	F: CGTGTACCTGTGCTGACTCCT R: CTTGGCACAATTATTGCATCC	qPCR
Ribosomal 16S RNA	*16s*	AY390777	F: CGTGCAAAAGCGAGGATAACATTAT R:AGTCTTTAGGGTAGTAAGTGTGATAGTTGAT	qPCR

The expression of the selected genes was analyzed by real-time PCR (qPCR), in the 40 selected turtles, using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), which was performed with an ABI PRISM 7500 instrument (Life Technologies) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 μL of 2 × SYBR Green supermix, 5 μL of primers (0.6 mM each) (Table 1) and 5 μL of cDNA template) were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and finally 15 s at 95°C, 1 min at 60°C and 15 s at 95°C. For each mRNA, gene expression was corrected by the ribosomal 16S (*16s*) (Chavez et al., 2009) RNA content in each sample. In all cases, each PCR was performed with triplicate samples.

3.3.2.4 Enzyme activity

3.3.2.4.1. Sample preparation

Enzymatic activities were determined in the 40 selected turtles. Whole blood/plasma, liver and kidney samples were homogenized in 500 μL of 50 mM phosphate buffer (pH 7.0) during 30 to 60 seconds in ice-cold conditions. Then, samples were vortexed for 10s and immediately centrifuged at 12,000 g for 10 min at 4˚C. Supernatants obtained were used for SOD, CAT and GR activity determination. Protein concentration in each sample was determined by the dye binding method of Bradford (Bradford, 1976), using bovine serum albumin (BSA, Sigma-Aldrich) as the standard. Samples were aliquoted and stored at -80°C until use.

3.3.2.4.2. Superoxide dismutase (SOD)

SOD activity was determined by was determined by the inhibition of the reduction of cytochrome c (McCord and Fridovich, 1969). A reaction solution was prepared containing EDTA 0.079 mM, xanthine 0.097 mM, cytochrome c 0.012 mM. The samples (50 μl from blood/plasma, liver or kidney extracts) and the reaction solution (925 μl) were added into a cuvette and the formation of superoxide radical anion ($O_2^{\cdot -}$) was detected by monitoring the formation of the reduced form of cytochrome c at 550 nm during 1 minute in a spectrophotometer (Thermo Scientific, Model Evolution 300). The maximum of the reaction

was calculated monitoring the reduction of the cytochrome c in a cuvette with 925 µl of the reaction solution and 25 µl of the xanthine oxidase 0.025 UI ml^{-1}. The reaction was probed using 1 ml of reaction solution with enough sodium dithionite to reduce all the cytochrome c from the cuvette. One unit of SOD activity was defined as the amount of enzyme that inhibited the reduction rate of cytochrome c for 50% under the described condition. Results were expressed as units of SOD per milligram of protein (U mg^{-1}).

3.3.2.4.3. Catalase activity (CAT)

The activity of CAT was determining by the decrease in absorbance at 240 nm due to the decomposition of hydrogen peroxide Aebi (1984). The amount of H_2O_2 converted into H_2O and O_2 in 1 min under standard conditions is accepted as the enzyme reaction rate. Thus, 950 µl of H_2O_2 solution (10 mM) in a 50mM phosphate buffer (pH=7) and 50 µl of sample were pipetted into a cuvette. The reduction of H_2O_2 was followed at a wavelength of 240 nm for 4 min against a blank containing 50mM phosphate buffer. The results are expressed as units of CAT per milligram of protein (U mg^{-1}).

3.3.2.4.4. Glutathione reductase (GR)

GR activity was measured by the standard method (Carlberg and Mannervik, 1975) with some modifications. The reaction was initiated by the addition of 100 µl 0.1 mM NADPH to 600 µl of 50 mM potassium phosphate buffer 2 mM EDTA pH 7.0, 200 µl of 0.5 mM glutathione oxidized (GSSG) solution and 50 µl of sample. Absorbance was monitored at 340 nm for 3 min by a UV–Vis Thermo Scientific Model Evolution 300 dual beam spectrophotometer. One unit of GR activity is defined as the amount of enzyme that catalyzes the reduction of 1 µmol of NADPH per minute (ε340 nm for NADPH 6.22 mM^{-1} cm^{-1}). The results are expressed as units of GR per milligram of protein (U mg^{-1}).

3.3.2.5 Statistical analysis

The software R (R Core Team, 2016) was used to analyze the data. The relationships between the enzyme activities, gene expression and metal concentrations for each tissue

were performed with a generalized linear model (GLM), using Gaussian distribution and an identity link from the package "stats" with the **glm()** function. The same function was used to check significant relationships in metal concentrations between the two years of sampling. Spearman correlations were also performed for activity and gene expressions in the different tissues. Principal components analysis (PCA) was perform using "Hmisc" package. The level of significance was fixed at 95%.

3.3.3. Results

3.3.3.1. Metal concentrations

Metal concentrations in tissues are summarized in Table 2 by tissue and by year. Significant differences between year 2013 and 2014 sampling were found in liver and kidney (Table 2, Figure 1). In the liver, Li had a significant increase from 2013 to 2014 (Table 2, Figure 1a). Kidney was the organ with more significant increments from year 2013 to 2014. Thus, kidney levels of As, Cd, Cr, Tl, Li, Cu and Fe increased in 2014 ($p<0.01$) (Table 2, Figure 1b). Thus, this finding may indicate an increase of some toxic metals, which must be carefully followed to see if it is a real increase of these metals availability or a one-time event, and if these elements may have a negative impact in the health of these turtles.

3.3.3.2. Biological markers

We successfully found and partially cloned Olive Ridley turtle *mt*, *sod*, *cat* and *gr* genes, with GenBank accession numbers KY777504, KY777501, KY777503 and KY777502, respectively; which are all very well conserved. Next, we evaluated their expression by qPCR in kidney, liver and blood samples. The highest transcription was of *mt* and the lowest corresponded to *gr* genes (Table 3). Among the tissues, *sod*, *cat* and *gr* genes showed the highest expression in the blood followed by similar transcription in the liver and kidney being very similar the expression of *mt* gene in all tissues (Table 3).

Regarding the enzymatic activities, SOD and CAT activities were detected in the three tissues with similar pattern (liver≈kidney>blood/plasma). Blood samples were all negative

for SOD activity and this only resulted positive in plasma samples. GR was highest in kidney and not analyzed in plasma due to the lack of enough sample (Table 3).

Table 2. Inorganic element concentrations (mean ± SD; n=7-20) in liver and kidney (dead turtles; 7 in 2013 and 13 in 2014) and blood (live turtles; 20 in 2014).

Element	Year	Liver	Kidney	Blood
Al	2013	9.03 ± 6.64	3.03 ± 1.10	
	2014	2.14 ± 3.43*	0.34 ± 0.45*	0.20 ± 0.25
As	2013	3.64 ± 2.66	0.55 ± 0.26	
	2014	5.93 ± 3.51	1.50 ± 0.77*	1.41 ± 1.62
Cd	2013	73.90 ± 54.3	78.1 ± 52.9	
	2014	95.65 ± 49.2	225 ± 103*	0.13 ± 0.08
Cr	2013	1.19 ± 1.03	0.06 ± 0.02	
	2014	0.80 ± 0.45	0.89 ± 0.41*	0.73 ± 0.10
Cu	2013	11.29 ± 4.89	1.07 ± 0.25	
	2014	11.11 ± 7.35	1.64 ± 0.61*	0.56 ± 0.35
Fe	2013	2,429 ± 2,124	24.8 ± 8.4	
	2014	2,127 ± 1,373	102 ± 86*	259 ± 152
Li	2013	BDL	BDL	
	2014	0.05 ± 0.04*	2.89 ± 1.73*	0.03 ± 0.02
Ni	2013	0.06 ± 0.05	0.02 ± 0.02	
	2014	0.06 ± 0.04	0.03 ± 0.01	0.06 ± 0.03
Pb	2013	0.20 ± 0.13	0.03 ± 0.01	
	2014	0.07 ± 0.05*	0.02 ± 0.02*	0.01 ± 0.01
Sb	2013	BLD	0.07 ± 0.01	
	2014	0.06 ± (0.08	0.04 ± 0.09	BDL
Se	2013	9.82 ± 6.64	1.49 ± 0.49	
	2014	11.01 ± 3.85	2.33 ± 1.46	7.26 ± 5.52
Sr	2013	0.79 ± 0.19	0.60 ± 0.25	
	2014	0.78 ± 0.78	0.80 ± 0.70	1.02 ± 0.62
Ti	2013	0.24 ± 0.16	0.98 ± 0.68	
	2014	0.15 ± 0.23	5.62 ± 9.39	0.02 ± 0.04
Tl	2013	1.58 ± 1.58	BDL	
	2014	1.36 ± 0.84	14.54 ± 7.09*	BDL
Zn	2013	56.4 ± 39.38	32.9 ± 10.1	
	2014	46.3 ± 10.3	42.8 ± 14.9	8.06 ± 4.70

*Elements with significant differences between 2013 and 2014 based in a GLM test. BDL=Below the detection limit (<0.01)

3.3.3.3. Relationships among biological markers and metal concentrations

Any significant correlation between *sod* gene expression and SOD activity in liver, kidney or blood was found (p=0.27, 0.11 and 0.49, respectively). Only *gr* gene expression and GR activity had a significant negative relationship in kidney (p<0.01). However, some

interesting relations resulted when the genes/enzymatic activities were compared with the metal concentrations (Table 4). After the application of GLM 7 significant relationships between *sod* gene expression and metals in liver (As, Cd, Cr, Fe, Ni, Pb, Zn) and one in kidney (Cr) were found. *Cat* had 3 negative in liver (Cr, Fe, Tl), 2 positive (Ni, Cr) and one negative (Cd) in kidney; and one positive in blood (Cd). *Gr* had only positive relationships, 5 in liver (As, Cr, Fe, Ni, Pb) and 3 in kidney (Cd, Zn, Ni). *Mt* had one positive (As) and one negative (Cu) relationships in kidney. Regarding the enzyme activity SOD had 6 negative relationships in liver (Cd, Cr, Fe, Pb, Ti, Tl), another negative in kidney (Cd) and one positive in plasma (Pb). CAT had only positive relationships: 5 in liver (Al, As, Cd, Se, Zn), 3 in kidney (As, Cd, Sb), and one in blood (Cu). Finally, GR activity also had only positive relationships: 7 in liver (Al, As, Cd, Cr, Fe, Sb, Ti) and one in kidney (Sb); GR activity was not determined in blood or plasma.

Table 3. Relative gene expression and enzyme activity (U mg^{-1} protein) in liver, kidney and blood/plasma. SOD activity was measured in plasma.

Gene expression		**MT**	**SOD**	**CAT**	**GR**
	mean	0.12162	0.00262	$3.2609E^{-04}$	$7.5583E^{-05}$
Liver	SD	0.15879	0.00321	$2.5971E^{-04}$	$5.0451E^{-05}$
	n=	19	18	10	18
	mean	0.01813	0.00151	$1.1398E^{-04}$	$7.0021E^{-05}$
Kidney	SD	0.01526	0.00207	$1.2968E^{-04}$	$9.3526E^{-05}$
	n=	20	20	16	20
	mean	0.06721	0.04285	0.00215	0.00419
Blood	SD	0.1074	0.04981	0.00640	0.00152
	n=	20	18	8	15
Activity (U mg^{-1} protein)					
	mean		7.20921	18.1907	0.02774
Liver	SD		3.70680	14.2682	0.02339
	n=		16	20	16
	mean		9.05795	16.36979	0.43084
Kidney	SD		3.60903	12.43439	0.15266
	n=		19	19	19
	mean		0.86096*	3.75863	
Blood/ Plasma*	SD		0.88181	2.67649	
	n=		18	20	

In order to integrate oxidative stress and metal interactions in the turtles a PCA was carried out. Gene expression (*sod, cat, gr* and *mt*), enzyme activity (SOD, CAT and GR), all the elements (Al, As, Cd, Cr, Cu, Fe, Li, Ni, Pb, Sb, Se, Sr, Ti, Tl and Zn), biometrics (CCL and CCW),

and sampling year information was used. The integration of all these variables in the PCA was represented by four principal components that explained the 73.76% of the variance in the original data set. The most important principal component, PC1 explained the 35.36% of the variance, follow by PC2 with 17.52%, PC3 with the 11.81% and finally the PC4 with only 9.07% of the total. PCA graphs show that gene expression and the enzyme activity are clearly grouped in different axis in a opposite way, except for GR gene expression and activity, which seems slightly related (Figure 2). Besides, most of the analyzed metals (As, Cr, Cu, Fe, Se, Pb, Zn, Ni, Cd, Tl and Al) are grouped in the same direction with the four gene expressions as well as the size of the individuals (CCL and CCW) and the year of the sampling. On the other side, all the enzymatic activities measured are grouped together along with Ti and Sb.

Figure 1. Metal concentration (µg g^{-1}) with significant differences between years 2,013 and 2,014 in liver (A) and kidney (B) from Olive Ridley turtles. Data are presented as box showing mean, SD, maximum and minimum, all these metals were above the limit of detection in all the samples.

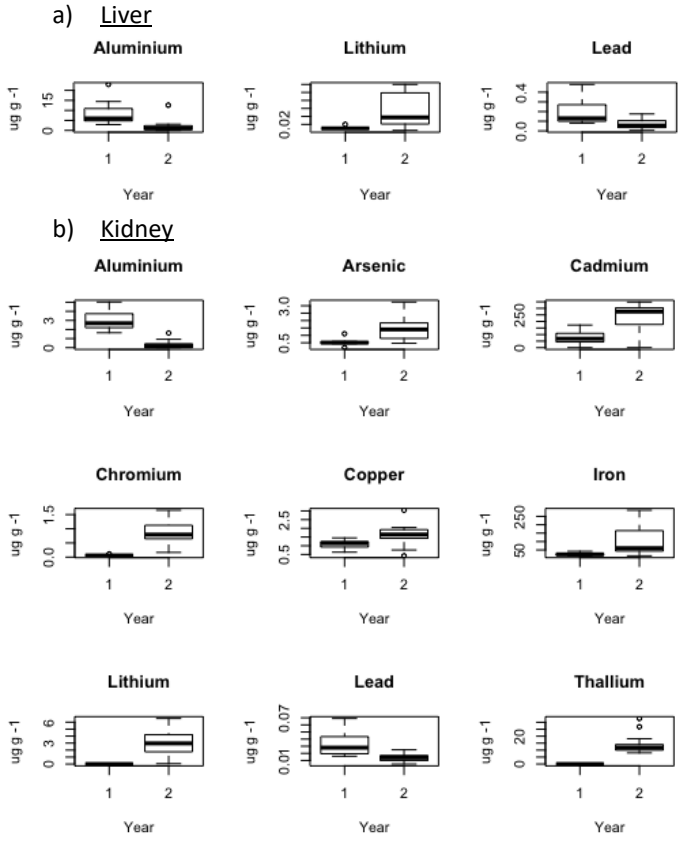
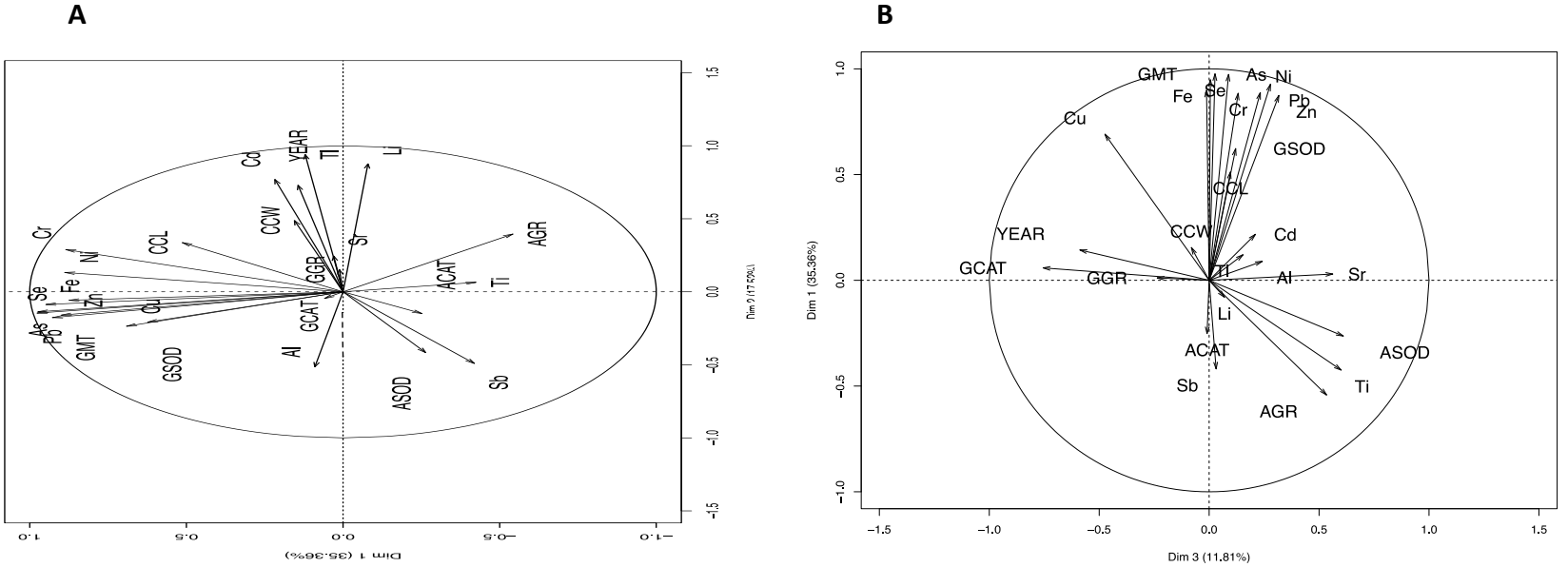


Table 4. Significant differences found by tissue for gene expression and enzyme activity and metals after the GLM. P= level of significance (95%). (+) are positive and (-) negative relationships.

	Liver	**Kidney**	**Blood/plasma**
Gene expression			
sod	**(+)** As, Cd, Cr, Fe **(P<0.05)** **(+)** Ni, Pb, Zn **(P<0.01)**	**(+)** Cr **(P<0.05)**	-
cat	**(-)** Cr, Fe, Tl **(P<0.05)**	**(+)** Ni, Cr **(P<0.01)** **(-)** Cd **(P<0.05)**	**(+)** Cd **(P<0.05)**
gr	**(+)** As, Cr, Fe, Ni **(P<0.05)** **(+)** Pb **(P<0.01)**	**(+)** Cd, Zn **(P<0.05)** **(+)** Ni **(P>0.001)**	-
mt	-	**(+)** As **(P=0.05)** **(-)** Cu **(P=0.05)**	-
Enzyme activity			
SOD	**(-)** Ti, Cd, Cr, Fe, Pb and Tl **(P<0.01)**	**(-)** Cd **(P<0.01)**	**(+)** Pb **(P<0.01)**
CAT	**(+)** As, Cd, Se, Zn **(P<0.05)** **(+)** Al **(P<0.001)**	**(+)** As, Sb, Cd **(P<0.05)**	**(+)** Cu **(P<0.05)**
GR	**(+)** As, Cd, Cr, Fe, Sb **(P<0.05)** **(+)** Al, Ti **(P<0.001)**	**(+)** Sb **(P<0.01)**	Not analyzed

Figure 2. Variables factor map from the Principal components analysis (PCA). Figure **A** with PC1 (Y, explaining 35.36% of the variance) and PC2 (X, 17.52%), and **B** with dimension PC1 (Y) and PC3 (X, 11.81%), explaining 65% of the variance in total.



3.3.4 Discussion

3.3.4.1 Cellular oxidative stress

Marine turtles must deal with a significant amount of ROS generated as a consequence of the ischemia–reperfusion associated with diving, like another hypoxia-tolerant marine species (Valdivia et al., 2007; Vazquez-Medina et al., 2006; Zenteno-Savín, 2002). Consequently, previous works have reported high O_2^- (the principal ROS) production in some species of sea turtles and other marine animals (Valdivia et al., 2007; Weydert and Cullen, 2010). Additionally, ROS production in marine ectotherms can be elevated during physiological stress (Abele and Puntarulo, 2004) what could cause oxidation of proteins and lipids, alterations in gene expression and changes in cell redox status (Sevcikova et al., 2011).

3.3.4.1.1 Superoxide dismutase

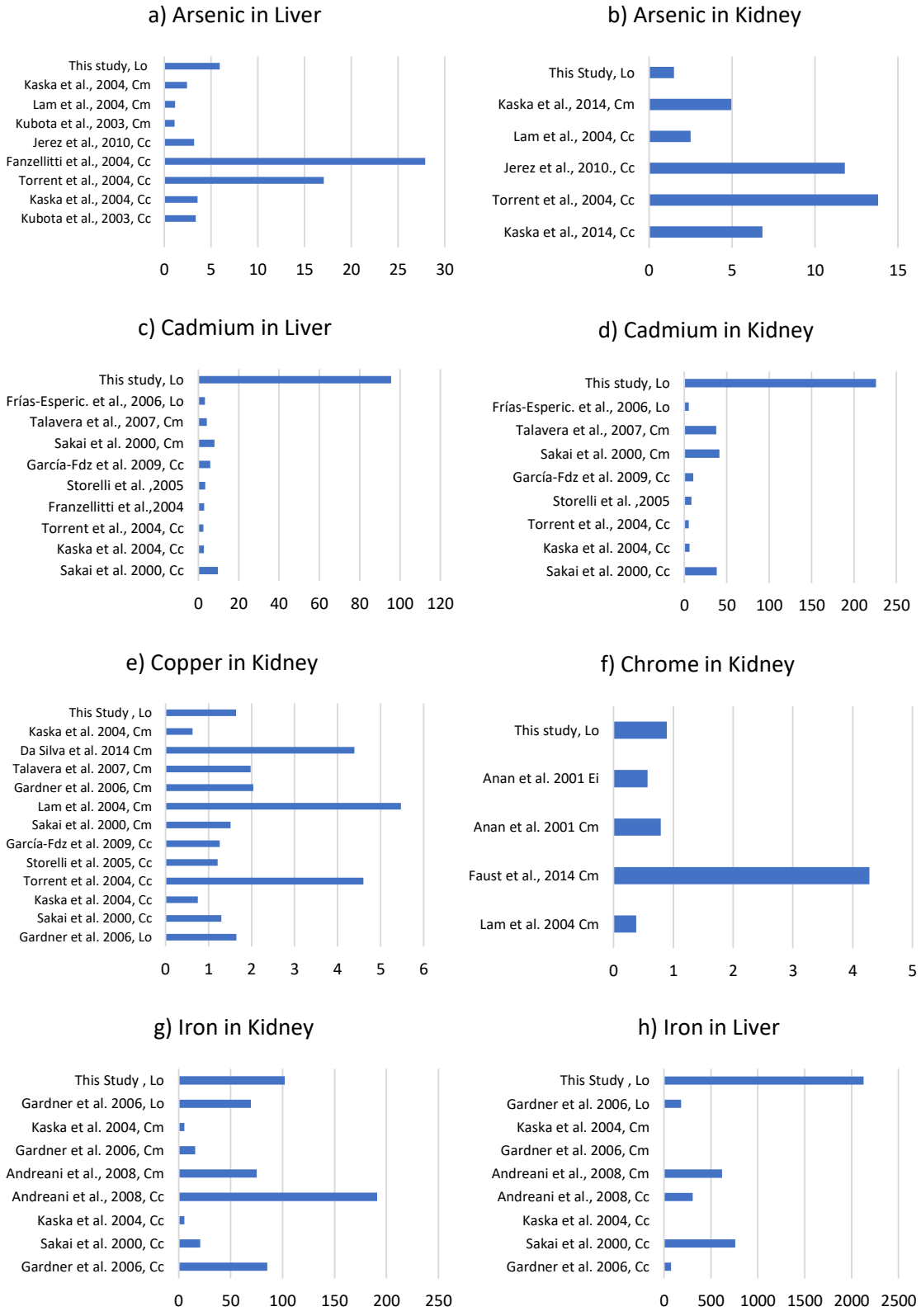
Gene expression of *sod* had positive relationships with As, Cd, Cr, Fe, Ni, Pb and Zn levels in liver; with Cr in kidney whilst failed to show any significant relationship in blood. Our data are consistent with *in vitro* and *in vivo* experiments in marine fish species and fish cell lines exposed to metals (Morcillo et al., 2015; Morcillo et al., 2016). Such studies showed that Cd, Pb and As exposure increased *sod* gene transcription in a dose dependent manner. On the other hand, Zn is closely related to SOD*,* as a part of the Cu/Zn SOD complex, the principal cytosolic isoform of SOD in eukaryotes (Lesser, 2006), and this could explain, at least in a part, this positive relationship. To our knowledge in sea turtles, there is any work about oxidative stress gene expression.

Conversely, SOD activity had only negative significant relationships in liver with Ti, Cd, Cr, Fe, Pb and Tl; also with Cd in kidney and positive with Pb in blood. Such apparently contradictory results have been previously observed in marine organisms, in the so called bell-shaped or biphasic response (Regoli and Giuliani, 2014). This response is produced when a limited (if any) responsiveness of enzymatic antioxidant activities might be observed after a medium/long term exposure (and the corresponding increase of oxidative pressure). After the initial increase response, enzyme activity is followed by a progressive decrease,

even in the presence of enhanced gene transcription (Matovic et al., 2015; Regoli and Giuliani, 2014). Other authors have also suggested that this contrary effect might result from a time delay between gene expression and enzyme production, suggesting that SOD synthesis is regulated at the transcriptional as well as at the translational levels (Lucia et al., 2012; Regoli and Giuliani, 2014). Complementary, some studies had shown that in a prolonged Pb and Cd exposure the enzyme activity (including SOD) decrease in liver; Cd also showed the same tendency in kidney (Jurczuk et al., 2004; Matovic et al., 2015). In addition, Cd concentration in liver and kidney of these turtles is considerably higher than any other population or species ever reported worldwide (Figure 3). In liver, this decrease of SOD activity has been related to Pb and Cd, since these metals bind to functional -SH groups rendering them nonfunctional and depressing their activities (Ahamed et al., 2005; Gurer-Orhan et al., 2004; Matovic et al., 2015). On the contrary, Pb in the hematological system is an important oxidative stress inductor, as a result of erythrocyte hemolysis induced by Pb exposure (Patra et al., 2011; Quintanar-Escorza et al., 2010), thus could explain our results.

Regarding the other significant relationships, Fe is a redox–active metal interconnected with SOD due to Fe ability catalyzing ROS formation (specially $O_2^{\cdot -}$) via the Fenton reaction (Emerit et al., 2001; Sevcikova et al., 2011). Chrome is another redox-active metal and Ni has also been connected with oxidative stress (Sevcikova et al., 2011). Iron dose-depended inhibition of hepatic SOD activity has been observed in Medaka Fish in the initial phase of Fe exposure (but increased with the time) (Sevcikova et al., 2011). Our data showed that turtles sampled in 2014 had significant higher Fe levels in the kidney (Figure 1b). Moreover, compared to other populations/species, Fe concentration in liver of this population is still very high. Regarding Cr, we did not find any specific work about its levels in turtle liver. However, concentrations of this element in liver, kidney and blood are still low/medium compared with other species. Finally, there is not bibliography about Ti and Tl in vertebrates, but they seem to be important since they increase in concentration in kidney from the first to the second year and show negative relationship with SOD activity in the liver.

Figure 3. Metals with significant differences found in this work compared to other works in different marine turtle species worldwide. Lo= *L. olivacea*; Cm= *C. mydas*; Cc= *C. caretta*. All concentrations are expressed in µg g^{-1}.



About enzyme activity, we found 3 works in different marine turtle species: one relating SOD and CAT activity to some metals in different tissues of liver, kidney, heart, lung and muscle (Valdivia et al., 2007); other in *Eretmochelys imbricata* relating blood SOD, CAT and GR to organochlorines (Tremblay et al., 2016); and the last in *Lepidochelys kempii* relating SOD activity to organochlorines in blood. Compared to those works, Valdivia (2005) and (Tremblay et al., 2016) reported higher levels of SOD activity in liver than in kidney, while we have the inverse results.

3.3.4.1.2 Catalase

In a broad sense, the overall reaction catalyzed by CAT is the degradation of hydrogen peroxide into water and oxygen (Chelikani et al., 2004; Kirkman and Gaetani, 2007). The highest *cat* expression was found in blood, followed by kidney and liver from Olive Ridley turtles (Table 3). Negative *cat* gene expression relationships were found in liver with Cr, Fe and Tl and in kidney with Cd, while in kidney we found only positive relationships with Ni and Cr and in blood with Cd. We had a negative relationship between *cat* transcription and Fe in liver but the opposite occurred with *sod* in the same organ. These significant relationships with Fe should be due to the important role that Fe plays during the oxidative stress cycle in general (Regoli and Giuliani, 2014). Although the mechanisms are still mostly unknown in aquatic species, experiments in other species are consistent, showing early induction and later depletion of *cat* in marine organisms exposed to different prooxidants (Regoli et al., 2011a; Regoli and Giuliani, 2014). This may also have some influence in these opposite relationships found with *cat* and *sod* in the different tissues.

Contrarily, we found that CAT activity followed a different importance order: liver>kidney>blood. This is different from the results of the only other paper about oxidative stress in tissues in marine turtles (*C. mydas*), where liver presented higher CAT activity than kidney (Valdivia et al., 2007). Comparatively, CAT enzyme activity was also higher in *C. mydas* than in *L. olivacea* (78 *vs* 7 U mg^{-1} in liver and 63 *vs* 9 U mg^{-1} in kidney) (Valdivia et al. (2007). This could be due to many factors, such as species, age, sex, diet, season or even

the technique used to measure the activity (McGraw et al., 2010; Metcalfe and Alonso-Alvarez, 2010). Additionally, this big inter-species difference has been also observed in some birds (Lucia et al., 2012). This CAT activity had only positive relationships in liver with Al, As, Cd, Se and Zn, in kidney with As, Sb and Cd, and in blood with Cu. The element with the strongest relationship with CAT was Al in liver ($p<0.000$), even though it had a significant decrease from the first to the second year in liver and kidney. Even if enzymatic activity of this study was low compared with Green turtles (Labrada-Martagon et al., 2011a; Valdivia et al., 2007), consistent significant positive relationships were found. We can also see that many of these elements with significant relationships increased their concentrations from the first to the second year. These positive relationships might explain, at least in part, a stimulant factor for CAT enzyme activity production, working as a protection mechanism towards these metal increments.

3.3.4.1.3 Glutathione

This was the only antioxidant with a significant relationship (negative) between gene expression and enzyme activity in kidney ($p<0.01$). This discrepancy has been previously observed in different glutathione molecules, and it has been attributed, at least in part, to the different methodological approaches used: molecular analysis measures some specific isoforms, while enzymatic analysis usually measure the total activity of several isoforms (Giuliani et al., 2013; Regoli and Giuliani, 2014; Regoli et al., 2011b). This antioxidant also was the only one with just positive relationships in both, gene expression and enzyme activity. We did not find any other work on this antioxidant in turtles to compare with. Interestingly, all the metals with negative relationships with *cat* gene expression (Cr, Fe, Tl and Cd) were positive in *gr*. This contrast, may be part of a physiological known compensatory adjustment for depletion of *cat*: the induction of another antioxidant defense. Then, there is a transcriptional and catalytic enhancement of glutathione: thus, when *cat* is inhibited, tissues become highly dependent on glutathione for removal H_2O_2 (Regoli et al., 2011a; Regoli and Giuliani, 2014; Regoli et al., 2011b).

Arsenic was another element with positive relationship with GR in both, gene expression and enzyme activity. This element has been studied towards its influence in oxidative stress in *in vitro* and *in vivo* works, including aquatic species (Barrera-Garcia et al., 2013; Morcillo et al., 2015; Morcillo et al., 2016; Sevcikova et al., 2011). Glutathione plays a key role in the cell redox status induced by As, since glutathione is an electron donor in the reduction of arsenate (more active form) to arsenite (Sevcikova et al., 2011). Even though, As was low compared with other studies worldwide in liver and kidney from marine turtles.

3.3.4.2 Cellular protection and stress

Metallothioneins are small cysteine-rich and metal-binding proteins, which participate in an array of protective stress responses; these proteins are among the most important in protecting cells against heavy metal toxicity, such as Cd, Zn, Cu, As and Pb (Klaassen et al., 2009; Ngu and Stillman, 2009; Ruttkay-Nedecky et al., 2013). In the past decades, MT have become very used as a monitoring biomarker, used as a "contaminant-specific" biochemical indicator of metal exposure in some species (Figueira et al., 2012). The study of this protein related to Cd has been an important focus of interest in the last years, due to Cd high toxicity in many species, also because it is the metal with the highest attraction to MT (Bondía et al., 2013; Klaassen et al., 2009; Lucia et al., 2012; Pedrini-Martha et al., 2016; Zorita et al., 2007). There are also *in vitro* and *in vivo* experiments proving the up-regulation of *mt* gene after the exposure of these metals (Morcillo et al., 2015; Morcillo et al., 2016; Zhang et al., 2013).

Metallothionein concentrations have been shown to be positively correlated with Cd in the liver and kidneys of some marine birds (Lucia et al., 2009; Lucia et al., 2012). Despite the very high Cd concentrations found in this population, any significant relationship was found in any tissue with *mt*. To our knowledge, there is only one work about MT enzyme activity in marine turtles in blood of two different species, *C. mydas* and *C. caretta* (Andreani et al., 2008b). The authors found positive significant correlations for MT and Cd and Cu. On the contrary, we had a negative relationship with Cu and another positive relationship with As,

both found in kidney. Finally, in the Table 3 we observed the differenced of Cd, Cu and As concentrations in different species worldwide, being Cd the element with the highest concentrations.

In summary and with all the above exposed, gene expression of *sod, cat* and *gr* was higher in blood than liver or kidney. However, most of the significant relationships were found in liver, not only for gene expression but also for enzyme activities. Thus, they must be related to the role of liver has as the first filter organ, then all metals pass through this organ before, some of them, go to their target organs and accumulate. It is also very important also to keep in mind that many complex factors and intricate relationships occurred between transcriptional and catalytic response of antioxidants. Even more, in wildlife animals, organisms are typically exposed to multiple natural and anthropogenic stressors; additionally, to a mixture of xenobiotics. All these factors interact each other and produce multiple biological effects. In this respect, we consider that the analysis of molecular and enzymatic variation must be very carefully taken and that cannot be routinely used as biomarkers of the biological impact of environmental pollutants by themselves. We suggest that more tools assessing stress response must be also used and related to these antioxidants (e.g. ROS measurements, apoptosis, lipid peroxidation, etc.) in order to stablish a clearer effect. We also recommend more laboratory/control experiments before use these antioxidants as biomarkers in wild turtle populations. Finally, even if oxidative damages with potential consequences on organisms and/or population performances is expected, the use of transcriptional or catalytic changes as suitable biomarkers for detecting short or long-term exposures and its consequences, would still not easy to interpret in wild populations exposed to a great number of stressors.

3.3.4 Conclusions

Several positive relationships of *sod, cat* and *gr* gene expression in the different tissues and the very high Cd concentrations were found in this population of Olive Ridley turtles. This could mean that these turtles are adapting to the metals-production of ROS and damage

through a high transcription of these antioxidants. Except for *cat* in kidney, where we had a negative relationship and the highest Cd concentrations. Multiple positive relationships with GR seems to be part of the compensatory effect of GR due to the decreased of SOD and CAT production against the high and chronic exposure to certain xenobiotics, such as Cd.

References

Abele, D., Puntarulo, S., 2004. Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. Comp Biochem Physiol A Mol Integr Physiol 138, 405-415.

Aebi, H., 1984. Catalase in vitro. Methods in enzymology 105, 121-126.

Ahamed, M., Verma, S., Kumar, A., Siddiqui, M., 2005. Environmental exposure to lead and its correlation with biochemical indices in children. Science of the total environment 346, 48-55.

Alava, J.J., Keller, J.M., Kucklick, J.R., Wyneken, J., Crowder, L., Scott, G.I., 2006. Loggerhead sea turtle (*Caretta caretta*) egg yolk concentrations of persistent organic pollutants and lipid increase during the last stage of embryonic development. Science of the Total Environment 367, 170-181.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman., D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403-410.

Andreani, G., Santoro, M., Cottignoli, S., Fabbri, M., Carpene, E., Isani, G., 2008. Metal distribution and metallothionein in Loggerhead (*Caretta caretta*) and Green (*Chelonia mydas)* sea turtles. Science of the Total Environment 390, 287-294.

Barrera-Garcia, A., O'Hara, T., Galvan-Magana, F., Mendez-Rodriguez, L.C., Castellini, J.M., Zenteno-Savin, T., 2013. Trace elements and oxidative stress indicators in the liver and kidney of the blue shark (*Prionace glauca*). Comp Biochem Physiol A Mol Integr Physiol 165, 483-490.

Bondía, S., Ribas, B., De la Torre, A., Ruiz, A.S., 2013. Zinc and cadmium metalloprotein induced by cadmium administration, Nutrition, Digestion, Metabolism: Proceedings of the 28th International Congress of Physiological Sciences, Budapest, 1980. Elsevier, p. 71.

Buitrago, J.n., 2009. El Rol de las Tortugas Marinas en los Ecosistemas. Fundacion La Salle de Ciencias Naturales, Venezuela.

Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathion reductase from rat liver. Journal of Biological Chemistry 250, 5475-5480.

Chavez, B., Ramos, L., Merchant-Larios, H., Vilchis, F., 2009. Cloning and expression of the estrogen receptor-alpha (Esr1) from the Harderian gland of the sea turtle (*Lepidochelys olivacea*). Gen Comp Endocrinol 162, 203-209.

Chelikani, P., Fita, I., Loewen, P.C., 2004. Diversity of structures and properties among catalases. Cell Mol Life Sci 61, 192-208.

CONANP, 2011. Ficha de la tortuga golfina. Dirección para especies prioritarias para la conservación, in: Marinas, P.N.p.l.C.d.l.T. (Ed.). Comisión Nacional Areas Naturales Protegidas, Mexico.
Cortés-Gómez, A.A., Fuentes-Mascorro, G., Romero, D., 2014. Metals and metalloids in whole blood and tissues of Olive Ridley turtles (L*epidochelys olivacea*) from La Escobilla Beach (Oaxaca, Mexico). Marine Pollution Bulletin 89, 367-375.
Cortés-Gómez, A.A., Romero, D., Girondot, M., 2017. The current situation of inorganic elements in marine turtles: A general review and meta-analysis. Environmental Pollution 229, 567-585.
Emerit, J., Beaumont, C., Trivin, F., 2001. Iron metabolism, free radicals, and oxidative injury. Biomed Pharmacother 55, 333-339.
Figueira, E., Branco, D., Antunes, S.C., Goncalves, F., Freitas, R., 2012. Are metallothioneins equally good biomarkers of metal and oxidative stress? Ecotoxicol Environ Saf 84, 185-190.
Giuliani, M.E., Benedetti, M., Arukwe, A., Regoli, F., 2013. Transcriptional and catalytic responses of antioxidant and biotransformation pathways in mussels, *Mytilus galloprovincialis*, exposed to chemical mixtures. Aquat Toxicol 134-135, 120-127.
Godley, B.J., Broderick, A.C., Moraghan, S., 1999. Short-term effectiveness of passive integrated transponder (PIT) tags used in the study of mediterranean marine turtles. Chelon Conserv Biol 3, 477-479.
Guirlet, E., Das, K., Girondot, M., 2008. Maternal transfer of trace elements in leatherback turtles (*Dermochelys coriacea)* of French Guiana. Aquatic Toxicology 88, 267-276.
Gurer-Orhan, H., Sabır, H.U., Özgüneş, H., 2004. Correlation between clinical indicators of lead poisoning and oxidative stress parameters in controls and lead-exposed workers. Toxicology 195, 147-154.
IUCN, 2012. Red List of Threatened Species, Version 2012.1. International Union for Conservation of Nature, http://www.iucnredlist.org/.
Jurczuk, M., Brzoska, M.M., Moniuszko-Jakoniuk, J., Galazyn-Sidorczuk, M., Kulikowska-Karpinska, E., 2004. Antioxidant enzymes activity and lipid peroxidation in liver and kidney of rats exposed to cadmium and ethanol. Food Chem Toxicol 42, 429-438.
Kirkman, H.N., Gaetani, G.F., 2007. Mammalian catalase: a venerable enzyme with new mysteries. Trends Biochem Sci 32, 44-50.
Klaassen, C.D., Liu, J., Diwan, B.A., 2009. Metallothionein protection of cadmium toxicity. Toxicology and Applied Pharmacology 238, 215-220.
Labrada-Martagon, V., Rodriguez, P.A., Mendez-Rodriguez, L.C., Zenteno-Savin, T., 2011. Oxidative stress indicators and chemical contaminants in East Pacific green turtles (*Chelonia mydas*) inhabiting two foraging coastal lagoons in the Baja California peninsula. Comparative Biochemistry and Physiology Part C 154, 65-75.
Lesser, M.P., 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. Annual Review of Physiology 68, 253-278.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.
Lucia, M., Andre, J.M., Gonzalez, P., Baudrimont, M., Gontier, K., Maury-Brachet, R., Davail, S., 2009. Impact of cadmium on aquatic bird Cairina moschata. Biometals 22, 843-853.

Lucia, M., Bocher, P., Cosson, R.P., Churlaud, C., Robin, F., Bustamante, P., 2012. Insight on trace element detoxification in the Black-tailed Godwit (*Limosa limosa*) through genetic, enzymatic and metallothionein analyses. Sci Total Environ 423, 73-83.
Matovic, V., Buha, A., Ethukic-Cosic, D., Bulat, Z., 2015. Insight into the oxidative stress induced by lead and/or cadmium in blood, liver and kidneys. Food Chem Toxicol 78, 130-140.
McCord, J.M., Fridovich, I., 1969. Superoxide dismutase an enzymic function for erythrocuprein (hemocuprein). Journal of Biological chemistry 244, 6049-6055.
McGraw, K.J., Cohen, A.A., Costantini, D., Hõrak, P., 2010. The ecological significance of antioxidants and oxidative stress: a marriage between mechanistic and functional perspectives. Functional Ecology 24, 947-949.
Metcalfe, N.B., Alonso-Alvarez, C., 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Functional Ecology 24, 984-996.
Misra, S., Niyogi, S., 2009. Selenite causes cytotoxicity in rainbow trout (*Oncorhynchus mykiss*) hepatocytes by inducing oxidative stress. Toxicology in Vitro 23, 1249-1258.
Morcillo, P., Cordero, H., Meseguer, J., Esteban, M.A., Cuesta, A., 2015. Toxicological in vitro effects of heavy metals on gilthead seabream (*Sparus aurata L*.) head-kidney leucocytes. Toxicol In Vitro 30, 412-420.
Morcillo, P., Esteban, M.A., Cuesta, A., 2016. Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. Chemosphere 144, 225-233.
Ngu, T.T., Stillman, M.J., 2009. Metal-binding mechanisms in metallothioneins. Dalton Trans, 5425-5433.
Patra, R.C., Rautray, A.K., Swarup, D., 2011. Oxidative stress in lead and cadmium toxicity and its amelioration. Vet Med Int 2011, 457327.
Pedrini-Martha, V., Niederwanger, M., Kopp, R., Schnegg, R., Dallinger, R., 2016. Physiological, Diurnal and Stress-Related Variability of Cadmium-Metallothionein Gene Expression in Land Snails. PloS one 11, e0150442.
Perrault, J.R., Schmid, J.R., Walsh, C.J., Yordy, J.E., Tucker, A.D., 2014. Brevetoxin exposure, superoxide dismutase activity and plasma protein electrophoretic profiles in wild-caught Kemp's ridley sea turtles (*Lepidochelys kempii*) in southwest Florida. Harmful Algae 37, 194-202.
Quintanar-Escorza, M.A., Gonzalez-Martinez, M.T., del Pilar, I.O., Calderon-Salinas, J.V., 2010. Oxidative damage increases intracellular free calcium [Ca2+]i concentration in human erythrocytes incubated with lead. Toxicol In Vitro 24, 1338-1346.
R Core Team, 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
Regoli, F., Benedetti, M., Giuliani, M.E., 2011a. Antioxidante defenses and acquisition of tolerance to chemical stress, in: Claude Amiard-Triquet , P.S.R.a.M.R. (Ed.), Tolerance to Environmental Contaminants. CRC Press, Boca Raton, Florida, pp. 153-173.
Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar Environ Res 93, 106-117.
Regoli, F., Giuliani, M.E., Benedetti, M., Arukwe, A., 2011b. Molecular and biochemical biomarkers in environmental monitoring: a comparison of biotransformation and antioxidant defense systems in multiple tissues. Aquat Toxicol 105, 56-66.

Ruttkay-Nedecky, B., Nejdl, L., Gumulec, J., Zitka, O., Masarik, M., Eckschlager, T., Stiborova, M., Adam, V., Kizek, R., 2013. The role of metallothionein in oxidative stress. Int J Mol Sci 14, 6044-6066.
Sakai, H., Saeki, K., Ichihashi, H., Suganuma, H., Tanabe, S., Tatsukawa, R., 2000. Species-specific distribution of heavy metals in tissues and organs of Loggerhead turtle (*Caretta caretta*) and Green turtle (*Chelonia mydas*) from Japanese Coastal Waters. Marine Pollution Bulletin 40, 701-709.
Sevcikova, M., Modra, H., Slaninova, A., Svobodova, Z., 2011. Metalas as cause of oxidative stress in fish: a review. Veterinarni Medicina 56, 537-546.
Silman, R., Vargas, I., Troëng, S., 2002. Tortugas Marinas. Guía Educativa, in: Corporation, C.C. (Ed.), 2a ed.
Tremblay, N., Ortiz Arana, A., Gonzalez Jauregui, M., Rendon-von Osten, J., 2016. Relationship between organochlorine pesticides and stress indicators in hawksbill sea turtle (*Eretmochelys imbricata*) nesting at Punta Xen (Campeche), Southern Gulf of Mexico. Ecotoxicology.
Valdivia, M., 2005. Intoxicación por Plomo, Rev. Soc. Per. Med. Inter. , Lima, Perú, pp. 22-27.
Valdivia, P.A., Zenteno-Savin, T., Gardner, S.C., Aguirre, A.A., 2007. Basic oxidative stress metabolites in eastern Pacific green turtles (*Chelonia mydas agassizii*). Comparative Biochemistry and Physiology, Part C 146, 111-117.
Vazquez-Medina, J.P., Zenteno-Savin, T., Elsner, R., 2006. Antioxidant enzymes in ringed seal tissues: potential protection against dive-associated ischemia/reperfusion. Comp Biochem Physiol C Toxicol Pharmacol 142, 198-204.
Venancio, L.P.R., Silva, M.I.A., da Silva, T.L., Moschetta, V.A.G., de Campos Zuccari, D.A.P., Almeida, E.A., Bonini-Domingos, C.R., 2013. Pollution-induced metabolic responses in hypoxia-tolerant freshwater turtles. Ecotoxicology and Environmental Safety 97, 1-9.
Wallace, B.P., DiMatteo, A.D., Bolten, A.B., Chaloupka, M.Y., Hutchinson, B.J., Abreu-Grobois, F.A., Mortimer, J.A., Seminoff, J.A., Amorocho, D., Bjorndal, K.A., Bourjea, J., Bowen, B.W., Dueñas, R.B., Casale, P., Choudhury, B.C., Costa, A., Dutton, P.H., Fallabrino, A., Finkbeiner, E.M., Girard, A., Girondot, M., Hamann, M., Hurley, B.J., Lopez-Mendilaharsu, M., Marcovaldi, M.A., Musick, J.A., Nel, R., Pilcher, N.J., Troëng, S., Witherington, B., Mast, R.B., 2011. Global conservation priorities for marine turtles. PLoS One 6, e24510.
Weydert, C.J., Cullen, J.J., 2010. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nat Protoc 5, 51-66.
Zenteno-Savín, T., 2002. Oxidative stress in marine organisms: a review. Oxidative Stress at Molecular, Cellular and Organ Levels. Research Signpost, Kerala, India, 68-76.
Zhang, J., Wang, J., Xiang, J., 2013. A cadmium metallothionein gene of ridgetail white prawn Exopalaemon carinicauda (Holthuis, 1950) and its expression. Chinese Journal of Oceanology and Limnology 31, 1204-1209.
Zorita, I., Bilbao, E., Schad, A., Cancio, I., Soto, M., Cajaraville, M.P., 2007. Tissue- and cell-specific expression of metallothionein genes in cadmium- and copper-exposed mussels analyzed by in situ hybridization and RT-PCR. Toxicol Appl Pharmacol 220, 186-196.

3.4 Biochemistry

3.4.1 CHAPTER V. P-nitrophenyl acetate esterase activity and cortisol as biomarkers of metal pollution in blood of Olive Ridley turtles (*Lepidochelys olivacea*)

3.4.1.1 Introduction

The Olive Ridley turtle (*Lepidochelys olivacea*) is a pantropical species and its largest populations are found in the Pacific and Indian Oceans (Marquez et al., 1996; Zug et al., 2006). In the State of Oaxaca (Mexico), "La Escobilla" is a nature sanctuary and one of the most important nesting beaches in the world for this species, with more than one million turtles arriving every year to nest (CONANP, 2011b). Like all marine turtles, the Ridley turtle is considered an endangered species (IUCN, 2012), and is listed in Appendix I of the Convention of International Trade in Endangered Species (CITES). In Mexico, this species is included within the regulation norm NOM-059-SEMARNAT-2010, and has been made a priority for conservation (CONANP, 2011b). The main threats that have brought turtles to this precarious situation are, among others, their exploitation for meat, eggs and shells in certain regions, by-catch, global warming, loss of nesting sites, and environmental pollution (Aridjis, 1990; Camacho et al., 2014a; Casale and Margaritoulis, 2010; MTSG, 2007; Wallace and Saba, 2009). According to several authors, anthropogenic-induced stressors such as coastal pollution can exacerbate other adverse impacts and seriously compromise the ability of sea turtles to adapt to climate change (Poloczanska et al., 2009). Furthermore, different turtle diseases such as fibropapillomatosis have been associated with coastal pollution (Aguirre and Lutz, 2004; Barile, 2004). In this context, it is necessary to study biological markers for the evaluation of pollution effects in sea turtles.

Heavy metals are contaminants of important environmental relevance due to their high risk of toxicity and persistence (Bjerregaard and Anders, 2007). Lead (Pb) and cadmium (Cd) are among the elements most studied in aquatic environments and both have been associated with different pathologies in many species, such as bone necrosis, endocrinological alterations, immune and reproductive problems among others (Boughammoura et al., 2013; Nunes et al., 2014; Rana, 2014; Sonne et al., 2009). High Cd concentrations in liver and kidney have been previously reported in nesting turtles from "La Escobilla" beach (Cortés-Gómez et al., 2014). On the other hand, Al, As, Se, and Zn play essential roles in

tissue metabolism and growth, but in most species it has been demonstrated that a concentration of these elements higher than their physiological threshold can cause diverse toxic effects, such as oxidative stress, endocrine disruption and neurological damage (ATSDR, 2003, 2007a, 2008). Recently, there has been growing concern regarding other elements, such as titanium (Ti) and strontium (Sr), that have been shown to cause biological alterations (e.g. oxidative stress, tumors or cancer) in some species after long-term exposure (Mei et al., 2006; Shi et al., 2013). Both elements are considered to be emerging chemicals from nanotechnology, and the adverse effects of nanomaterials in marine life should be analysed (Blaise et al., 2008; Kahru and Dubourguier, 2010; Wise et al., 2011).

In marine organisms, reactive oxygen species (ROS) production has been described as one of the main components of organic response against environmental stressors, such as pollutants, including heavy metals and metalloids (Bears et al., 2006; Labrada-Martagon et al., 2011a; Lesser, 2006; Morcillo et al., 2016; Valdivia et al., 2007). It has been demonstrated, both in *in vivo* and *in vitro* studies, that ROS have the potential to alter proteins, lipids and nucleic acids, resulting in tissue damage, metabolic dysfunction, and apoptosis (Labrada-Martagon et al., 2011a; Morcillo et al., 2016). In marine turtles, few reports on oxidative stress exist (Gregory and Schmid, 2001; Labrada-Martagon et al., 2011a; Perrault et al., 2014; Richardson et al., 2009b; Valdivia et al., 2007). These evaluated oxidative stress biomarkers such as superoxide dismutase (SOD), catalase (CAT), and glutathione. However, to the best of our knowledge there is only one study evaluating the relationship between oxidative stress markers (SOD, CAT, GST, TBARS) and chemical element concentrations in green turtles (*Chelonia mydas*) (Labrada-Martagon et al., 2011a).

Esterase activity (EA) has been widely studied due to its multifunctional roles in diverse biochemical pathways, such as protection against oxidative damage, lipid peroxidation, contribution to innate immunity, detoxification of reactive molecules, modulation of endoplasmic reticulum stress and regulation of cell proliferation/apoptosis, metabolization and detoxification of organophosphorus compounds, *inter alia* (Ceron et al., 2014; Franco

et al., 2015; Laird et al., 2014; Li et al., 2006). In humans, it has been reported that some metals could modulate activity of this enzyme (Hernandez et al., 2009; Laird et al., 2014; Li et al., 2006), and thus we hypothesized that EA could be altered in serum of the turtles exposed to heavy metal pollution.

Finally, hypothalamic–pituitary–adrenal (HPA) axis was shown to be sensitive to stress in reptiles (Gregory and Schmid, 2001). Elevation of plasma corticosteroids in reptiles as a result of stress exposure has been demonstrated in many species including marine turtles (Aguirre et al., 1995; Gregory et al., 1996; Gregory and Schmid, 2001). Toxicants, such as metals and metalloids, can be perceived as stressors by the HPA and affect the stress response, inducing changes in cortisol levels (Brodeur et al., 1997). However, to the authors' knowledge, no studies have been undertaken to evaluate the possible relationship between pollution and cortisol levels in turtles.

The aim of this work was to determine the concentrations of EA and cortisol in serum samples of Olive Ridley turtles (*Lepidochelys olivacea*) from a Mexican Pacific population, and to evaluate the possible relation of toxic metals (Pb and Cd), essential but potentially toxic elements (Al, As, Se, and Zn) and emerging chemicals (Ti and Sr) with these biomarkers.

3.4.1.2 Material and methods

3.4.1.2.1 Sample collection

Samples were collected at Escobilla Beach, in the State of Oaxaca (Southeast Mexico, Eastern Pacific, 96°44'W and 15°47'N). Beach monitoring was performed in August 2013 (nesting season, third arribada event). Since no method for age determination in marine turtles has yet been established, biometrics such as curve carapace length (CCL), curve carapace width (CCW), straight carapace length (SCL) and body mass were taken and used to determine size as a relative indicator of age as previously described (Frazier, 1983). Permission for taking samples was SGPA/DGVS/00905/13 and for exporting samples were CITES MX 67725 (07/10/2013) and PFPA/471/00061/2013.

Forty-four apparently healthy nesting female turtles were randomly selected. Blood samples were carefully taken from the dorsal cervical sinus, using a 5 mL syringe with 21" needles. The turtles showed no alteration in their behaviour after sampling. Before blood extraction, the neck region was carefully cleaned using ethanol. For EA and cortisol analysis, blood samples were immediately transferred into empty tubes and centrifuged at 2000 rpm for 10 min at room temperature to obtain serum, which was transferred into *Eppendorf*® microtubes and stored at -20°C. For metal analysis, the complete blood samples were stored into 1.5 *Eppendorf*® microtubes at -20°C until analyses.

3.4.1.2.2 Chemical element analysis

Blood samples were pre-treated as previously described (Cortés-Gómez et al., 2014). In brief, 0.5 g of sample was submitted to acid digestion using 4 mL of trace mineral grade HNO3 (69%) and 1 mL of H2O2 (33%) in a microwave digestion system (Ultra Clave-Microwave Milestone) at 220 °C for 20 min. Finally, samples were diluted with 25 mL of double deionized water (Milli-Q). Metal and metalloid concentrations were determined using an Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES, ICAP 6500 Duo Thermo Scientific, with One Fast System). All concentrations are expressed in microgram per gram in wet weight. Detection limits (DL) for all elements were 0.01 $\mu g\ g^{-1}$.

3.4.1.2.3 EA and cortisol analysis

EA was analysed in an automated clinical chemistry analyser (Olympus AU 2700; Olympus Diagnostica GmbH, Hamburg, Germany) by measuring the hydrolysis of *p-nitrofenolacetate* to *p-nitrophenol* as described by Haagen and Brock (1992), with some modifications (Tvarijonaviciute et al., 2012). Intra and inter coefficient of variation (CV) were below 2% and 9%, respectively. Recovery ranged between 95% and 99%. The DL was 0.1 IU mL^{-1}.

Cortisol was analysed in an automated chemiluminescent immunoassay (Immulite System; Siemens Health Diagnostics, Deerfield, Illinois, USA) as per the manufacturer's instructions.

Intra and inter CV were below 8% and 10%, respectively. Recovery ranged between 97% and 112%. The DL was 0.05 µg dL^{-1}.

3.4.1.2.4. Data analysis

Statistical analyses were performed with GRAPHPAD PRISM, GraphPad Software, San Diego, CA, USA. Results of biochemical parameters and chemical elements were reported as arithmetic median, minimum, maximum, mean and standard deviation, in micrograms per gram on a wet weight (ww) basis (metalloid and metals), IU mL^{-1} (EA) and µg dL^{-1} (cortisol). Biometric data were reported as mean and standard deviation (SD). The Shapiro-Wilk test was used to check the distribution of our data, giving us a non-parametric distribution. Then Spearman's rank correlation coefficient test for non-parametric variables was applied to establish correlation coefficients between EA, cortisol, metal and metalloid concentrations and turtle biometrics as a relative indicator of age. In all cases, *p* values of less than 0.05 were considered statistically significant.

3.4.1.3 Results

Descriptive data of biometric measures are given in Table 1. The detected concentrations of EA, cortisol and chemical elements in blood are given in Table 2. All of the samples analysed exhibited values above the DL for all elements, except cortisol that was bellow DL in three cases, and Cd, Ti and As in one case each. Zn was the metal with highest concentrations in blood, while Pb was the element with lowest concentration.

Table 1. Biometric parameters of Olive Ridley turtles (*Lepidochelys olivacea*) from "La Escobilla" beach (Mexican Pacific). Mean, standard deviation, minimum and maximum of curve carapace length (CCL), curve carapace width (CCW), straight carapace length (SCL) and body mass.

CCL (cm)	CCW (cm)	SCL (cm)	Mass (Kg)
65.89±3.06	60.68±2.96	60.70±2.99	33.69±3.30
(58.00-72.00)	(54.00-71.40)	(54.00-71.40)	(25.00-42)

Descriptive data of biometric measures are given in Table 1. The detected concentrations of EA, cortisol and chemical elements in blood are given in Table 2. All of the samples analysed exhibited values above the DL (Table 2) for all elements, except cortisol, which was below DL in three cases, and Cd, Ti and As in one case each. Zn was the metal with highest concentrations in blood, while Pb was the element with lowest concentrations.

Table 2. Esterase Activity (EA, IU mL^{-1}), cortisol (µL dL^{-1}) and chemical elements (µg g^{-1} wet weight) in blood of 44 Olive Ridley turtles (*Lepidochelys olivacea*) from "La Escobilla" beach (Mexican Pacific). BDL= below the detection limit. DL= detection limit

	EA	Cortisol	Cd	Pb	Ti	Sr	Se	Al	As	Zn
Median	0.87	0.24	0.15	0.06	0.17	0.99	6.17	1.32	0.94	10.70
Min	0.36	ND	ND	0.01	ND	0.56	1.58	0.01	ND	3.93
Max	3.95	2.54	0.32	0.24	7.47	1.80	22.23	35.05	2.82	19.52
BDL	0	3	1	0	1	0	0	0	1	0
DL	0.001	0.05	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

The percentage of blood samples with high concentrations (outliers) were 6.82 for EA, (three samples with more than 2.57 IU ml-1), 4.55 for cortisol (two samples with more than 2.53 µg dL-1), 2.27 for Pb (one sample with 0.24 µg g-1), 13.64 for Ti (six samples with more than 2.78 µg g-1), 2.27 for Se (one sample with 22.23 µg g-1) and 2.27 for Al (one sample with 35.06 µg g-1). Outliers were not found in the case of Cd, Sr, As and Zn. We did not find outliers in blood samples with low element concentrations.

3.4.1.4 Discussion

The population of Olive Ridley turtles is of particular concern with regards to heavy metal pollution (Cortés-Gómez et al., 2014). In this work, the EA and cortisol, metalloid and metal concentrations were evaluated in these turtles, and the possible relation with metal pollution with these biomarkers was studied.

3.4.1.4.1 Chemical element concentrations

Concentrations of chemical elements have been reported in sea turtles by several authors (Table 3). Nevertheless, there are few works published on the subject of heavy metals and

metalloids in *L. olivacea* turtles, and fewer still from the Mexican Pacific, two of which (Cortés-Gómez et al., 2014; Páez-Osúna et al., 2010b) are from the same turtle population from **"**La Escobilla" Beach. Pb concentration in blood was higher than the preceding year on the same beach (mean 0.07 vs 0.02 μg g^{-1} ww) (Cortés-Gómez et al., 2014), indicating an increase in this element in the environment from 2012 to 2013. Since Pb is a component of petroleum (Ancheta and Speight, 2007), this increase could be attributed to several oil spills in the nesting and feeding areas (Mexico, Central America and part of South America). In 2012, for instance, one of the most important oil refineries in Salina Cruz, Oaxaca (located about 200 km from "La Escobilla"), had an significant spill in the area, which had a direct impact on Ridleys from Oaxaca (Moreno, 2012).

In contrast, Cd concentration in blood did not significantly vary over the two years of this biomonitoring program (mean 0.17 and 0.15 μg g^{-1} ww in samples from 2012 and 2013 respectively). However, our concentrations were 9 times lower than those found in Ridleys from the north of Mexico in a study by Zavala-Norzagaray et al. (2014), which reported the highest concentrations of this metal in blood with 1.33±0.20 μg g^{-1}.

Concentrations of Se, Zn, and As detected in blood of turtles included in the present study were intermediate when compared with data reported previously, while Al mean concentration was the highest (Table 3). To the authors' knowledge, no works have been published regarding Ti, Sr or Al in blood of *L. olivacea*. In captive hawksbill (*Eretmochelys imbricate*) and wild green sea turtles (*Chelonia mydas*), Suzuki et al. (2012)a, b) reported 0.28 μg g^{-1} and 0.44 μg mL^{-1} of Ti, higher concentrations than the median found in our study (0.17 μg g^{-1}), although in those studies the samples were plasma, not whole blood. We also found an important percentage of samples (13.64) with high Ti concentration (2.78 to 7.47 μg g^{-1}). With regards to this metal, it is important to highlight that Ti is one of the most used inorganic nanomaterials in industry (Aitken et al., 2006; Trouiller et al., 2009). Additionally, marine microplastics, a global problem that affects vertebrate groups such as fish, birds and sea turtles (Costa and Barletta, 2015), may act as a TiO_2 source (Fries et al., 2013). According

to different authors (Guebert-Bartholo et al., 2011; Tourinho et al., 2010), microplastics have been found in the stomach contents of sea turtles, so it is possible that Ti concentrations are increasing in this species, although this situation has not yet been fully considered in the scientific literature. Currently, the possible accumulation of Ti in the marine environment is indicated by (Clemente et al., 2011). Furthermore, Ti is considered class 2B by the International Agency of Research on Cancer (possibly carcinogenic to humans), with a genotoxic effect by generation of oxidative stress (Chen et al., 2014). In our study, Sr concentrations were higher than those reported in blood by several authors in *C. mydas* and plasma of *E. imbricata* (Faust et al., 2014a; Suzuki et al., 2012), but similar or lower than those reported in plasma by Suzuki et al (2012b) in *C. Mydas*, *E. Imbricata* and *C. Caretta* (captive and wild sea turtles). Sr is the most abundant trace element in ocean water (Cohen-Solal, 2002), and it has been considered a hazardous metal in aquatic organisms (Mei et al., 2006), and associated with skeletal abnormalities (Moiseenko and Kudryavtseva, 2001). Since Sr is widely used in the nanomaterials industry, further research is necessary to know if it could become a problem for marine turtles. Finally, Al mean concentration was higher than those found in other studies (Table 3). Al is considered a potential threat to aquatic ecosystem health at high concentrations (Golding et al., 2015), but there is little information about Al concentrations in sea turtles and its potential effects on these species (Sparling et al., 2010). Although McFadden et al (2014) do not exclude the possibility that this element may be abnormally high in *C. mydas*, the concentration is lower than those found in our study.

3.4.1.4.2 EA and cortisol

Serum EA and cortisol in turtles of this study were detected in most of the samples analysed, with the exception of 3 samples that showed concentrations below DL. To our knowledge, this is the first report describing *p-nitrophenyl* acetate EA in marine turtles, while some studies about cortisol have been performed in several turtle species by endocrinology and stress studies (Fazio et al., 2014; Ikonomopoulou et al., 2006; Sanford and Stephens, 1988; Zhou et al., 2004; Zhou et al., 2003), but not by environmental pollution studies. Plasma

cortisol detected in *L. olivacea* was partially in agreement with the range reported by Zhou et al. (2003)) between stressed and unstressed soft-shelled turtles (*Pelodiscus sinensis*).

In serum of some species, the main EA *p-nitrophenyl* acetate esterase is paraoxonase 1 (PON1). PON1 has been described to be an antioxidant and anti-inflammatory molecule (Ceron et al., 2014) and furthermore, it has been described to participate in an adrenal regulation with an important role in the production of corticosteroids, such as cortisol (Gamliel-Lazarovicha et al., 2010). Since pollutants can stimulate cortisol production as a stress response of the organism (Cockrem, 2013), it could be expected that, during an acute stress episode, cortisol and EA levels increase, and that both biomarkers would present positive correlation. However, a strong negative correlation between the two markers was observed. Taking in account the previous pollutant information in this turtle population (Cortés-Gómez et al., 2014), it could be assumed that these animals were chronically exposed to the different inorganic elements as heavy metals, suggesting that after a prolonged period of time under a stressful condition generated by pollution, the esterase is highly consumed, resulting in decreased its levels, although further studies are needed to clarify this topic.

3.4.1.4.3 EA correlations

A statistically significant negative correlation between EA and Cd and Pb was found in this study (p>0.05). Different works have demonstrated that Cd inhibits EA *in vivo* and *in vitro* (Costa et al., 2005; Hernandez et al., 2009; Laird et al., 2014; Pollack et al., 2014), and the association between EA and Pb agrees with that previously described in other species by different authors (Costa et al., 2005; Hernandez et al., 2009; Laird et al., 2014; Li et al., 2006). Considering both that the evaluated turtle population presents very high liver and kidney concentrations of Cd (Cortés-Gómez et al., 2014) and the increase of Pb from 2012 to 2013, we might be led to suspect that both heavy metals are responsible for the observed EA decrease in *L. olivacea* from “La Escobilla” beach. However, Ti and Al also showed negative correlation with EA in serum. Recent works have attributed a genotoxicity activity

to Ti, as well as the generation of reactive oxygen species and oxidative damage (Chen et al., 2014; Hao et al., 2009; Reeves et al., 2008; Tay et al., 2014). Al is a trace metal in vertebrates, although elevated concentrations of this element can cause, among other things, neurological, immunological and reproductive disorders, as well as oxidative stress (ATSDR, 2008). Thus, further experimental studies would be required in order to clarify if this effect is due to Cd and Pb or because of co-contamination with other pollutants.

On the other hand, it is understood that most metals and metalloids tend to accumulate in the tissues of living organisms. This means that in older animals, higher concentrations of certain metals (for instance Cd, Pb) could be found in tissues from polluted areas (Camacho et al., 2014a; Cortés-Gómez et al., 2014; Garcia-Besne et al., 2015). As we have indicated, one would expect a decrease of esterases by chronic pollutant exposure, so this could also explain the negative correlation between EA and CCL, SCL, and mass that was observed in the present study.

3.4.1.4.4 Cortisol correlations

Hypothalamus-pituitary-adrenal (HPA) endocrine products, such as cortisol, are commonly used as physiological indicators of stress in different species (Cockrem, 2013; Gregory and Schmid, 2001). It has been reported that exposure to even sub-lethal contaminants can interfere with the endocrine function (Marentette et al., 2013). For this reason, in recent years the evaluation of the effects of pollutants on the endocrine system in wild endangered species has been gaining attention (Cockrem, 2013; Marentette et al., 2013). For instance, it has been described that toxicants such as metals and metalloids can influence the glucocorticoid stress response in two different pathways: (1) they can act as stressors themselves, activating the HPA axis; or (2) they can have a direct impact in the HPA itself (Brodeur et al., 1997). Alterations in the HPA axis, such as elevated cortisol levels, were shown in the short-term to provide protection against toxic effects (Bury et al., 1998; DeBoeck et al., 2003). Nevertheless, a prolonged cortisol elevation can result in immunosuppression, reduced reproductive investment and/or reduced growth, among

other things (Barton et al., 1987; Gregory and Wood, 1999; Marentette et al., 2013). With regards to metals and metalloids in our study, a positive correlation, weak but statistically significant, between cortisol and Sr was observed in blood of Olive Ridley. Although Sr is a trace element in marine waters, it is considered a hazardous contaminant in oceans (Mei et al., 2006). Sr is released into the environment in particular by the nuclear industry as sludge wastes, and more recently in nanomaterials. It is known that this element causes disease at higher concentrations (>300 μg g^{-1}), such as endemic osteoarthritis deformans, because this metal replaces calcium in bones (ATSDR, 2004; Mei et al., 2006). However, information concerning its chronic effects is lacking, and further studies would be needed to clarify the pathogenic mechanisms responsible for the observed relationship between cortisol and Sr.

In contrast, serum cortisol showed weak negative but statistically significant correlations with As and Se, and there was a positive and significant correlation between both elements (data not shown). It is known that As is accumulated at high levels in marine fauna (ATSDR, 2007a; Fujihara et al., 2003; Saeki et al., 2000), and it has been previously reported that marine turtles presented a higher concentration of As in tissues compared with marine mammals (Fujihara et al., 2003; Kubota et al., 2002). The As concentrations found in the present study were intermediate with respect to other populations (Table 3). However, it is also reported that As, even at low concentrations (0.001-0.1 μg g^{-1}), may become an important endocrine disrupter in other marine species (Shaw et al., 2007), and that the As toxicity involved oxidative damage (El-Demerdash et al., 2009; Izquierdo-Vega et al., 2006; Kitchin and Ahmad, 2003). Several authors have reported that Se may protect against the toxic effects of As (Biswas et al., 1999; Xu et al., 2013), as this element has a key role in many enzymatic activities, being essential for humans and animals (Perrault et al., 2013). For instance, Se is a component of glutathione peroxidase, which is an important antioxidant that protects cells against ROS damage (Miller et al., 2009), but in fish it has been demonstrated that Se induces a decrease in cortisol levels due to a disruption on the HPI axis (Miller and Hontela, 2011). This could explain the correlations mentioned above. On the other hand, in most species Se can become toxic in concentrations above those

needed to preserve homeostasis (Dyc et al., 2015; Hopkins et al., 2005a; Miller and Hontela, 2011). Thus, in aquatic species, selenite and selenomethionine are the most important Se toxic forms, acting both as ROS, leading to an increase in oxidative damage (Misra and Niyogi, 2009; Rigby et al., 2010). However, selenite mechanism pathways vary among different species, from disruption of one specific step in steroidogenesis to impairment of cortisol secretion at multiple sites in cells (Miller and Hontela, 2011). Therefore and taken all together, all these elements should be included in future biomonitoring programs, to establish the physiological levels of these elements in order to better understand their effects in turtles.

3.4.1.5 Conclusions

The results of the presented work indicate that some metals and metalloids could have an influence on *p-nitrophenyl* acetate esterase activity and on cortisol in Olive Ridley turtles, and could be considered as useful biomarkers of contamination in this species. The lack of studies regarding concentrations of understudied elements in this species, such as Ti and Sr in marine turtles, and the weak but significant correlations detected between EA and Cd, Pb and Ti, and those between cortisol and Sr, As and Se, highlight the necessity for further research in this field in order to identify the biological responses to each one of these elements.

Table 3. Metal and metalloid concentration (mean ± standard deviation, or median) in blood of sea turtles from different locations. §=µg g^{-1}; #= µg mL^{-1}; ¥=dry weight; (*)=ng g^{-1} or ng mL^{-1}; (**)=The mean range are included if the study gives more than one set of results; *bdl*=below detection limit; *na*=no analysed.

Reference	Species	Location	n	Pb	Cd	Se	Zn	As	Ti	Sr	Al
Wang (2005)§	*L. kempii*	USA and Mexico	18 to 58	0.01 to 0.05(**)	0.6 to 14.9 (*) (**)	*na*	3.90 to 22.70(**)	*na*	*na*	*na*	*na*
Deem et al. (2006)§	*D. coriacea*	Gabon	9	0.09± 0.03	*na*	*na*	*na*	*bdl*	*na*	*na*	*na*
Guirlet et al. (2008)§	*D. coriacea*	French Guiana	78	0.18±0.05	0.08±0.03	9.98±0.05	11.10±0.28	*na*	*na*	*na*	*na*
Deem et al. (2009a) §	*C. caretta*	USA	12	0.05 to 0.1 (*) (**)	*na*	*na*	*na*	1.82 to 7.92 (*) (**)	*na*	*na*	*na*
Innis et al. (2010)#	*D. coriacea*	USA	16	0.11 to 0.14(**)	0.07±0.02	7.55±2.83	*na*	*na*	*na*	*na*	*na*
Jerez et al. (2010a)§	*C. caretta*	Spain	5	0.31±0.31	0.12±0.21	2.8±1.29	7.07±2.85	6.99±9.28	*na*	*na*	*na*
Paez-Osuna et al. (2010a, b; 2011)§	*L. olivacea*	Mexico	25	0.95±0.18¥	*na*	*na*	*na*	*na*	*na*	*na*	*na*
Paez-Osuna et al. (2010a, b; 2011)§	*L. olivacea*	Mexico	25	*na*	0.45±0.20¥	*na*	58.40±4.70¥	*na*	*na*	*na*	*na*
van de Merwe et al. (2010b)#	*C. mydas*	Australia	16	0.02± 0.01	0.04±0.01	2.45±0.63	7.92±0.67	4,3618 ± 1,4149	*na*	*na*	*na*
Ikonomopoulou et al. (2011)#	*N. Depressus*	Australia	20	*bdl*	*bdl*	*na*	151.15±1.45(*)	*na*	*na*	*na*	*na*
Labrada-Martagon et al. (2011b)§	*C. mydas*	Mexico	11 to 42	*na*	0.03 to 0.06(**)	1.59 to 1.81(**)	13.58 to 13.92(**)	*na*	*na*	0.18 to 0.28(**)	*na*
Ley-Quiñónez et al. (2011)§	*C. caretta*	Mexico	22	*na*	1.8±0.63	6.14±3.58	44.81±17.53	4.09±2.56	*na*	*na*	*na*
Komoroske et al. (2011)§	*C. mydas*	USA	19 to 30	1.26±0.22	0.01±0.00	0.78±0.25	*na*	0.16±0.03	*na*	0.73±0.06	0.15±0.03

Camacho et al. (2013)§	*C. caretta*	Cape Verte	201	0.06±0.02	0.29±0.25	2.53±2.21	4.97±2.9	0.58±0.95	*na*	*na*	1.07±1.21
Ley-Quiñónez et al. (2013b)§	*C. mydas*	Mexico	12	*na*	0.99±0.35	7.66±3.19	63.58±17.06	*na*	*na*	*na*	*na*
Camacho et al. (2014)§	*C. mydas* *E. imbricata*	Cape Verte	21 13	0.07±0.02 0.03±0.02	0.30±0.07 0.32±0.06	0.61±0.25 0.59±0.40	1.04±0.45 2.60±2.11	0.44±0.10 0.48±0.23	*na* *na*	*na* *na*	1.95±2.35 0.78±0.29
Cortés-Gómez et al. (2014)§	*L. olivacea*	Mexico	41	0.02±0.01	0.17±0.08	5.75±2.48	10.55±3.68	1.16±0.70	*na*	*na*	*na*
Ehsanpour et al. (2014)§	*E. imbricata*	Iran	12	0.56±0.25¥	0.34±0.08¥	*na*	37.60±3.98¥	*na*	*na*	*na*	*na*
Faust et al. (2014b)§	*C. mydas*	USA	12	0.06±0.01	0.10±0.01	0.73±0.07	11.40±0.60	2.57±0.26	*na*	0.47±0.02	*na*
McFadden et al. (2014b)§	*C. mydas*	USA	79 to 105	0.02±0.01	0.03±0.03	1.58±3.53	*na*	*na*	*na*	*na*	1.06±0.94
Zavala-Norzagaray et al. (2014)§	*L. olivacea*	Mexico	19	*bdl*	1.33±0.20	11.15±0.26	37.12±3.67	2.44±0.45	*na*	*na*	*na*
Villa et al. (2015)#	*C. mydas*	Australia	2	0.04	2.9(*)	0.28	11.00	0.28	*na*	0.71	0.02
da Silva et al. (2016b)§	*C. mydas*	Brazil	13	0.95 to 2.41(**)	0.04 to 0.08(**)	*na*	0.47 to 1.14(**)	*na*	*na*	*na*	*na*
Present Study	*L. olivacea*	Mexico	44	0.07±0.04 (0.45±0.26)¥	0.15±0.06 (0.97±0.39)¥	6.54±3.10 (42.19±20.00)¥	10.88±2.33 (70.19±15.03)¥	1.05±0.53 (6.77±3.42)¥	0.99±1.84 (6.39±11.87)¥	1.03±0.29 (6.65±1.87)¥	3.89±6.23 (25.10±40.19)¥

References

Aguirre, A.A., Balazs, G.H., Spraker, T.R., Gross, T.S., 1995. Adrenal and hematological responses to stress in juvenile green turtles (Chelonia mydas) with and without fibropapillomas. . Physiol. Zool. 68, 831-854.

Aguirre, A.A., Lutz, P.L., 2004. Marine turtles as sentinels of ecosystem health: Is fibropapillomatosis an indicator? Ecohealth%1.702% 1, 275-283.

Aitken, R., Chaudhry, M., Boxall, A., Hull, M., 2006. Manufacture and use of nanomaterials: current status in the UK and global trends. Occupational medicine 56, 300-306.

Ancheta, J., Speight, J.G., 2007. Hydroprocessing of Heavy Oils and Residua.

Aridjis, H., 1990. Mexico Proclaims Total Ban on Harvest of Turtles and Eggs, Marine Turtle Newsletter, pp. 1-3.

ATSDR, 2003. Toxicological Profile for Selenium. U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service
Agency for Toxic Substances and Disease Registry, Atlanta, Georgia.

ATSDR, 2004. Toxicological Profile for Strontium. U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service
Agency for Toxic Substances and Disease Registry, Atlanta, Georgia.

ATSDR, 2007. Toxicological Profil for Arsenic, Agency for Toxic Substances and Disease Registry. Agency for Toxic Substances and Disease Registry, Atlanta, Georgia, p. 500.

ATSDR, 2008. Toxicological Profile for Aluminium. U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service
Agency for Toxic Substances and Disease Registry, Atlanta, Georgia.

Barile, P.J., 2004. Evidence of Anthropogenic Nitrogen Enrichment of the Littoral Waters of East Central Florida. Journal of Coastal Research, 1237-1245.

Barton, B.A., Schreck, C.B., Barton, L.D., 1987. ffects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. Dis Aquat O%2.201% 2, 173-185.

Bears, H., Richards, J.G., Schulte, P.M., 2006. Arsenic exposure alters hepatic arsenic species composition and stress-mediated gene expression in the common killifish (Fundulus heteroclitus). Aquatic Toxicology 77, 257-266.

Biswas, S., Talukder, G., Sharma, A., 1999. Prevention of cytotoxic effects of arsenic by short-term dietary supplementation with selenium in mice in vivo. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 441, 155-160.

Bjerregaard, P., Anders, O., 2007. Ecotoxicology of Metals—Sources, Transport, and Effects in the Ecosystem.

Blaise, C., Gagné, F., Férard, J., Eullaffroy, P., 2008. Ecotoxicity of selected nano - materials to aquatic organisms. Environmental Toxicology 23, 591-598.

Boughammoura, S., Kessabi, K., Chouchene, L., Messaoudi, I., 2013. Effects of cadmium and high temperature on some parameters of calcium metabolism in the killifish (Aphanius fasciatus). Biol Trace Elem Res 154, 73-80.
Brodeur, J.C., Sherwood, G., Rasmussen, J.B., Hontela, A., 1997. Impaired cortisol secretion in yellow perch (Perca flavescens) from lakes contaminated by heavy metals: in vivo and in vitro assessment. . Can J Fish Aquat Sci 54.
Bury, N.R., Jie, L., Flik, G., Lock, R.A.C., Wendelaar Bonga, S.E., 1998. Cortisol protects against copper induced necrosis and promotes apoptosis in fish gill chloride cells in vitro. Aquat Toxicol 40:193–202. Aquatic Toxicology 40, 193.202.
Camacho, M., Boada, L.D., Oros, J., Lopez, P., Zumbado, M., Almeida-Gonzalez, M., Luzardo, O.P., 2014. Monitoring organic and inorganic pollutants in juvenile live sea turtles: results from a study of Chelonia mydas and Eretmochelys imbricata in Cape Verde. Sci Total Environ 481, 303-310.
Casale, P., Margaritoulis, D., 2010. Sea turtles in the Mediterranean: Distribution, threats and conservation priorities. IUCN-MTSG, Gland, Switzerland, p. 304.
Ceron, J.J., Tecles, F., Tvarijonaviciute, A., 2014. Serum paraoxonase 1 (PON1) measurement: an update. BMC Veterinary Research 10:74.
Chen, T., Yan, J., Li, Y., 2014. Genotoxicity of titanium dioxide nanoparticles. journal of food and drug analysis 22, 95-104.
Clemente, Z., Castro, V., Jonsson, C., Fraceto, L., 2011. Ecotoxicology of nano-TiO2–an evaluation of its toxicity to organisms of aquatic ecosystems. International Journal of Environmental Research 6, 33-50.
Cockrem, J.F., 2013. Individual variation in glucocorticoid stress responses in animals. General and Comparative Endocrinology 181, 45-58.
Cohen - Solal, M., 2002. Strontium overload and toxicity: impact on renal osteodystrophy. Nephrology Dialysis Transplantation 17, 30-34.
CONANP, 2011. Ficha de la tortuga golfina. Dirección para especies prioritarias para la conservación, in: Marinas, P.N.p.l.C.d.l.T. (Ed.). Comisión Nacional Areas Naturales Protegidas, México.
Cortés-Gómez, A.A., Fuentes-Mascorro, G., Romero, D., 2014. Metals and metalloids in whole blood and tissues of Olive Ridley turtles (Lepidochelys olivacea) from La Escobilla Beach (Oaxaca, Mexico). Mar Pollut Bull 89, 367-375.
Costa, L.G., Vitalone, A., Cole, T.B., Furlong, C.E., 2005. Modulation of paraoxonase (PON1) activity. Biochem Pharmacol 69, 541-550.
Costa, M.F., Barletta, M., 2015. Microplastics in coastal and marine environments of the western tropical and sub-tropical Atlantic Ocean. Environmental Science: Processes & Impacts 17, 1868-1879.
DeBoeck, G., DeWachter, B., Vlaeminck, A., Blust, R., 2003. ffect of cortisol treatment and/or sublethal copper exposure on copper uptake and heat shock protein levels in

common carp, Cyprinus carpio. Environmental Toxicology and Chemistry 22, 1122-1126.
Dyc, C., Covaci, A., Debier, C., Leroy, C., Delcroix, E., Thomé, J.-P., Das, K., 2015. Pollutant exposure in green and hawksbill marine turtles from the Caribbean region. Regional Studies in Marine Science.
El-Demerdash, F.M., Yousef, M.I., Radwan, F.M., 2009. Ameliorating effect of curcumin on sodium arsenite-induced oxidative damage and lipid peroxidation in different rat organs. Food and Chemical Toxicology 47, 249-254.
Faust, D.R., Hooper, M.J., Cobb, G.P., Barnes, M., Shaver, D., Ertolacci, S., Smith, P.N., 2014. Inorganic elements in green sea turtles (Chelonia mydas): relationships among external and internal tissues. Environ Toxicol Chem 33, 2020-2027.
Fazio, E., Medica, P., Bruschetta, G., Ferlazzo, A., 2014. Do Handling and Transport Stress Influence Adrenocortical Response in the Tortoises (Testudo hermanni)? ISRN Vet Sci 2014, 798273.
Franco, L., Romero, D., García-Navarro, J.A., Teles, M., Tvarijonaviciute, A., 2015. Esterase activity (EA), total oxidant status (TOS) and total antioxidant capacity (TAC) in gills of Mytilus galloprovincialis exposed to pollutants: Analytical validation and effects evaluation by single and mixed heavy metal exposure. Marine Pollution Bulletin.
Frazier, J.G., 1983. Analisis estadistlco de la tortuga golfina *Lepidochelys olivacea* (Eschscholtz) de Oaxaca, Mexico. CIencia Pesquera 4, 49-75.
Fries, E., Dekiff, J.H., Willmeyer, J., Nuelle, M.-T., Ebert, M., Remy, D., 2013. Identification of polymer types and additives in marine microplastic particles using pyrolysis-GC/MS and scanning electron microscopy. Environmental Science: Processes & Impacts 15, 1949-1956.
Fujihara, J., Kunito, T., Kubota, R., Tanabe, S., 2003. Arsenic accumulation in livers of pinnipeds, seabirds and sea turtles: subcellular distribution and interaction between arsenobetaine and glycine betaine
. Comparative Biochemistry and Physiology Part C 136, 287-296.
Gamliel-Lazarovicha, A., Gantmana, A., Shinera, M., Colemanb, R., Avirama, M., Keidar, S., 2010. Paraoxonase 1 deficiency in mice is associated with reduced steroid biosynthesis: Effects on HDL binding, cholesteryl ester accumulation and scavenger receptor type BI expression. Atherosclerosis 211, 130-135.
Garcia-Besne, G., Valdespino, C., Rendon-von Osten, J., 2015. Comparison of organochlorine pesticides and PCB residues among hawksbill (Eretmochelys imbricata) and green (Chelonia mydas) turtles in the Yucatan Peninsula and their maternal transfer. Mar Pollut Bull 91, 139-148.
Gregory, L.F., Gross, T.S., Bolten, A.B., Bjorndal, K.A., Guillette, L.J.J., 1996. Plasma corticosterone concentrations associated with acute captivity stress in wild loggerhead sea turtles (*Caretta caretta*). General and Comparative Physiology 104, 312-320.

Gregory, L.F., Schmid, J.R., 2001. Stress responses and sexing of wild Kemp's ridley sea turtles (*Lepidochelys kempii*) in the northeastern Gulf of Mexico. General and Comparative Endocrinology 124, 66-74.
Gregory, T.R., Wood, C.M., 1999. The effects of chronic plasma cortisol elevation on the feeding behaviour, growth, com- petitive ability, and swimming performance of juvenile rainbow trout. Physiol Biochem Zool 72, 268-295.
Guebert-Bartholo, F.M., Barletta, M., Costa, M.F., Monteiro-Filho, E.L.A., 2011. Using gut contents to assess foraging patterns of juvenile green turtles *Chelonia mydas* in the Paranagua Estuary, Brazil. Endangered Species Research 13, 131-143.
Haagen, L., Brock, A.A., 1992. New Automated Method for Phenotyping Arylesterase (EC 3.1.1.2) Based Upon Inhibition of Enzymatic Hydrolysis of 4-Nitrophenyl Acetate by Phenyl Acetate. . European Journal of Clinical Chemistry and Clinical Biochemistry 30, 391-395.
Hao, L., Wang, Z., Xing, B., 2009. Effect of sub-acute exposure to TiO 2 nanoparticles on oxidative stress and histopathological changes in juvenile carp (Cyprinus carpio). Journal of Environmental Sciences 21, 1459-1466.
Hernandez, A.F., Gil, F., Leno, E., Lopez, O., Rodrigo, L., Pla, A., 2009. Interaction between human serum esterases and environmental metal compounds. Neurotoxicology 30, 628-635.
Hopkins, W.A., Snodgrass, J.W., Baionno, J.A., Roe, J.H., Staub, B.P., Jackson, B.P., 2005. Functional relationship among selenium concentration in the diet, target, tussues and nondestructive tissue samples of two species of snakes. . Environmental Toxicology and Chemistry 24, 344-351.
Ikonomopoulou, M.P., Bradley, A.J., Whittier, J.M., Ibrahim, K., 2006. Identification and properties of steroid-binding proteins in nesting Chelonia mydas plasma. J Comp Physiol B 176, 775-782.
IUCN, 2012. Red List of Threatened Species, Version 2012.1. International Union for Conservation of Nature, http://www.iucnredlist.org/.
Izquierdo-Vega, J.A., Soto, C.A., Sanchez-Peña, L.C., De Vizcaya-Ruiz, A., Del Razo, L.M., 2006. Diabetogenic effects and pancreatic oxidative damage in rats subchronically exposed to arsenite. Toxicology letters 160, 135-142.
Kahru, A., Dubourguier, H.-C., 2010. From ecotoxicology to nanoecotoxicology. Toxicology%3.681% 269, 105-119.
Kitchin, K.T., Ahmad, S., 2003. Oxidative stress as a possible mode of action for arsenic carcinogenesis. Toxicology letters 137, 3-13.
Kubota, R., Kunito, T., Tanabe, S., 2002. Occurrence of Several Arsenic Compunds in the Liver of Birds, Cetacean, Pinnipeds and Sea Turtles. Environmental Toxicology and Chemistry 22, 1200-1207.
Labrada-Martagon, V., Rodriguez, P.A., Mendez-Rodriguez, L.C., Zenteno-Savin, T., 2011. Oxidative stress indicators and chemical contaminants in East Pacific green turtles

(Chelonia mydas) inhabiting two foraging coastal lagoons in the Baja California peninsula. Comp Biochem Physiol C Toxicol Pharmacol 154, 65-75.
Laird, B.D., Goncharov, A.B., Ayotte, P., Chan, H.M., 2014. Relationship between the esterase paraoxonase-1 (PON1) and metal concentrations in the whole blood of Inuit in Canada. Chemosphere%3.206% 120C, 479-485.
Lesser, M.P., 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. Annu Rev Physiol 68, 253-278.
Li, W.-F., Pan, M.-H., Chung, M.-C., Ho, C.-K., Chuang, H.-Y., 2006. Lead Exposure Is Associated with Decreased Serum Paraoxonase 1 (PON1) Activity and Genotypes. Environmental Health Perspectives 114, 1233-1236.
Marentette, J.R., Tong, S., Balshine, S., 2013. The cortisol stress response in male round goby (*Neogobius melanostomus*): effects of living in polluted environments? Environ Biol Fish 96, 723-733.
Marine Turtle Specialist Group, 2007. *Lepidochelys olivacea* – Red List Assessment 2007 Marine Turtle Specialist Group revised doc May 01 2007.
Márquez, M.R., Peñaflores, C., Vasconcelos, J.C., 1996. Olive ridley turtles (*Lepidochelys olivacea*) show signs of recovery at La Escobilla, Oaxaca. Mar. Turt. Newsl. 73, 5-7.
Mei, L., Xitao, X., Renhao, X., Zhili, L., 2006. Effects of strontium-induced stress on marine microalgae Platymonassubcordiformis(Chlorophyta: Volvocales)*. Chinese Journal of Oceanology and Limnology 24, 154-160.
Miller, D.L., Wyneken, J., Rajeev, S., Perrault, J., Mader, D.R., Weege, J., Baldwin, C.A., 2009. Pathologic findings in hatchling and posthatchling leatherback sea turtles (*Dermochelys coriacea*) from Florida. Journal of Wildlife Diseases 45, 962–971.
Miller, L.L., Hontela, A., 2011. Species-specific sensitivity to selenium-induced impairment of cortisol secretion in adrenocortical cells of rainbow trout (Oncorhynchus mykiss) and brook trout (Salvelinus fontinalis). Toxicology and Applied Pharmacology 253, 137-144.
Misra, S., Niyogi, S., 2009. Selenite causes cytotoxicity in rainbow trout (Oncorhynchus mykiss) hepatocytes by inducing oxidative stress. Toxicol In Vitro 23, 1249-1258.
Moiseenko, T., Kudryavtseva, L., 2001. Trace metal accumulation and fish pathologies in areas affected by mining and metallurgical enterprises in the Kola Region, Russia. Environmental Pollution 114, 285-297.
Morcillo, P., Esteban, M.A., Cuesta, A., 2015. Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. Chemosphere%3.206% 144, 225-233.
Moreno, H., 2012. Derrama Pemex miles de litros de petróleo al mar en Salina Cruz, La Jornada. http://www.jornada.unam.mx/2012/08/24/estados/033n1est.
NOM-059-SEMARNAT-2010, 2010. NORMA Oficial Mexicana NOM-059-SEMARNAT-2010, Protección ambiental-Especies nativas de México de flora y fauna silvestres-

Categorías de riesgo y especificaciones para su inclusión, exclusión o cambio-Lista de especies en riesgo. SEMARNAT, México.
Nunes, B., Capela, R.C., Sergio, T., Caldeira, C., Goncalves, F., Correia, A.T., 2014. Effects of chronic exposure to lead, copper, zinc, and cadmium on biomarkers of the European eel, Anguilla anguilla. Environ Sci Pollut Res Int 21, 5689-5700.
Paez-Osuna, F., Calderon-Campuzano, M.F., Soto-Jimenez, M.F., Ruelas-Inzunza, J.R., 2010. Trace metals (Cd, Cu, Ni, and Zn) in blood and eggs of the sea turtle Lepidochelys olivacea from a nesting colony of Oaxaca, Mexico. Arch Environ Contam Toxicol 59, 632-641.
Perrault, J.R., Miller, D.L., Garner, J., Wyneken, J., 2013. Mercury and selenium concentrations in leatherback sea turtles (Dermochelys coriacea): Population comparisons, implications for reproductive success, hazard quotients and directions for future research. Science of The Total Environment 463-464, 61-71.
Perrault, J.R., Schmid, J.R., Walsh, C.J., Yordy, J.E., Tucker, A.D., 2014. Brevetoxin exposure, superoxide dismutase activity and plasma protein electrophoretic profiles in wild-caught Kemp's ridley sea turtles (Lepidochelys kempii) in southwest Florida. Harmful Algae 37, 194-202.
Pollack, A.Z., Sjaarda, L., Ahrens, K.A., Mumford, S.L., Browne, R.W., Wactawski-Wende, J., Schisterman, E.F., 2014. Association of cadmium, lead and mercury with paraoxonase 1 activity in women. Plos One%4.092% 9, e92152.
Poloczanska, E.S., Limpus, C.J., Hays, G.C., 2009. Chapter 2. Vulnerability of marine turtles to climate change. Advances In Marine Biology 56, 151-211.
Rana, S.V., 2014. Perspectives in endocrine toxicity of heavy metals-a review. Biol Trace Elem Res 160, 1-14.
Reeves, J.F., Davies, S.J., Dodd, N.J., Jha, A.N., 2008. Hydroxyl radicals (OH) are associated with titanium dioxide (TiO 2) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 640, 113-122.
Richardson, K.L., Gold-Bouchot, G., Schlenk, D., 2009. The characterization of cytosolic glutathione transferase from four species of sea turtles: Loggerhead (Caretta caretta), green (Chelonia mydas), olive ridley (Lepidochelys olivacea), and hawksbill (Eretmochelys imbricata). Comparative Biochemistry and Physiology, Part C 150, 279-284.
Rigby, M.C., Deng, X., Grieb, T.M., Teh, S.J., Hung, S.S., 2010. Effect threshold for selenium toxicity in juvenile splittail, Pogonichthys macrolepidotus A. Bull Environ Contam Toxicol 84, 76-79.
Saeki, K., Sakakibaea, H., Sakai, H., Kunito, T., TANABE, S., 2000. Arsenic accumulation in three species of sea turtles
241. Biometals%2.823% 13, 241-250.

Sanford, B., Stephens, G.A., 1988. The effects of adrenocorticotropin hormone and angiotensin II on adrenal corticosteroid secretions in the freshwater turtle Pseudemys scripta. General and comparative endocrinology 72, 107-114.
Shaw, J.R., Gabor, K., Hand, E., Lankowski, A., Durant, L., Thibodeau, R., Stanton, C.R., Barnaby, R., Coutermarsh, B., Karlson, K.H., Sato, J.D., Hamilton, J.W., Stanton, B.A., 2007. Role of glucocorticoid receptor in acclimation of killifish (Fundulus heteroclitus) to seawater and effects of arsenic. Am J Physiol Regul Integr Comp Physiol 292, R1052-1060.
Shi, H., Magaye, R., Castranova, V., Zhao, J., 2013. Titanium dioxide nanoparticles: a review of current toxicological data. Particle and Fibre Toxicology, 10:15.
Sonne, C., Aspholm, O., Dietz, R., Andersen, S., Berntssen, M.H., Hylland, K., 2009. A study of metal concentrations and metallothionein binding capacity in liver, kidney and brain tissues of three Arctic seal species. Sci Total Environ 407, 6166-6172.
Suzuki, K., Noda, J., Yanagisawa, M., Kawazu, I., Sera, K., Fukui, D., Asakawa, M., Yokota, H., 2012. Relationships between Carapace Sizes and Plasma Major and Trace Element Status in Captive Hawksbill Sea Turtles (Eretmochelys imbricata). Journal of Veterinary Medical Science 74, 1677-1680.
Tay, C.Y., Fang, W., Setyawati, M.I., Chia, S.L., Tan, K.S., Hong, C.H.L., Leong, D.T., 2014. Nano-hydroxyapatite and nano-titanium dioxide exhibit different subcellular distribution and apoptotic profile in human oral epithelium. ACS applied materials & interfaces 6, 6248-6256.
Tourinho, P.S., do Sul, J.A.I., Fillmann, G., 2010. Is marine debris ingestion still a problem for the coastal marine biota of southern Brazil? Marine Pollution Bulletin 60, 396-401.
Trouiller, B., Reliene, R., Westbrook, A., Solaimani, P., Schiestl, R.H., 2009. Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Cancer Research%7.856% 69, 8784-8789.
Tvarijonaviciute, A., Tecles, F., Caldin, M., Tasca, S., Ceron, J.J., 2012. Validation of spectrophotometric assays for serum paraoxonase type-1 measurement in dogs. American Journal of Veterinary Research 73, 34-42.
Valdivia, P.A., Zenteno-Savin, T., Gardner, S.C., Aguirre, A.A., 2007. Basic oxidative stress metabolites in eastern Pacific green turtles (Chelonia mydas agassizii). Comp Biochem Physiol C Toxicol Pharmacol 146, 111-117.
Wallace, B.P., Saba, V.S., 2009. Environmental and anthropogenic impacts on intra-specific variation in leatherback turtles: opportunities for targeted research and conservation. Endangered Species Research 7, 11-21.
Wise, J.P., 2011. A global assessment of gold, titanium, strontium and barium pollution using sperm whales (Physeter macrocephalus) as an indicator species. Journal of Ecosystem & Ecography.
Xu, Z., Wang, Z., Li, J.-j., Chen, C., Zhang, P.-c., Dong, L., Chen, J.-h., Chen, Q., Zhang, X.-t., Wang, Z.-l., 2013. Protective effects of selenium on oxidative damage and oxidative

stress related gene expression in rat liver under chronic poisoning of arsenic. Food and chemical toxicology 58, 1-7.
Zhou, X., Niu, C., Sun, R., 2004. The effects of vitamin E on antiacid stress ability in juvenile soft-shelled turtles (Pelodiscus sinensis). Comp Biochem Physiol C Toxicol Pharmacol 137, 299-305.
Zhou, X., Xie, M., Niu, C., Sun, R., 2003. The effects of dietary vitamin C on growth, liver vitamin C and serum cortisol in stressed and unstressed juvenile soft-shelled turtles (Pelodiscus sinensis). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 135, 263-270.
Zug, G.R., Chaloupka, M., Balazs, G.H., 2006. Age and growth in Olive Ridley sea turtles (*Lepidochelys olivacea*) from the North-central Pacific: a skeletochronological analysis. Marine Ecology 27, 263–270.

3.4.2 CHAPTER VI. Second year of biochemistry: ALT, AST, ALP, albumin, creatinine, glucose, urea, cholesterol, esterase activity and cortisol related to metals

3.4.2.1 Introduction

Olive Ridley (*Lepidochelys olivacea*) is the world most abundant sea turtle, with nesting beaches in the Atlantic, Pacific, and Indian Ocean basins. Ridleys have a remarkable aggregation behavior that results in synchronized mass-nesting of thousands of turtles nesting at the same time in the same beach over few consecutive nights during the so called *arribadas*. *La Escobilla*, in Mexico, among 3 of the most important *arribada* beaches in the world for this species. Here, hundreds of thousands of females nesting during the *arribadas* season every year (from June to January). Despite being the most abundant species of sea turtles, Olive Ridleys is listed as Vulnerable in the IUCN Red List (IUCN, 2012); is considered as Endangered Species in Mexico and is included within the NOM-ECOL-059-2010, as well as is included in the *Priority Species for Conservation* list.

Many anthropogenic causes of sea turtle mortality have contributed to the decline in local and global numbers of this species and more generally of all marine turtles. The deleterious effect of environmental pollutants is currently included among the top 20 research topics for sea turtle conservation (Hamann et al., 2010) and for the experts in the turtle toxicology field (Finlayson et al., 2016). Although environmental pollutants in sea turtles have been assessed by several authors throughout the world (see chapter 1.2), very few studies have documented biomarkers or health effects derived from these exposures (Camacho et al., 2013b; Keller and McClellan-Green, 2004; Keller et al., 2004; Keller et al., 2006a; Labrada-Martagon et al., 2011a), two of them has been performed in *L. olivacea* (Santillana, 2013; Santoro and Meneses, 2007). For sea turtle conservation purposes, it is essential to determine how these reptiles respond to different types of anthropogenic contaminants (Camacho et al., 2013b). In order to do this, a database about normal health clinical parameters for species and for population are needed, additionally to a regular pollutants biomonitoring in the areas.

Serum biochemistry is commonly used as a help for diagnostic to assess health in domestic and wild animals. These parameters can vary depending on species, gender, age, diet,

pathological process and analysis techniques (Anderson et al., 2011; Casal et al., 2009; Fazio et al., 2011). Due to all these variations, it is not easy to establish normal parameters in marine turtles, and it is recommended to have a database by species and by populations (nesting/feeding areas). This database would then, provide indications of health, diseases, nutrition and even evaluate habitat quality (Camacho et al., 2013b). All this information would help to make a better assessment on the health of different populations and also provide an important tool to make a better diagnosis and veterinary care in rescue animals. Among the different causes that can alter the health of marine turtles, as in other species, is the exposure to pollutants. One of the most studied due to their toxicity, are heavy metals (see chapter I). These elements are natural components of the heart crust, but their multiple anthropogenic uses in industrial, domestic, medical, agricultural, mining and electronic applications have led to their wide distribution in the environment. Due to their toxicity risk, cadmium, lead, arsenic and chromium are among the most studied metals (see chapter 1.2). *La Escobilla* population has been previously reported with very high concentration of certain metals, specially cadmium (Cortés-Gómez et al., 2014), but the impact of these elements in these turtle's health is still unknown.

As a result of the lack of information existing on this subjects in Olive Ridleys turtles from *La Escobilla*, the aims of this work were: (1) establish the range of normal values for the selected biochemical parameters (ALP, AST, ALT, creatinine, albumin, cholesterol, glucose, proteins, triglycerides, urea and P-nitrophenyl acetate esterase activity; *n*= 100); (2) assess As, Cd, Cr, Mn, Ni, Pb, Sr, Ti, Zn and Se concentration in whole blood of the same individuals; and (3) search relationships between biochemical parameters and the inorganic elements selected.

3.4.2.2 Material and methods

3.4.2.2.1 Sample collection

Samples were collected from 100 random apparently healthy nesting turtles at *La Escobilla* beach in the state of Oaxaca (Southeast Mexico, Eastern Pacific, 96°44'W and 15°47'N).

Beach monitoring was performed during 3 different *arribadas* from August to November 2014. Before blood extraction, the neck region was carefully cleaned using ethanol. Samples were taken when a turtle was on their way out to nest, during the pause the turtle takes during its displacement towards its selected nest area. Any restricted management was used during the sampling, all the process usually lasted between 15 and 30 seconds and the turtles did not show any alteration on their behaviour after sampling. Blood samples were taken from the dorsal cervical sinus, using a 5-mL syringe with 21" needle.

For the biochemical parameters, blood was placed in repose into a Vacutainer® tube without anticoagulant (red top) during approximately 2 hours to separate serum from the red cellular package. Then, serum was transferred into *Eppendorf*® microtubes. For metal analysis, the whole blood samples were stored into 1.5 *Eppendorf*® microtubes, all samples were keep at -20°C until analyses. Permission for this study to collect the samples was SGPA/DGVS/00774/14 and CITES for exportation was MX 72799.

3.4.2.2.2 Metal analysis

The concentration of arsenic (As), cadmium (Cd), chrome (Cr), manganese (Mn), nickel (Ni), lead (Pb), strontium (Sr), titanium (Ti), zinc (Zn) and selenium (Se) were determined. Prior metal analysis, an acid digestion was performed using 0.5 g of the sample into 4 mL of HNO3 (69%) and 1 mL of H2O2 (33%) mixed in special Teflon reaction tubes in a microwave digestion system (UltraClave-Microwave Milestone) for 20 min at 220°C and finally diluted with 25 mL of double deionized water (Milli-Q). Metal concentrations were determined using an Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES, ICAP 6500 Duo Thermo) following descriptions from Cortés-Gómez et al. (2014). All concentrations are expressed in microgram per gram in wet weight. Detection limit (DL) for all elements was 0.01 $\mu g\ g^{-1}$.

3.4.2.2.3 Biochemical analysis

The biochemical constituents of the serum were measured using an automated clinical chemistry analyser (Olympus AU 2700; Olympus Diagnostica GmbH, Hamburg, Germany). The biochemical panel included alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, albumin, cholesterol, glucose, proteins, triglycerides, urea and P-nitrophenyl acetate esterase activity (EA). All these parameters, except EA, were measured according with the manufacture instructions. EA was measured by the hydrolysis of *p-nitrofenolacetate* to *p-nitrophenol* as described by Haagen and Brock (1992), with some modifications (Tvarijonaviciute et al., 2012). Intra and inter coefficient of variation (CV) were below 10% and recovery ranged between 90% and 110% for all the parameters.

Cortisol was analysed in an automated chemiluminescent immunoassay (Immulite System; Siemens Health Diagnostics, Deerfield, Illinois, USA) as per the manufacturer instructions. Intra and inter CV were below 8% and 10%, respectively. Recovery ranged between 97% and 112%. The DL was 0.05 µg dL^{-1}.

3.4.2.2.4. Data analysis

The relationship between the biochemical parameters (BP) and the selected elements was assessed using a Gaussian distribution and an identity link. The selection of the variable linked to the BP was performed using the Akaike information criterion (AIC). AIC is a measure of quality of fit of a model penalized by the number of variables in the model (Burnham and Anderson, 2002). All statistical analyses were performed using R 3.3.0 (R Core Team, 2016).

3.4.2.3 Results

The results regarding the concentration of BP measured are shown in Table 1. Most of the parameters were detected in almost all the samples (*n*= 100), except cortisol, which was measured only in 33 samples.

All inorganic elements in whole blood included in this study (*n*=100) were above the detection limit (<0.01 µg g^{-1}). Zinc presented the highest concentration with a mean ± standard deviation of 7.7±2.4, it was follow for Se with 6.72±3.0, As 1.27±0.9, Sr 1.02±0.45, Cu 0.52±0.2, Mn 0.41±0.12, Cr 0.17±0.07, Cd 0.12±0.05, Ni 0.07±0.07, Ti 0.03±0.08, and finally Pb, with 0.02±0.01. In Table 2 are represented minimum and maximum of each element Finally, in the Figures 1 to 9, all the significant relationships found are represented in the graphics.

Table 1. Selected biochemical parameters and cortisol in *Lepidochelys olivacea* (mean ± SD).

ALP (UI/L)	*AST (UI/L)*	*ALT (UI/L)*	*Creatinine (mg/dL)*	*Albumin (g/dL)*	*Cholesterol (mg/dL)*
42±35 n=100	58±32 n=100	5.5±7.4 n=98	0.2±0.06 n=99	0.9±0.2 n=100	220±62 n=100
Glucose (mg/dL)	*Proteins (g/dL)*	*Triglycerides (mg/dL)*	*UREA (mg/dL)*	*EA (UI/mL)*	*Cortisol (µg/dL)*
79±18 n=100	3.4±0.5 n=100	453±208 n=100	19±6 *n*=100	1.6±1.1 *n*=100	1.2±2.7 *n*=33

Table 2. Metals and metalloids in whole blood of *Lepidochelys olivacea* (minimum-maximum, µg g^{-1} ww).

As	*Cd*	*Cr*	*Cu*	*Mn*	*Ni*
0.13-7.59	0.02-0.29	0.01-0.50	0.11-1.79	0.07-1.30	0.01-0.64
Pb	*Se*	*Sr*	*Ti*	*Zn*	
0.01-0.12	1.01-27.71	0.18-3.13	0.01-0.61	1.46-25.48	

Significant relationships found between metals and biochemical parameters are shown in the following graphics:

Figures 1 a and b. <u>AST</u> significant effects

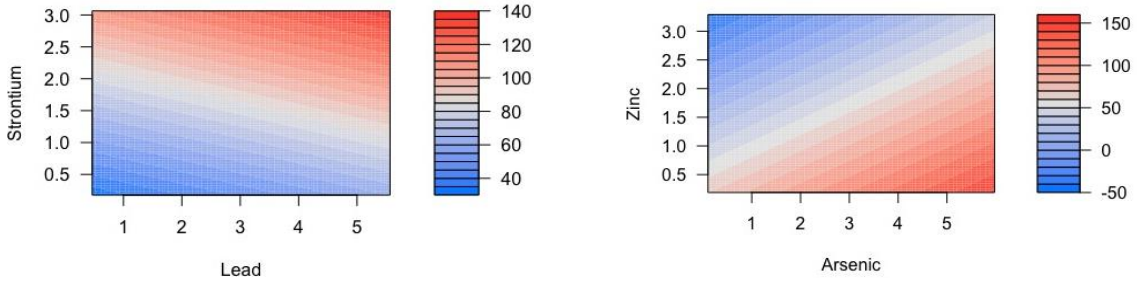


Figure 2. ALT significant effects

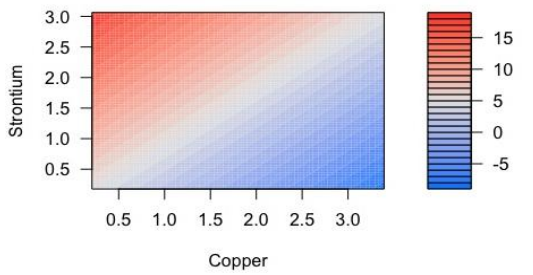


Figure 3. Albumin significant effects

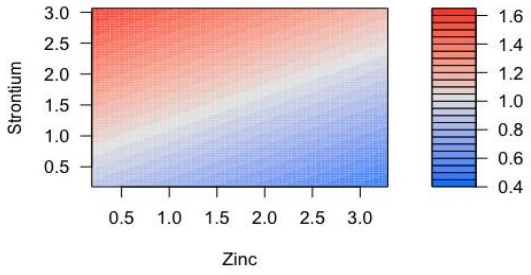
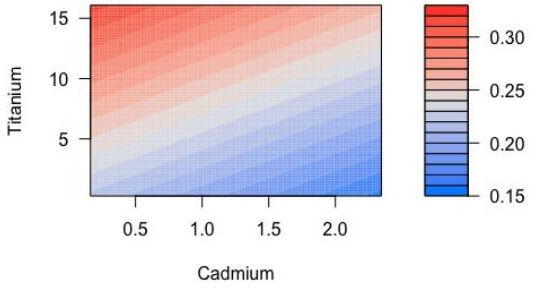


Figure 4 a and b. Creatinine significant effects



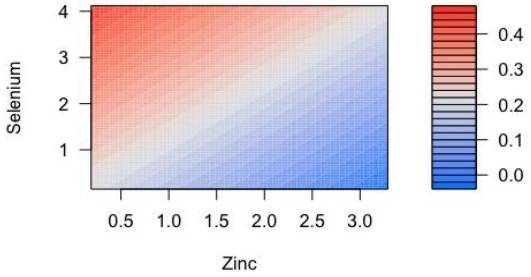
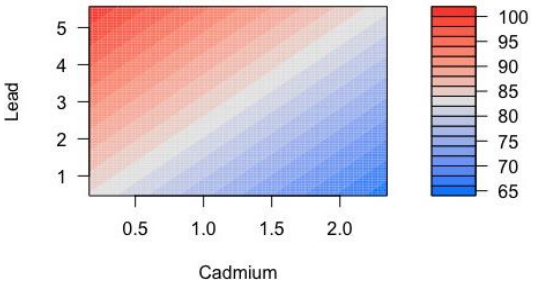


Figure 5. Glucose significant effects



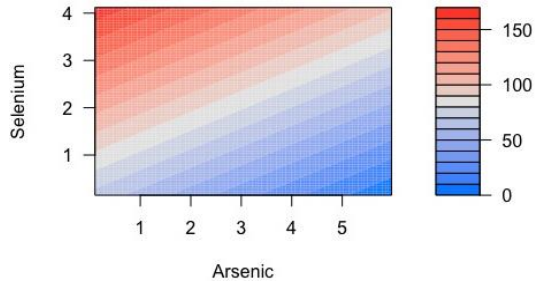
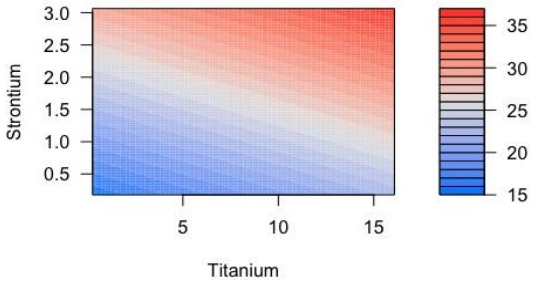


Figure 6 a and b. Urea significant effects



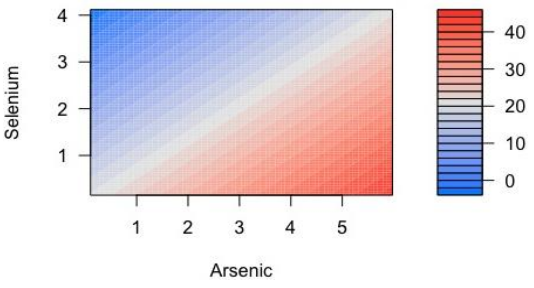
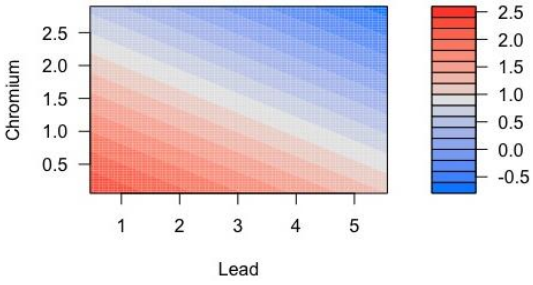


Figure 7 a and b. EA significant effects



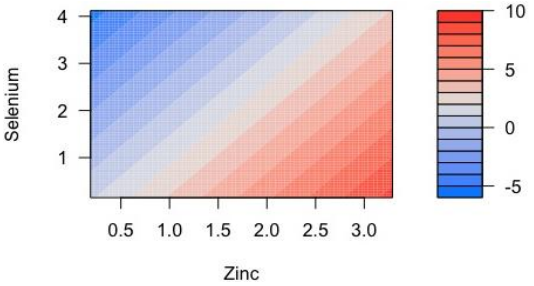


Figure 8. <u>Cholesterol</u> significant effects

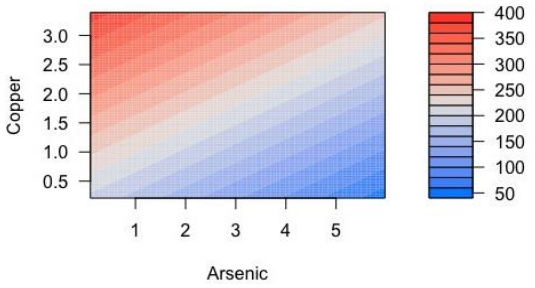
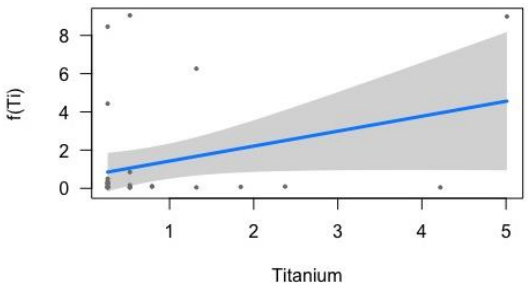


Figure 9. <u>Cortisol</u> significant effects



3.4.2.4. Discussion

Defining a baseline for health and monitoring programs has always been a challenged working with wildlife. In marine turtles, it has been demonstrated that most of health or pollutants parameters can change depending on the age, feeding habits, species, location and even the methodology used (Anderson et al., 2011; Casal et al., 2009; Finlayson et al., 2016; Kakizoe et al., 2007; Wolf et al., 2008). Then, it is important that each population is characterized with their own baseline data to evaluate the health of the individuals and to follow the xenobiotics concentration in the populations. Thus, this information is valuable for recovering centers, conservation programs, ecotoxicological studies, among others.

High concern exists regarding the possible adverse effects of anthropogenically-derived inorganic elements, including those considered essential (Finlayson et al., 2016). However, their toxic action on wildlife is not well understood yet. An assessment of the environmental impact of these trace elements requires data from a large series of animals. However, there is an important lack of information about the deleterious effects of elements, such as Hg, Cd, and Pb, in marine wildlife. The use of blood to assess the levels of exposure to elements is an attractive option for studies on wildlife health because blood can be non-lethally collected and allows the simultaneous determination of environmental anthropogenic pollutants and clinical parameters that could be adversely modulated by these contaminants.

Several studies have reported biochemical parameters from different sea turtle species (Anderson et al., 2011; Bolten et al., 1992; Camacho et al., 2013a; Camacho et al., 2013b; Casal et al., 2009; Fazio et al., 2011; Innis et al., 2009), but only two of them in *Lepidochelys olivacea* (Santillana, 2013; Santoro and Meneses, 2007). Many other studies have reported metal concentrations in blood of different sea turtle species and populations (See Chapter I). However, only 4 of them have related metals (or other pollutants) with classic biochemical parameters routinely used to assessed the health of animals and humans (Camacho et al., 2013b; da Silva et al., 2016a; Day et al., 2007; Komoroske et al., 2011).

Regarding the biochemical analytics, in the Table 3 are compared our results with those other 2 works existing in *L. olivacea*. The three works were done in adult nesting females from the Western Pacific: from El Salvador (Santillana, 2013) and from Ostional, another *arribada* beach in Costa Rica (Santoro and Meneses, 2007). Our study is similar to Santoro and Meneses (2007) from Ostional, Costa Rica. Santillana (2013) reported, in general, higher concentrations of all the comparable analytics, especially glucose; on the contrary, cholesterol was the only analytic which was significant lower than the other 2 works. These important differences could be due, *a priori*, to the number of individuals used. However, the biggest difference in number of samples was between Santoro and our results, not explain the differences among these 2 works and that reported by Santillana (2013). Another factor previously reported as important and to take into account is the analytical methodology used (Wolf et al., 2008). The three works were done in a very different way, going from a manual (Santoro and Meneses, 2007), semi-automatic (Santillana, 2013) and automatic (this work) spectrophotometers. Despite Santoro and Meneses (2007) and our work values still similar, this could be a valid explanation about the differences reported. In regard to glucose vast difference with Santillana (2013), we may also suspect that turtles from the *arribada* beaches are longer distance travelers than turtles from El Salvador, which are solitary nesters and then, their energy supplies may be lower.

Table 3. Comparative table of biochemical parameters in serum of adult *L. olivacea* females. Mean (SD).

	This Study *n*=100	Santoro et al, 2007; *n*=21	Santillana, 2013 *n*= 38
ALP (UI/L)	42 (35)	29.5 (30.2)	NA
AST (UI/L)	58 (32)	73.4 (29.3)	115 (41)
ALT (UI/L)	5.5 (7.4)	8.2 (5.5)	11.2 (9.7)
Creatinine (mg/dL)	0.2 (0.06)	0.4 (0.2)	1.24 (0.8)
Albumin (g/dL)	0.9 (0.2)	0.7 (0.1)	NA
Cholesterol (mg/dL)	220 (62)	275 (55)	65 (49)
Glucose (mg/dL)	79 (18)	63 (12)	219 (193)
Proteins (g/dL)	3.4 (0.5)	3.9 (0.9)	NA
Triglycerides (mg/dL)	453 (208)	480 (302)	NA
UREA (mg/dL)	19 (6)	NA	34 (12)

Many of the biochemical health markers are different when compared with those of other species, age differences, feeding diets and with physiological alterations (Anderson et al., 2011; Camacho et al., 2013b; Casal and Orós, 2009; Fazio et al., 2011; Snoddy et al., 2009; Wrobel GoldbergI et al., 2011). Thus, it is important to stablish a database for this population to better understand any change against different xenobiotics or to different pathological process.

Concerning the significant relationships found between these biochemical parameters (BP) and metals (Figures 1 to 9), previously we mention that Pb and Cd are the most studied metals in many species due to its toxicity and worldwide presence. Pb has been present in this turtle population for long time (Paez-Osuna et al., 2010), and we found a positive relationship between this metal and AST (Figure 1). This metal has been proved cause an increase in the AST in humans and experimental species (Kuzmichevaa et al., 2014; Matovic et al., 2015). Pb also had a positive relationship with glucose, this relationship has been previously observed at low doses of Pb (similar to ours) due to the up-regulated of a glucose-regulated protein (GRP78) (Qian et al., 2001). A negative relationship of Pb with EA is consistent with a previous work in the same population (see Chapter 3.4.1). This

association between EA and Pb also agrees with those previously described in other species by different authors (Costa et al., 2005; Hernandez et al., 2009; Laird et al., 2014; Li et al., 2006). Then, after 2 studies with the same association, it looks that Pb may have a negative influence of EA in these turtles.

Cadmium shows two negative relationships (with creatinine and glucose; figures 4 and 5). According to a previous publication on the same population (Cortés-Gómez et al., 2014), we know that these turtles present very high and chronic Cd concentrations in its accumulation organs (liver and kidney). BP are usually found in high levels in acute Cd expositions, but in chronic expositions, some of them tend to diminish due to the incapacity of the organisms to produce high levels of these BP for a long term (Kowalczyk et al., 2003; Massanyi et al., 2014). The high and chronic exposition to Cd could explain, at least in a part, the negative relationships found between creatinine and glucose with Cd.

Among the other and less studied elements, Ti shows three positive relationships, being the only element with a significant relationship with cortisol (Figures 4a, 6a and 9). Regarding this association with cortisol, in humans has been demonstrate that the nanoparticles of Ti promote a chronic inflammation through B cell activation and IgE production (Park et al., 2009). Then, it could be the role of cortisol as an anti-inflammatory corticosteroid the relation with inflammation cause by Ti (Rhen and Cidlowski, 2005). Additionally, even if in blood mean concertation of Ti in this work is not very important, very high concentration of this metal had been detected in some individuals when its been analyzed in different tissues (*unpublished data*). Strontium had 4 positive relationships with the BP (Figures 1a, 2, 3 and 6a). Neither Sr nor Ti has been previously evaluated in this population, however this does not mean that they were not present, and all the positive relationships found may suggest that these elements have being introduced recently to these turtles ecosystem, or at least they have now a higher availability.

Arsenic and Se had both, two positive and two negative relationships (Figures 1b, 5b, 6b, 8 and 4b, 5a, 6b, 7b respectively). As had positive relationships with AST and urea; AST is regarded as biomarker for liver function and urea is used to estimate glomerular filtration and renal function. Studies in experiment rats had reported the increase of these metabolites in a chronical exposure to As (Nandi et al., 2006). On the other hand, Se has been also linked to an increase of glucose in rats as well (Çay and Naziroğlu, 1999), one of the 2 positive relationships of Se and the BP. We did not find more information about the correlations reported in this work, but all this information leads us to think that these elements are important for the function of most of the BP measured. Both, As and Se have been previously reported with high concentrations in this Ridleys population (Cortés-Gómez et al., 2014).

To our knowledge, only two works relating metals and BP in marine turtles have been published (Camacho et al., 2013b; Komoroske et al., 2011) in *Caretta caretta* and *Chelonia mydas*. Komoroske et al. (2011) measured, comparable with us, ALP, ALT, AST, glucose, albumin and cholesterol; contrarily to us, they found more correlations with ALP (As, Cd, Pb and Se), and we did not find any and with Albumin (As, Cd, Cu and Se), where we found only Zn and Sr related to it. With the work of Camacho et al. (2013b), we only could compared urea, and they had 3 negative relationships with Pb, Ni and As, while we had one negative (Se) and 3 positive (Sr, Ti and As). These differences must be due to the different concentrations of these metals and the different studied species.

3.4.2.5 Conclusions

This study represents the first evaluation of the possible relationship between metals and the biochemical parameters here studied in Olive Ridleys from this important *arribada* beach, and in this species. Evidence suggests several associations between metal concentrations and biochemical parameters. Then, this study reinforces the utility of the blood of sea turtles for the simultaneous monitoring of environmental contaminants and clinical parameters. The several correlations detected in the present study between the

elements analyzed and biochemical parameters, additionally to the previous high concentrations reported, indicate that these contaminants may have a negative effect on the health of these turtles. Finally, with the important number of turtles sampled (n=100, except Cortisol with *n*=33), these results can be used as baseline guide for future researches and to assess the health of rescue individuals in this area.

References

Anderson, E.T., Minter, L.J., Clarke, E.O., 3rd, Mroch, R.M., 3rd, Beasley, J.F., Harms, C.A., 2011. The Effects of Feeding on Hematological and Plasma Biochemical Profiles in Green (Chelonia mydas) and Kemp's Ridley (Lepidochelys kempii) Sea Turtles. Vet Med Int 2011, 890829.

Bolten, A.B., Jacobson, E.R., Bjorndal, K.A., 1992. Effects of anticoagulant and autoanalyzer on blood biochemical values of loggerhead sea turtles (*Caretta caretta*). American Journal of Verterinary Research 53, 2224-2227.

Burnham, K.P., Anderson, D.R., 2002. Model selection and multimodel inference: A practical information-theoretic approach. Springer-Verlag, New York.

Camacho, M., Luzardo, O.P., Boada, L.D., Lopez Jurado, L.F., Medina, M., Zumbado, M., Oros, J., 2013a. Potential adverse health effects of persistent organic pollutants on sea turtles: evidences from a cross-sectional study on Cape Verde loggerhead sea turtles. Sci Total Environ 458-460, 283-289.

Camacho, M., Oros, J., Boada, L.D., Zaccaroni, A., Silvi, M., Formigaro, C., Lopez, P., Zumbado, M., Luzardo, O.P., 2013b. Potential adverse effects of inorganic pollutants on clinical parameters of loggerhead sea turtles (Caretta caretta): results from a nesting colony from Cape Verde, West Africa. Marine Environmental Research 92, 15-22.

Casal, A.B., Camacho, M., Lopez-Jurado, L.F., Juste, C., Oros, J., 2009. Comparative study of hematologic and plasma biochemical variables in Eastern Atlantic juvenile and adult nesting loggerhead sea turtles (Caretta caretta). Vet Clin Pathol 38, 213-218.

Casal, A.B., Oros, J., 2009. Plasma biochemistry and haematology values in juvenile loggerhead sea turtles undergoing rehabilitation. Veterinary Record 164, 663-665.

Çay, M., Naziroğlu, M., 1999. Effects of intraperitoneally-administered vitamin E and selenium on the blood biochemical and haematological parameters in rats. Cell biochemistry and function 17, 143-148.

Cortés-Gómez, A.A., Fuentes-Mascorro, G., Romero, D., 2014. Metals and metalloids in whole blood and tissues of Olive Ridley turtles (L*epidochelys olivacea*) from La Escobilla Beach (Oaxaca, Mexico). Marine Pollution Bulletin 89, 367-375.

Costa, L.G., Vitalone, A., Cole, T.B., Furlong, C.E., 2005. Modulation of paraoxonase (PON1) activity. Biochemical Pharmacology 69, 541-550.

da Silva, C.C., Klein, R.D., Barcarolli, I.F., Bianchini, A., 2016. Metal contamination as a possible etiology of fibropapillomatosis in juvenile female Green Sea turtles *Chelonia mydas* from the southern Atlantic Ocean. Aquatic Toxicology 170, 42-51.

Day, R.D., Segars, A.L., Arendt, M.D., Lee, A.M., Peden-Adams, M.M., 2007. Relationship of blood mercury levels to health parameters in the loggerhead sea turtle (Caretta caretta). Environ Health Perspect 115, 1421-1428.
Fazio, E., Liotta, A., Medica, P., Bruschetta, G., Ferlazzo, A., 2011. Serum and plasma biochemical values of health loggerhead sea turtles (Caretta caretta). Comparative Clinical Pathology 21, 905-909.
Finlayson, K.A., Leusch, F.D., van de Merwe, J.P., 2016. The current state and future directions of marine turtle toxicology research. Environ Int 94, 113-123.
Haagen, L., Brock, A.A., 1992. New Automated Method for Phenotyping Arylesterase (EC 3.1.1.2) Based Upon Inhibition of Enzymatic Hydrolysis of 4-Nitrophenyl Acetate by Phenyl Acetate. . European Journal of Clinical Chemistry and Clinical Biochemistry 30, 391-395.
Hamann, M., Godfrey, M.H., Seminoff, J.A., Arthur, K., Barata, P.C.R., Bjorndal, K.A., Bolten, A.B., Broderick, A.C., Campbell, L.M., Carreras, C., Casale, P., Chaloupka, M., F.Chan, S.K., Coyne, M.S., Crowder, L.B., Diez, C.E., Dutton, P.H., Epperly, S.P., FitzSimmons, N.N., Formia, A., Girondot, M., Hays, G.C., Jiunn, C.I., Kaska, Y., Lewison, R., Mortimer, J.A., Nichols, W.J., Reina, R.D., Shanker, K., Spotila, J.R., Tomás, J., Wallace, B.P., Work, T.M., Zbinden, J., Godley, B.J., 2010. Global research priorities for sea turtles: informing management and conservation in the 21st century. Endangered Species Research 11, 245–269.
Hernandez, A.F., Gil, F., Leno, E., Lopez, O., Rodrigo, L., Pla, A., 2009. Interaction between human serum esterases and environmental metal compounds. Neurotoxicology 30, 628-635.
Innis, C., Ravich, J.B., Tlusty, M.F., Hoge, M.S., Wunn, D.S., Boerner-Neville, L.B., Merigo, C., Weber, E.S.I., 2009. Hematologic and plasma biochemical ndings in cold-stunned Kemp's ridley turtles:
176 cases (2001–2005). Journal of the American Veterinary Medical Association 235, 426-432.
IUCN, 2012. Red List of Threatened Species, Version 2012.1. International Union for Conservation of Nature, http://www.iucnredlist.org/.
Kakizoe, Y., Sakaoka, K., Kakizoe, F., Yoshii, M., Nakamura, H., Kanou, Y., Uchida, I., 2007. Successive changes of hematologic characteristics and plasma chemistry values of juvenile loggerhead turtles (Caretta caretta). Journal of Zoo and Wildlife Medicine 38, 77-84.
Keller, J., McClellan-Green, P., 2004. Effects of organochlorine compounds on cytochrome P450 aromatase activity in an immortal sea turtle cell line. Marine Environmental Research 58, 347-351.
Keller, J.M., Kucklick, J.R., Stamper, M.A., Harms, C.A., McClellan-Green, P.D., 2004. Associations between organochlorine contaminant concentrations and clinical health parameters in loggerhead sea turtles from North Carolina, USA. Environ Health Perspect. 112, 1074-1079.
Keller, J.M., McClellan-Green, P.D., Kucklick, J.R., Keil, D.E., Peden-Adams, M.M., 2006. Effects of organochlorine contaminants on loggerhead sea turtle immunity: Comparison of a correlative field study and in vitro exposure experiments. Environmental Health Perspectives 114, 70-76.

Komoroske, L.M., Lewison, R.L., Seminoff, J.A., Deheyn, D.D., Dutton, P.H., 2011. Pollutants and the health of green sea turtles resident to an urbanized estuary in San Diego, CA. Chemosphere 84, 544-552.
Kowalczyk, E., Kopff, A., Fijatkowski, P., Kopff, M., Niedworok, J., Btaszczyk, J., Kêdziora, J.z., Tycelerowicz, P., 2003. Effect of anthocyanins on selected biochemical parameters in rats exposed to cadmium. Acta Biochimica Polonica 50, 543-548.
Kuzmichevaa, L.V., Lopatnikovaa, E.A., Maksimov, G.V., 2014. Biochemical changes in blood at lead intoxication and pectin correction. Moscow University Biological Science Bulletin 69, 51-56.
Labrada-Martagon, V., Rodriguez, P.A., Mendez-Rodriguez, L.C., Zenteno-Savin, T., 2011. Oxidative stress indicators and chemical contaminants in East Pacific green turtles (*Chelonia mydas*) inhabiting two foraging coastal lagoons in the Baja California peninsula. Comparative Biochemistry and Physiology Part C 154, 65-75.
Laird, B.D., Goncharov, A.B., Ayotte, P., Chan, H.M., 2014. Relationship between the esterase paraoxonase-1 (PON1) and metal concentrations in the whole blood of Inuit in Canada. Chemosphere 120C, 479-485.
Li, W.-F., Pan, M.-H., Chung, M.-C., Ho, C.-K., Chuang, H.-Y., 2006. Lead Exposure Is Associated with Decreased Serum Paraoxonase 1 (PON1) Activity and Genotypes. Environmental Health Perspectives 114, 1233-1236.
Massanyi, P., Stawarz, R., Halo, M., Formicki, G., Lukac, N., Cupka, P., Schwarcz, P., Kovacik, A., Tusimova, E., Kovacik, J., 2014. Blood concentration of copper, cadmium, zinc and lead in horses and its relation to hematological and biochemical parameters. J Environ Sci Health A Tox Hazard Subst Environ Eng 49, 973-979.
Matovic, V., Buha, A., Ethukic-Cosic, D., Bulat, Z., 2015. Insight into the oxidative stress induced by lead and/or cadmium in blood, liver and kidneys. Food Chem Toxicol 78, 130-140.
Nandi, D., Patra, R.C., Swarup, D., 2006. Oxidative stress indices and plasma biochemical parameters during oral exposure to arsenic in rats. Food Chem Toxicol 44, 1579-1584.
Park, E.J., Yoon, J., Choi, K., Yi, J., Park, K., 2009. Induction of chronic inflammation in mice treated with titanium dioxide nanoparticles by intratracheal instillation. Toxicology 260, 37-46.
Qian, Y., Falahatpisheh, M.H., Zheng, Y., Ramos, K.S., Tiffany-Castiglioni, E., 2001. Induction of 78 kD glucose-regulated protein (GRP78) expression and redox-regulated transcription factor activity by lead and mercury in C6 rat glioma cells. Neurotoxicity Research 3, 581-589.
R Core Team, 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
Rhen, T., Cidlowski, J.A., 2005. Antiinflammatory action of glucocorticoids—new mechanisms for old drugs. New England Journal of Medicine 353, 1711-1723.
Santillana Segovia, P.R., 2013. Valores Hematológicos y bioquímicos sanguíneos de tortugas anidantes de Golfina (*Lepidochelys olivacea*) en El Salvador. BIOMA, 11-17.
Santoro, M., Meneses, A., 2007. Haematology and plasma chemistry of breeding olive ridley sea turtles (*Lepidochelys olivacea*). The Veterinary Record 161, 818-819.
Snoddy, J.E., Landon, M., Blanvillain, G., Southwood, A., 2009. Blood biochemistry of sea turtles captured in gillnets in the Lower Cape Fear River, North Carolina, USA. Journal of Wildlife Management 73, 1394-1401.

Tvarijonaviciute, A., Tecles, F., Caldin, M., Tasca, S., Ceron, J.J., 2012. Validation of spectrophotometric assays for serum paraoxonase type-1 measurement in dogs. American Journal of Veterinary Research 73, 34-42.
Wolf, K.N., Harms, C.A., Beasley, J.F., 2008. Evaluation of ve clinical chemistry analyzers for use in health assessment in sea turtles. Journal of the American Veterinary Medical Association 233, 470-475.
Wrobel GoldbergI, D., Wanderlinde, J., Alexandre Freire, I.M., Pereira da Silva, L.C., Pereira Almosny, N.R., 2011. Serum biochemistry profile determination for wild loggerhead sea turtles nesting in Campos dos Goytacazes, Rio de Janeiro, Brazil. Ciência Rural, Santa Maria 41, No. 1, 143-148.

3.5 Developmental Instability Index

CHAPTER VII. Carapace asymmetry: A possible biomarker for metal accumulation in adult Olive Ridleys marine turtles?

3.5.1 Introduction

The impact of anthropogenic pollutants on marine ecosystems is of global concern (Depledge et al., 2013; Duarte, 2014; Ericson et al., 2013). Among the many different kinds of contaminants, some of the most studied are heavy metals and metalloids due to their toxicity. In marine environments, these elements occur naturally at low concentrations; however, anthropogenic activities can increase their concentrations to high levels that may pose a threat to marine ecosystems with deleterious results (Hansen et al., 2016). Regarding heavy metals, Pb and Cd are among the most studied and associated with diverse pathologies in different marine species (Boughammoura et al., 2013; Nunes et al., 2014; Rana, 2014; Sonne et al., 2009).

In addition, other heavy metals such as Ti, Tl, Li, and Sr are a growing concern due to their tendency to induce physiological effects after long-term exposure (Lehmann and Lee, 2013; McPherson et al., 2014; Rodriguez-Mercado and Altamirano-Lozano, 2013; Shi et al., 2013). Meanwhile, metalloids (e.g., Al, As, Co, Cr, Cu, Se) play an essential role in certain metabolic processes. However, when their concentrations accumulate beyond physiological requirements, they become toxic in many species such as fish (Bears et al., 2006), rats (Mahieu et al., 2005), primates (Clauss and Paglia, 2012), and reptiles (Marco et al., 2004; Perrault et al., 2013). For instance, Se, being essential for cell functions, is recommended for daily consumption in humans, but at higher concentrations, it may cause cardiovascular disease, arrest the cell cycle, and even break the DNA structure (Sun et al., 2014).

Developmental stability (DS) is the ability of an individual to withstand perturbations during its development (Băncilă et al., 2012; Beasley et al., 2013; Bishop et al., 1998; Mast and Carr, 1989). DS has been used as an indicator index of individual fitness and is considered an environmental stress biomarker in different species related, among others, to certain pollutants such as organochloride and metals (Băncilă et al., 2012; Beasley et al., 2013; Ho et al., 2009). A common approach for assessing DS is through fluctuating asymmetry (FA) analysis (Beasley et al., 2013; Dongen, 2006; Parsons, 1989; Sanchez-Chardi et al., 2013).

For instance, Eeva et al. (2000) reported increased levels of FA in the tarsus of *Parus major* (passerine birds) populations exposed to heavy metals compared to other sites with less metal exposure. Bishop et al. (1998) also found higher rates of developmental abnormalities in eggs and hatchlings of Common Snapping turtles exposed to a higher concentration of organochlorine and total mercury. In this sense, geometric morphometric tools have also proved to be better and more sensitive tools and detect a larger signal of FA compared to studies that use only linear/meristic measures (Beasley et al., 2013).

In sea turtles, a high frequency of carapace bilateral asymmetry has been described in Olive Ridley (*Lepidochelys olivacea*) on the Indian Ocean (Deraniyagala, 1939), Western Africa (Carr, 1957), British Guiana (Pritchard, 1966), Surinam (Hill, 1971) and on the Pacific coast of Mexico (Frazier, 1983). This quite general pattern for this species (Wyneken, 2001) is remarkable, as developmental instability generally reflects a non-normal development caused by perturbations of the developmental process, while developmental stability reflects the capacity to avoid or reduce such perturbations by developmental means (Moller, 2006). The asymmetric condition of *L olivacea* carapace can be considered fixed at the hatching stage, because costal carapace scutes do not show any significant allometric variance during the different life stages of marine turtles (Mast and Carr, 1989; Wyneken, 2001). In Olive Ridleys, egg manipulation for protection purposes and high incubation temperatures during embryonic development may promote asymmetry in turtles (Ayres-Fernández and Cordero-Rivera, 2004; Davis and Grosse, 2008; Lazic et al., 2013; Mast and Carr, 1989; Navarro Sánchez, 2015). Regarding developmental instability and pollutants, two studies on chelonian have been published (Bishop et al., 1991; Bishop et al., 1998). These works focused on eggs, developing embryos, hatchlings, and the FA of snapping turtles (*Chelydra serpentina serpentina*), with high rates of developmental abnormalities related to higher polychlorinated aromatic hydrocarbon concentrations in eggs.

High metal and metalloid concentrations in Olive Ridley marine turtle (*Lepidochelys olivacea*) populations from the Southern Pacific coast of Mexico have been previously

documented (Cortés-Gómez et al., 2014). In many species, the ability of an individual to escape pollutant threats differs based on behavioral or physiological adaptations. For example, the individual may have some physiological capabilities to be more or less affected by pollutants based on its capacity to concentrate them more or less or to detoxify better (Walker et al., 2005). In wildlife, to assess the effect that pollutants can pose to individuals, is imperative to anticipate and try to settle different biomarkers, either of exposure, effect or sensibility (Aguirre and Lutz, 2004; Labrada-Martagon et al., 2011a). Additionally, there is a demand for species that can serve as bioindicators of pollutants and for nondestructive sampling techniques (Beasley et al., 2013; Burger et al., 2007). As previously mentioned, the influence of pollutants on developmental instability and one of its consequences, the asymmetry of individuals, has been demonstrated in several species, but the reverse link has never been explored: can carapace asymmetry at the adult stage be used as a biomarker of contamination in individuals?

To explore this premise, the aims of this paper were: (1) to establish the concentrations of 15 metals and metalloids in liver, kidney, muscle, brain, bone, blood, and egg components (albumin, yolk and eggshell) from stranded dead Olive Ridley turtles; (2) to develop a method to use carapace morphologies obtained from photographs of *L. olivacea* to quantify the morphological plasticity that could be a marker of developmental instability; and (3) to examine if there is a relationship between metals and metalloids and asymmetry of the carapace of this species, and if this information can be used as a biomarker for these pollutants in this species.

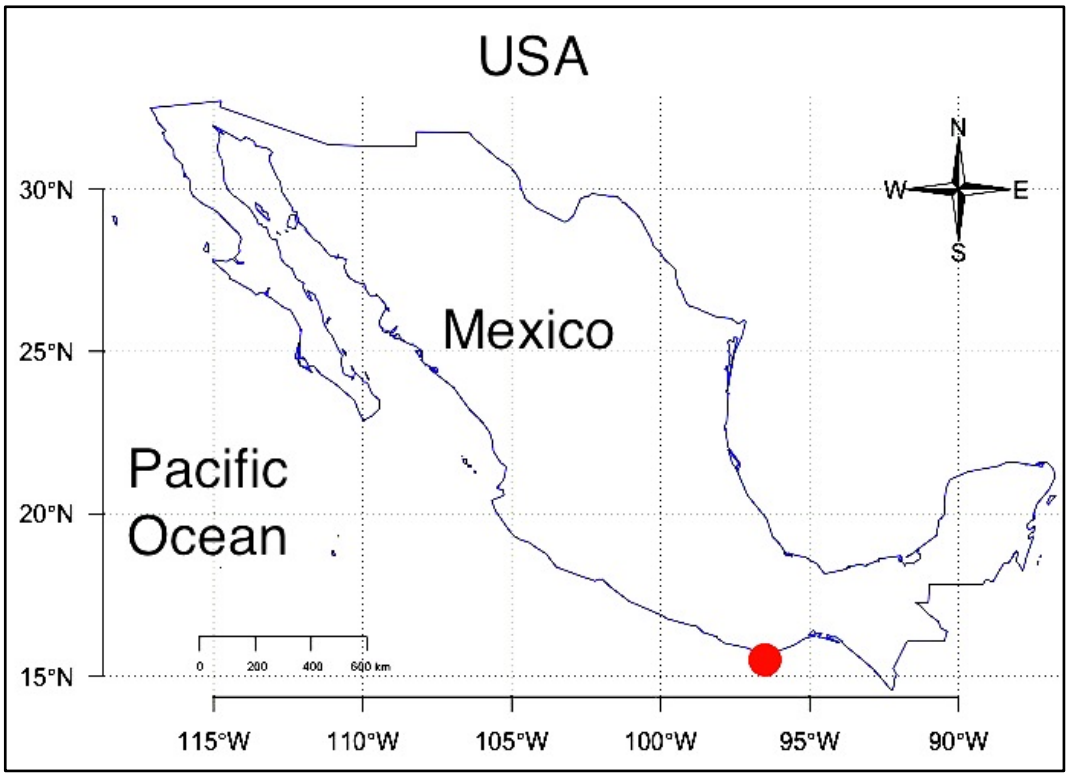
3.5.2. Material and methods

3.5.2.1 Sample collection

La Escobilla beach is located in the state of Oaxaca on the Mexican Pacific coast (Fig. 1). Beach monitoring was performed between August and October 2014, during the third, fourth, and fifth “arribadas”. Seventeen recently dead (less than 12 h after death) turtles were found along the beach during this period (nine died from head trauma; the cause of

death for the remaining eight is unknown). After collecting the carcasses (during the 6 am patrol, using a quad to collect them), each one was photographed, and the curved carapace length and width were measured. Finally, blood samples from the heart, liver, kidney, muscle, brain/spinal cord, bone, and eggs were taken.

Figure 1. La Escobilla beach location (circle).



To make the sampling as accurate as possible, it was always performed it in the same way, by taking the same tissue portion. Blood was always the first sample taken, the heart was carefully opened with a scalpel, and 1.5 mL of blood was taken from the atrium. Then the other tissue samples were collected, usually in the follow order: pectoral muscle, right lobe of liver, and right kidney (a cut from the exterior to the deepest part of the organ), a proximal part of the humerus, and one egg transferred from the oviduct to a plastic bag. Finally, the head was separated from the body to be able to open it and take part of the brain/spinal cord. To avoid contamination, samples were directly transferred into the microtubes after they were taken to avoid contact with any surface. Once in the laboratory,

the three egg compartments (yolk, albumin, and eggshell) were separated and placed into three distinct tubes. Again, to avoid contamination, during the fieldwork, all the materials used to collect samples were new (Eppendorf tubes, blade scalpel, syringes, and bags). All the samples from all the carcasses except for 3 brains and 4 eggs were obtained, because all but three carcasses were fresh (these carcasses were in a more advanced state of decomposition, around 12 hours after death). All samples were kept frozen at -20 °C until processed. Permissions for taking and transporting samples were SGPA/DGVS/07774/14 and CITES MX 72799/ 2014.

3.5.2.2 Metal analysis

Metal concentrations of 15 inorganic elements (Bi, Cd, Li, Pb, Sb, Sr, Ti, Tl, Al, As, Co, Cr, Cu, Ni, and Se) were determined. As there are different classifications for these inorganic elements, in the present work, we classified as heavy metals all non-essential elements for most living organisms (Bi, Cd, Li, Pb, Sb, Sr, Ti, and Ti) and as metalloids all elements that are essential, but in low concentrations, for certain metabolic pathways (Al, As, Co, Cr, Cu, Ni, and Se) (Cortés-Gómez et al., 2014; González Muñoz and Meseguer Soler, 2009). All the samples and laboratory materials were thoroughly rinsed with deionized water (Milli-Q) to avoid contamination. A pre-treatment of the samples was then performed: 0.5 g of each sample (blood, liver, and kidney in wet weight) was taken for an acid digestion using 4 mL of HNO_3 (69%) and 1 mL of H_2O_2 (33%) mixed in special Teflon reaction tubes in a microwave digestion system (UltraClave-Microwave Milestone®) for 20 min at 220 °C and finally diluted with 25 mL of double deionized water (Milli-Q). All the elements were determined using an inductively coupled plasma optical emission spectrophotometer (ICP-OES, ICAP 6500 Duo Thermo®) (Cortés-Gómez et al., 2014; Marin et al., 2011). All concentrations were expressed in micrograms per gram in wet weight (µg g^{-1} ww). Minimum detection limits for all elements were 0.01 µg g^{-1} ww. Two readings were made for each sample. To check for possible contamination, 1 blank sample for every 11 samples was also analyzed in the ICP-OES.

Element calibration standards were prepared with different concentrations of inorganic elements, taking as reference the UNE-EN ISO 11885 for the determination of elements by inductively coupled plasma atomic emission spectroscopy. In addition, intermediate patterns of all elements were prepared. The equipment calibration was established by batch. Calibration was established with a minimum of three points for each lot. Each run began with the calibration standards and continued with samples and intermediate patterns, finishing the series with intermediate patterns (10% variation coefficient). The wavelengths were as follows:

Element	**Bi**	**Cd**	**Li**	**Pb**	**Sb**	**Sr**	**Ti**	**Tl**
λ (nm)	223.061	214.438	670.784	220.353	217.581 206.833	421.552	336.121 334.941	190.856
Element	**Al**	**As**	**Co**	**Cr**	**Cu**	**Ni**	**Se**	
λ (nm)	167.079 396.152	193.759	228.616	205.552	324.754 224.700	231.604	196.090 203.985	

The uncertainty percentages of the elements were as follows: Bi=5.47, Cd=4.56, Li=6.78, Pb=6.14, Sb=7.61, Sr=7.04, Ti=8.12, Tl=7.05, Al=5.38, As=5.56, Co=5.97, Cr=4.3, Cu=4.12, Ni=4.83, Se=6.43. When one element was below the detection limit (BDL) in more than 50% of the samples for one tissue (for instance, Bi in yolk and albumin), it was not used to perform the statistical analysis. When the proportion of BDL was less than 50%, half of the limit of detection (0.005) was used (Helsel, 2011).

3.5.2.3 Photographs and measurements

A standardized procedure was used to take photographs at a vertical height of 1.5 m above the carapace of each turtle before the tissue sampling was performed. Color contrasts were enhanced using Graphic Converter for Mac OSX version 9.5.1 to better visualize the sutures between the scutes. Left and right costal scutes were counted, and each scute was measured from the medial edge along the suture with the vertebral scutes to avoid, at best, the effect of the curvature of the carapace. Measurements of the j scutes on the left ($L_{1:j}$ in cm) and k on the right ($R_{1:k}$ in cm) were then transformed into proportions using $l_i=L_i/\Sigma L$ and

$r_i=R_i/\Sigma R$. The use of proportions rather than actual size allowed us to correct for uncertainty in the total size of the left and right side of the carapace due to perspective distortion when the surface of the camera lens was not perfectly parallel to the ground.

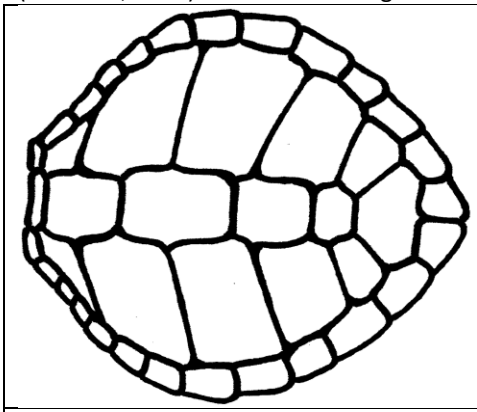
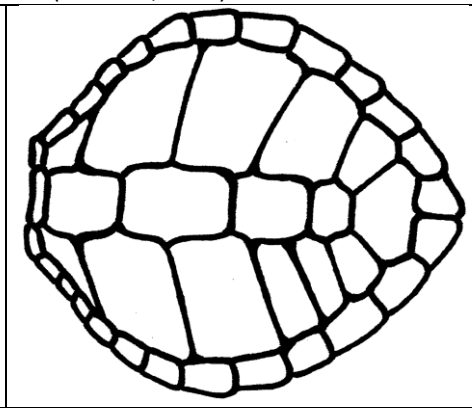
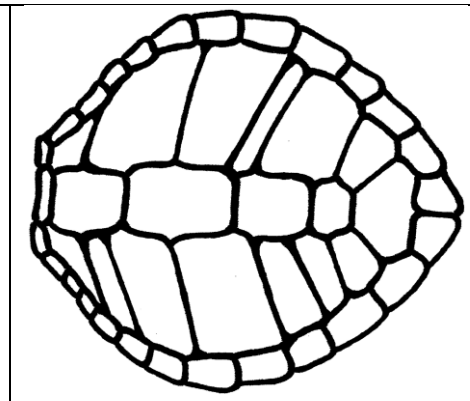
3.5.2.4 Index for the diversity of scutes

Carapace scute asymmetry is apparent not only in the relationship between the number of left and right costal scutes, but also in the sizes and shapes of these scutes. To quantify this condition and facilitate comparisons and analyses, a new index to better describe the scutes' heterogeneity within an individual was developed. Two different measures of shape heterogeneity in a single metric were combined: (i) the diversity of the width of scutes was measured by the geometric averaged Shannon H entropy for each side (Shannon, 1948), and (ii) the difference in coastal scute size between both sides (*i.e.*, asymmetry) was measured using Edwards' angular distance (Edwards, 1971). Both measures were chosen because they are standard measures of diversity and require only relative proportions. They were weighted equally in the final index using the geometric mean, as we had no reason to give more importance to the intra- (using Shannon Index) *versus* inter-side (using Edwards distance) diversity. This integrative index gave the expected relative values when tested in different situations, being higher for (a) greater asymmetry of the scutes or their relative widths, (b) a greater number of scutes on the left or right side, and (c) more diverse widths of scutes on the right or left side (Fig. 2). The R function DIx () developed for this project is available in the package HelpersMG (≥1.7) available in CRAN (https://CRAN.R-project.org/package=HelpersMG). We named it the developmental instability index (DIx).

Asymmetry on L. olivacea carapace based on the number of right and left scutes has been described by Deraniyagala (1939) n=37, Carr (1957) n=31, Pritchard (1966) n=14, Hill (1971) *n*= 250, Frazier (1983) *n*=76. We also add information about 25 live and apparently healthy individuals from the same beach where samples for this work were taken. Finally, we included the observations of Carr (1957) on *Lepidochelys kempii* turtles (*n*=100), the phylogenetically closest species to L. olivacea. Afterwards, comparison between frequency

of asymmetrical individuals in different studies has been performed using selection of two models: one with a series-specific probability of asymmetrical individuals and one with a single probability of asymmetrical individuals for all series. The maximum likelihood for each case has been estimated from binomial distribution and AIC has been estimated. AIC is a measure of quality. y of fit that penalized for too high number of parameters in the model (Akaike, 1974). The probability that all series are similar or not has then been calculated using Akaike weight which give the relative support for both models (Burnham and Anderson, 1998).

Figure 2. Examples of the developmental instability index (DIx) with low, medium and high variations based on the Shannon H. entropy (Shannon, 1948) and Edward's angular distance (Edwards, 1971).

		
Low DIx: 1.3314 Edward's distance: 0.8943 Shannon entropy: 1.488	Medium DIx: 1.417 Edward's distance: 0.9127 Shannon entropy: 1.5529	High DIX: 1.6094 Edward's distance: 0.9257 Shannon entropy: 1.7385

3.5.2.5 Relationship between DIx and pollutants

Fifteen metal and metalloid concentrations in 9 tissues from 17 individuals were collected, any common multivariate method would fail because the information available is lower than the explanatory variables. Thus, a multivariable classification using the random forest method (Breiman, 1999) was performed to simplify the datasets. The principle of random forests is to aggregate many binary decision trees coming from two random perturbation mechanisms: the bootstrap sampling of observations (growing step) and the random choice of a subset of explanatory variables at each node (pruning step). The original random forest method cannot be used when the number of variables is nearly equal to the number of elements used for classification (17 *vs.* 15). A derivative method available in R package

VSURF (Genuer et al., 2015) used variable bootstrapping to build the forest of trees and solved this constraint: first, in the growing step, at each node, a fixed number of input variables are randomly chosen, and the best split is calculated only among them; second, no pruning step is performed, so all the trees of the forest are maximal trees. The order of the variables contributing the most often to the decision trees was used as a guide for further analysis. The 10 most important elements (heavy metals or metalloids) were retained to perform a generalized linear model (GLM). This method allowed us to avoid any arbitrary element selection and make a more stable GLM selection afterwards.

Tissue	Elements
Liver	Al+Sr+Pb+Ni+Cd+Se+Li+Ti+Tl+Bi
Kidney	Cd+Li+Tl+Ni+Sb+Se+Co+Bi+Ti+Cu
Muscle	Li+Ni+Pb+As+Tl+Al+Se+Cu+Sr+Cr
Brain	Se+Li+Tl+Al+As+Sb+Sr+Cd+Pb
Bone	Al+Sb+Ni+Cu+Ti+Se+Pb+Tl+Cd+Cr
Blood	Ni+Li+Pb+Ti+Al+As+Co+Cu+Sr
Yolk	Cd+ Li+ Pb+ Cr+ Ti+ Tl+ Al+ As+ Cu+ Se
Albumin	Pb+Ti+Cd+Al+Li+As+Sr+Se+Ti+Cu
Eggshell	Bi+Cd+Li+Sr+Tl+Al+Cr+Cu+Ni+Se

Table 1. Random forest selection by tissue

The relationship between DIx and the selected metals and metalloids for each tissue was assessed using a Gaussian distribution and an identity link. The selection of the variable linked to DIx was performed using the Akaike information criterion (AIC). AIC is a measure of quality of fit of a model penalized by the number of variables in the model (Burnham and Anderson, 2002). All statistical analyses were performed using R 3.3.0 (R Core Team, 2016).

3.5.3 Results

All the metal and metalloid concentrations in yolk, albumin, eggshell, liver, kidney, muscle, brain, bone, and blood taken from the 17 individuals are summarizes in the Table 2. Elements with more than 50% of results below the detection limit were considered below the limit results, and were not used for statistical analysis. Regarding heavy metals, Cd, Li, Pb, and Sr were detected in all tissues and egg components (yolk, albumin, and eggshell), while Ti and Tl were detected in all but eggshell and blood, respectively. Sb was under the detection limit in the three egg components, liver, and blood, while Bi was only detected in eggshell, liver, kidney, and bone. Among the remaining elements, Al, Cr, Cu, and Se were detected in all tissues; As was detected in all tissues except eggshell, and Ni in all tissues except yolk and albumin; Co was only present in liver, kidney, and blood samples.

Table 2. Metal and metalloid concentrations. Mean (standard deviation) in different tissues of *Lepidochelys olivacea*. Values are expressed in μg g^{-1} wet weight. BDL = Below the detection limit.

	Yolk	Albumin	Eggshell	Liver	Kidney	Muscle	Brain	Bone	Blood
Heavy metals									
Bi	BDL	BDL	5.19(1.0)	0.99(0.9)	0.62(0.3)	BDL	BDL	4.01(1.6)	BDL
Cd	0.18(0.09)	0.02(0.05)	0.43(0.7)	74(46)	118(83)	0.75(0.3)	0.87(1.6)	0.79(0.3)	7.04(20)
Li	0.03(0.02)	0.04(0.02)	3.75(0.8)	0.02(0.02)	0.01(0.1)	0.02(0.04)	0.03(0.06)	2.72(1.2)	0.04(0.1)
Pb	0.03(0.03)	0.01(0.0)	BDL	0.17(0.1)	0.04(0.03)	0.04(0.03)	0.13(0.1)	0.89(0.7)	0.08(0.1)
Sb	BDL	BDL	BDL	BDL	0.42(0.3)	0.05(0.02)	0.11(0.08)	0.35(0.25)	BDL
Sr	27.1(10)	3.47(4.3)	453(115)	0.7	0.77(0.4)	0.79(0.7)	4.36(6.3)	994(367)	1.27(1.8)
Ti	0.03(0.02)	0.02(0.01)	BDL	0.56(1.0)	1.23(1.1)	2.18(3.1)	9.18(11.8)	9.67(7.35)	7.75(24)
Tl	1.92(0.5)	0.13(0.4)	6.18(1.2)	5.74(5.8)	0.04(0.1)	0.18(0.2)	0.24(0.5)	18.7(6.47)	BDL
Other elements									
Al	0.66(0.2)	0.10(0.1)	9.29(3.8)	3.6(3.4)	7.2(7.9)	12.9(19)	100(123)	87(94)	3.51(5.7)
As	0.17(0.07)	0.14(0.1)	BDL	12.9(10)	1.22(1.3)	3.79(1.8)	1.53(4.4)	0.66(0.5)	0.92(0.6)
Co	BDL	BDL	BDL	0.21(0.16)	0.07(0.06)	BDL	BDL	BDL	0.03(0.05)
Cr	0.11(0.1)	0.03(0.02)	0.20(0.1)	1.01(0.8)	0.09(0.03)	0.17(0.1)	1.71(4.7)	1.88(2.1)	0.54(0.91)
Cu	1.26(0.4)	0.24(0.2)	8.61(2.3)	13.7(8.3)	1.38(0.6)	0.83(0.2)	3.46(9.7)	1.58(4.0)	1.00(0.9)
Ni	BDL	BDL	0.02(0.0)	0.05(0.04)	0.02(0.01)	0.06(0.1)	0.19(0.2)	0.20(0.1)	0.06(0.06)
Se	2.83(1.1)	0.81(0.5)	6.66(3.3)	9.19(4.7)	1.63(0.5)	7.76(3.1)	7.27(17)	1.29(0.4)	7.15(4.5)

In Table 3, all relative costal scute measurements and DIx are summarized. Comparing the DIx results to our example/control in Figure 2.1 (low DIx), which has an almost perfectly

symmetric pattern for its costal scutes, we find that this DIx value from the example is lower than all the real individuals shown in Table 3. This result shows us that this almost perfectly symmetric carapace is not very often found in real-life Olive Ridleys. It is also a good statement about the classification of our DIx logarithm.

Table 4 presents the elements with significant results after GLM and AIC selection. We observe that most heavy metals had positive relationships with DIx in tissues (except for Pb) and mostly negative in the three egg components (except for Ti and Cd in albumin). Unlike heavy metals, metalloids did not show any clear tendency in their different significant relationships. However, most of these relationships are consistent with the organ where these elements tend to accumulate in animal organisms after chronic exposure to both heavy metals and metalloids.

The results of a previous monitoring study (samples taken in 2012) are shown with some of the same elements and tissues used in the present study (samples taken in 2014) in Table 5. We observe that in blood, all the element concentrations increase from 2012 to 2014 (except for As). In liver, three elements had a higher concentration (Pb, As, and Se) and three a lower concentration (Cd, Cu, and Ni) when comparing 2012 and 2014 samples. In addition, kidney showed a decrease in six element concentrations over the two-year period

Table 3. Measures of the relative carapace costal scutes, and the developmental instability index (DIx) for each turtle. CCL = curved carapace length

ID	Relative CCL	Relative right costal scutes	Relative left costal scutes	DIx	ID	Relative CCL	Relative right costal scutes	Relative left costal scutes	DIx
1	29.13	3.03 3.95 7.19 4.9 2.22 1.79	2.85 4.33 6.8 5.25 1.94 1.34 2.29	1.6181	**9**	28.9	1.9 4.3 7.12 5.15 4.2	3.1 5.32 6.35 6.85 3.0	1.3817
2	24.97	2.71 4.69 8.07 5.96 2.01 2.08	2.46 4.33 8.39 6.03 1.94 2.25 1.97	1.5930	**10**	25.3	1.83 3.42 5.68 4.5 1.69 1.76	1.51 3.49 6.13 4.23 1.62 1.55	1.5145
3	26.07	4.19 6.63 6.17 4.41 2.68 1.65	2.82 5.82 3.38 3.59 5.3 2.08 2.32	1.6558	**11**	25.71	2.75 3.7 6.52 5.22 2.0 1.58 1.72	2.43 4.05 6.56 5.36 2.5 1.44	1.6141
4	23.56	3.28 4.16 4.97 2.71 1.34	3.47 4.33 5.08 2.78 1.25	1.3667	**12**	31.11	2.43 4.45 3.5 4.48 5.46 3.31	2.78 4.41 3.73 4.48 4.12 2.92 2.36	1.6800
5	23.84	2.85 4.83 7.12 2.61 2.68 4.19	2.85 5.04 7.37 2.92 2.5 1.55 1.58	1.6271	**13**	30.2	2.57 4.23 7.3 5.32 1.55 2.68	2.43 4.3 7.37 5.92 3.28	1.1463
6	26.5	2.3 4.6 6 4.8 2.1 2.0	1.5 1.2 3.8 6.3 4.8 1.3 2.5 2.0	1.6853	**14**	32.45	4.37 5.11 7.47 5.68 1.87 1.94	4.19 4.69 7.65 5.92 3.91 1.83	1.5510
7	25.7	3 3.9 5.8 4.5 1.9 1.4	2.5 3.5 5.75 2.4 2.25 1.6 1.7	1.6364	**15**	30.75	2.32 5.15 6.35 6.03 2.01	2.32 3.09 7.4 5.75 2.29	1.3438
8	27	2.4 3.9 6.3 4.8 1.95 1.5	2.3 3.5 6.5 2.5 2.7 1.87 1.5	1.6198	**16**	32.56	2.46 4.44 7.76 6.1 2.15 1.83 3.17	2.43 4.83 8.8 3.24 3.73 2.15 2.25	1.6810
					17	32.2	2.78 4.41 7.97 5.92 3.59 2.43	2.85 4.62 4.23 3.35 3.24 4.9 3.17	1.6781

Table 4. Generalized linear model/Akaike information criterion results. Elements with significant relationships with the developmental instability index (DIx) by tissue from *L. olivacea*.

	Elements with positive relationship		**Elements with negative relationship**	
Tissue	Heavy metal	Metalloids	Heavy metal	Metalloids
Liver	Bi	Al		Cr
Kidney	Cd			Cu
Muscle	Sr			Se
Brain	Li	As		Al
Bone	Tl	Cr	Pb	
Blood		Co		Cu, Al
Yolk		Cr, As	Li, Cd	Se
Albumin	Ti, Cd	Se	Sr, Pb	
Shell		Cu	Cd	Al, Se

Table 5. Comparison between samples taken in 2012 (Cortés-Gómez et al., 2014) and the current study (samples taken in 2014) in turtles from the same nest population in blood, liver and kidney. Mean (SD), all results are expressed in μg g^{-1} wet weight.

Element	**Blood**		**Liver**		**Kidney**	
	2012	**2014**	**2012**	**2014**	**2012**	**2014**
Pb	0.02 (0.1)	0.08 (0.1)	0.11 (0.08)	0.17 (0.1)	0.06 (0.03)	0.04 (0.03)
Cd	0.17 (0.09)	7.04 (20)	82.8 (36)	74.4 (46)	150 (110)	118 (83)
As	1.16 (0.69)	0.92 (0.6)	3.34 (1.5)	12.9 (10)	1.38 (0.85)	1.22 (1.3)
Cu	0.61 (0.12)	1.00 (0.9)	16.37 (10.3)	13.7 (8.3)	1.41 (0.69)	1.38 (0.6)
Ni	0.03 (0.02)	0.06 (0.06)	0.08 (0.06)	0.05 (0.04)	0.07 (0.04)	0.02 (0.01)
Se	5.73 (2.50)	7.15 (4.5)	8.24 (2.47)	9.19 (4.7)	1.66 (0.78)	1.63 (0.5)

3.5.4 Discussion

Biomarkers have been defined as functional measures of exposure to various stressors such as pollutants (Beasley et al., 2013). Developmental instability through FA has become a popular method for measuring phenotypic response to environmental stress (Beasley et al., 2013; Leamy and Klingenberg, 2005). The strongest aspect of FA as a potential biomarker in wildlife is that it is a non-lethal tool. It has also been associated with different biological systems, stressors, and life history traits and fitness (Lens and Eggermont, 2008; Lens et al., 2002). Furthermore, compared with other biomarkers, FA is relatively easy to obtain using

measurements or photographs (Beasley et al., 2013; Buică and Cogălniceanu, 2013; Davis and Grosse, 2008; Dongen, 2006).

A common problem when working with wild animals is the access to fresh carcasses. For this reason, it is important to obtain as much information as possible when different samples are available. Thanks to the new techniques for assessing pollutants such as the ICP-OES (Marin et al., 2011), we can measure a large quantity of elements with only a small sample. However, a very large quantity of information from only a few individuals confronts us with a new statistical problem: too few variables to explain, or dependent variables (e.g., individual asymmetry), *versus* too many explicative variables, or independent variables (e.g., inorganic elements from many tissues), which can lead to false positive results and the impossibility of performing model selection due to the highest number of independent variables as compared to dependent ones.

Until now, to our knowledge in the ecotoxicology field, no adequate solution for these statistical problems has been proposed. Each element and tissue can be analyzed separately, but this approach is not satisfactory because the number of tests can be very large, with many false positives being obtained. Some elements in some tissues can be chosen based on prior information from other studies, but this risk failing to detect some important and significant relationships if one particular element or tissue was not included in the analyzed set of data. To solve this problem, we used a random forest, which is a classification algorithm. Recent advances in the use of the random forest statistical technique use bootstrapping to reduce the number of co-variables for one particular tree. With this model, we can improve a biomarker (DIx) to understand the possible links between these pollutants and their effects.

3.5.4.1 Developmental instability index (DIx)

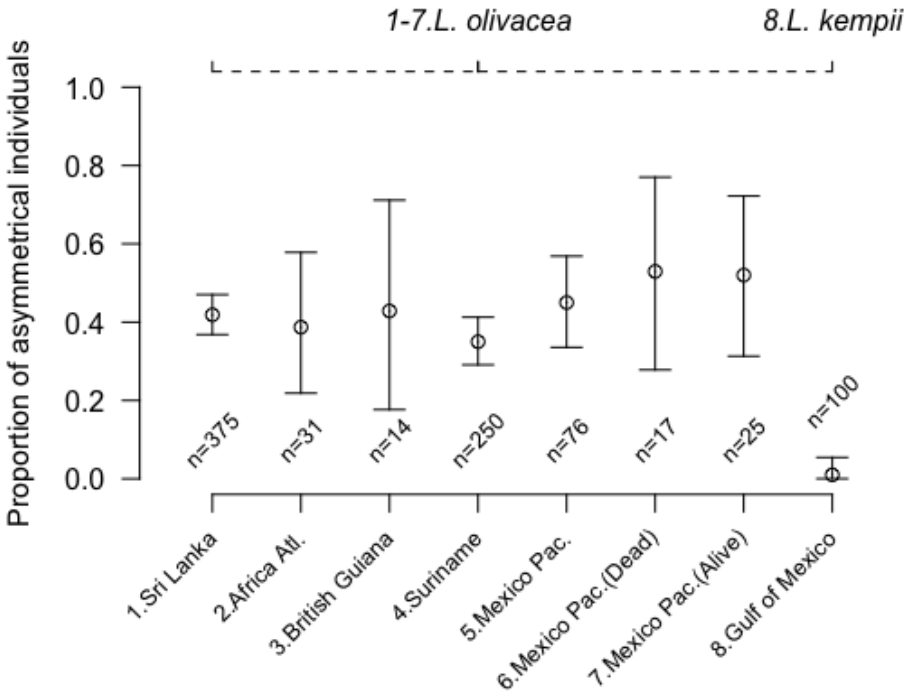
Developmental instability (DI) as a biomarker has attracted the attention of ecologists due to the relative ease of identifying the optimal levels of perfect symmetry (Allenbach, 2010;

Tarlow and Blumstein, 2007). However, this DI is more difficult to determine in wildlife than in laboratory experiments, as these wild populations are exposed to many different stresses with unknown effects on the study species (Beasley et al., 2013). Thus, the interpretation of this DI should be carefully considered within the current understanding of the population's life history and the study species' adaptive potential. Nevertheless, DI has been positively linked to high heavy metal exposure in different wildlife species, usually determined through fluctuating/bilateral asymmetry (FA). For instance, FA in the wild brown trout (*Salmo trutta fario*) has been positively related to a high heavy metal pollution in a protected area with a heavy mining activity (Monna et al., 2011).

Regarding FA, to our knowledge, there is not many or recent works about the number of scutes on Ridley's carapace despite it's well know its scutes asymmetry (Wyneken, 2001). We just found 5 works about left and right scutes number in adults *L. olivacea*: the first work on this subject was done with turtles from Ceylon (now Sri Lanka) by Deraniyagala (1939) with a n=375; followed by Carr (1957) in Western Africa (n=31), Pritchard (1966) in British Guiana (now Guyana) (n=14), Hill (1971) worked with in Surinam (Atlantic, *n*= 250), Frazier (1983) with turtles from the same population as the present study (La Escobilla beach) in the Mexican Pacific (*n*= 76). To complete this comparison we add information about 25 live and apparently healthy individuals from "La Escobilla" beach, and also the observations of Carr (1957) on *Lepidochelys kempii* turtles from the Gulf of Mexico (*n*=100). A similar 95% confidence interval for the 7 populations of *L. olivacea* were overlapping (figure 3), even if some populations were from different oceans (for comparative purposes, we used only adult females in all possible cases). The proportion in asymmetrical individuals for *L. olivacea* was similar in all the studies (Akaike weight, p=0.93). *A contrario*, it falls to be 10^{-18} when *L. olivace*a was compared to *L. kempii* which was expected based in the previous knowledge on this species (Wyneken, 2001). Our study offers new and interesting data about this subject, but more studies through the time and with more individuals are needed to determine if there may be a carapace asymmetrical differences between populations and through the time and if this may have any influence in these turtle's fitness, as is the case

of some fish, birds or insects in different FA cases (Beasley et al., 2013). Or if may be, as in some bird cases, asymmetrical individuals may be less attractive and therefore be less likely to mate (Dongen, 2006; Tarlow and Blumstein, 2007). Or if this situation has no effect at all in Olive Ridley marine turtles.

Figure 3. Confidence interval (CI) of asymmetrical individuals from 7 different studies in *L. olivacea* compared with one in *L. kempii*. The works are represented in the follow order: 1)Deraniyagala, 1939; 2)Carr, 1957; 3)Pritchard, 1966; 4)Hill, 1971; 5)Frazier, 1983; 6)This study, 2014; 7)Unpublished data from the same beach than this study, 2016 and 8) Carr, 1957.



3.5.4.2 Metal and metalloid concentrations

There are several works about the concentration of metals and metalloids in different tissues and species of marine turtles (Abdallah and Abd-Allah, 2011; Adel et al., 2015; Barbieri, 2009; Burger et al., 2010; Caurant et al., 1999; Cortés-Gómez et al., 2014; da Silva et al., 2014; Faust et al., 2014a; Gardner et al., 2006; Ikonomopoulou et al., 2011). However, most of these works focus on bioaccumulation by biomonitoring, while only a few examine the effect/relationship of some of these elements on the turtle's health (Aguirre et al., 1994b; Andreani et al., 2008b; da Silva et al., 2016a; Labrada-Martagon et al., 2011a).

Furthermore, some of the elements analyzed in our paper are not usually studied, not even in reptiles or wildlife in general (Bi, Sb, Sr, Ti, and Tl). Thus, throughout this discussion section, it was often impossible to compare our results with other species and hypothesize about the effects of certain elements, found in high concentrations, on the turtles' health.

Regarding accumulation, some metals and metalloids presented in this paper (Pb, Cd, As, Cu, Ni, and Se) were assessed in a previous ecotoxicological approach on this turtle population using blood from live turtles and kidney and liver from dead individuals (Cortés-Gómez et al., 2014). Compared with the previous liver and kidney analysis (Table 5), we can observe that the concentrations of some elements in kidney were very similar to those reported in our current study, including Cd, an element with the highest kidney concentration ever reported worldwide in this organ (150 and 118 μg g^{-1} ww, respectively).

In liver, most of the elements were also constant over the two years separating the studies, except for Pb and As: the first was 1.5 times higher and the second almost 4 times higher in our study (Table 5). In the two years following our first biomonitoring publication (Cortés-Gómez et al., 2014), an increase in Pb and As concentrations was observed in different tissues (unpublished data). We think that this rise in measured Pb could be related to several oil spills (Duyck et al., 2007; Osuji and Onojake, 2004) that occurred in the habitat of this population during the intervening years. For instance, in 2012 and 2014, one of the most important oil refineries in Salina Cruz, Oaxaca, had two major spills in the area, with a direct impact on Ridleys from Oaxaca (Briseño and Rodríguez, 2014; Moreno, 2012). Regarding As, this element is widely distributed throughout all oceans (ATSDR, 2007a) and tends to bioaccumulate, especially in carnivorous species; in turtles, for instance, Loggerheads (Kunito et al., 2008; Saeki et al., 2000) and Olive Ridleys (Cortés-Gómez et al., 2014) were reported to have greater concentrations than herbivorous turtles (Fujihara et al., 2003; Saeki et al., 2000). This increase in As concentration in this turtle population could be related to different prey availability in recent years. For example, it is known that jellyfish

have high As concentrations (Kunito et al., 2008), and a rise in jellyfish has been reported in all oceans due to warmer temperatures (Brotz et al., 2012; Gibbons and Richardson, 2013).

Blood was the tissue showing the largest variations between 2012 and 2014 samples. We observed that Cu, Se, and Ni showed around 50% higher concentrations in the current study (Table 5). Pb varied only slightly over this two-year period: 0.019 µg g^{-1} ww in 2012 and 0.02 µg g^{-1} ww in 2014. However, Cd concentrations were significantly higher in the current study (7.04±20.6 µg g^{-1} ww, with a range of 0.08-81.53 µg g^{-1} ww) compared with the former (0.14±0.09 g^{-1} ww, with a range of 0.06-0.41 µg g^{-1} ww). Here, it is important to mention that blood from the first study was from live turtles, while blood from the present study was from dead turtles. This is a very interesting result, although we did not find references about lethal Cd concentration in sea turtles. Further studies are thus needed to evidence if Cd concentrations in blood are higher in dead turtles as compared to live and apparently healthy turtles, and then to verify if Cd contributes to the turtle's death. Meanwhile, As was the only element in blood that had slightly lower concentrations in dead turtles compared with live turtle samples from two years prior (1.19±0.92 and 0.92±0.6 µg g^{-1} ww, respectively). This different As tendency in liver and blood may suggest a possible chronic exposure to this element with a recent decrease in exposure, or a higher As concentration in the feeding area of these turtles than around the reproductive beach area.

3.5.4.3 DIx and metals

There are few studies on the relationship between asymmetry and pollutants in vertebrates, such as shrew bones and heavy metals (Sanchez-Chardi et al., 2013), and fish and diverse stressors (Allenbach, 2010). These experimental studies identified some positive relationships between pollutant concentrations and fluctuating asymmetry. In marine turtles, it was found that asymmetry is a characteristic developed at the embryo stage with different causes (Mast and Carr, 1989; Navarro Sánchez, 2015). With facial scales in *Chelonia* mydas, there seems to be little change in asymmetry during the individual's development in adult life (Carpentier et al., 2016). Hence, asymmetry recorded in adult

turtles probably cannot be attributed to the effects of pollutants during this life stage. Through DIx, it was feasible to determine if the load of pollutants was higher in asymmetrical adult individuals than in individuals with less asymmetry in their carapace. If this were the case, asymmetry in marine turtles would be a characteristic effect of counter-selection, being related, at least partly, to contamination, of course, if higher contamination levels had an adverse fitness effect.

3.5.4.3.1 Tissues

Cadmium does not perform any known role in vertebrate organisms and is only poorly excreted, resulting in long-term storage, especially in kidney (Andreani et al., 2008b; Cortés-Gómez et al., 2014; D'Ilio et al., 2011; Frias-Espericueta et al., 2006; Storelli et al., 2008). As mentioned previously, these turtles had very high Cd concentrations in kidney (118 μg g^{-1} ww). In previous experimental studies on asymmetry, Cd was associated with bone limb asymmetry, other bone mineralization, and deformities in experimental animals such as rats, guinea pigs, fish, chicks, *inter alia* (ATSDR, 2012; Boughammoura et al., 2013; Levin, 2005; Rana, 2014). We have no explanation for the increase in renal Cd concentration in these turtles, but as far as we can see, there is a relationship between both results, and so we could consider that more asymmetric turtles could have greater problems with Cd detoxification and/or excretion mechanisms than less asymmetric ones.

Regarding less studied elements like Bi, there is a substantial lack of information on the impact of this metal on marine fauna, and it has never been studied in marine turtles. In humans, 0.1 μg g^{-1} ww in blood has been proposed as an alarm level from a toxicological point of view (Lall and Lewis-McCrea, 2007), and it has been related to disturbances in bone mineralization, hepatitis, and renal disorders in humans and birds (Jayasinghe et al., 2004; Lall and Lewis-McCrea, 2007). In this study, Bi was detected in bone (4.01±1.6 μg g^{-1}), liver (0.99±0.9 μg g^{-1}), and kidney (0.62±0.3 μg g^{-1}), with a positive relationship with DIx in liver. We did not find any other study of this element in turtles or other reptiles to compare our

results, so we cannot evaluate if this is a high or normal concentration, although we observed that concentrations are much higher than the human alarm level.

In this study, Sr was the metal with the highest concentrations in bone and eggshell (994±367 and 453±115 µg g^{-1}, respectively). According to different animal studies, Sr can substitute for Ca in many physiological processes, and it may accumulate in bone and transfer to eggs in place of Ca (Budis et al., 2013; Pors Nielsen, 2004; Yalçin-Özdilek et al., 2011). However, there is no comparative publication about this element in turtle bone. In other turtle species, Rheingold and Hues (1983) reported lower Sr concentrations in the bone of herbivorous (400-500 µg g^{-1} ww), omnivorous (150-400 µg g^{-1}), and carnivorous turtles (100-300 µg g^{-1}) than those reported in our Ridley turtles. At high and chronic exposure levels and due to Sr-Ca interactions, Lall and Lewis-McCrea (2007) suggest that this element might have impairment effects on calcified tissue. Regarding muscle, Faust et al. (2014a) conducted the only study on Sr concentration in this tissue in turtles, reporting a concentration of 0.33±0.03 µg g^{-1} ww (Green turtles). In our study, we found 0.79±0.7 µg g^{-1} ww. Polak-Juszczak (2011) mentions that high Sr concentrations in muscle in Baltic cod can slow the process of incorporating macro- and microelements into this tissue, driving changes in the normal physiological functions of the organism, leading to muscle cramps, limited movement, and even paralysis. Currently, we cannot say whether the observed concentration of Sr in muscle is high, but we did find a positive relationship between this tissue and DIx; it would thus appear that asymmetry in adult turtles could be related to a failure of the Sr detoxification mechanism or to Sr storage. It is therefore very important to establish normal levels of Sr concentration in bone, muscle, and other tissues for turtles, and thus, to be able to know if we have recorded high Sr accumulation.

Regarding Li, we found a positive relationship between this element and DIx in brain samples. There are many experimental works about Li in laboratory animals (Khandwala and Van Uum, 2006; Lehmann and Lee, 2013; O'Donnell and Gould, 2007), although we did not find information about the concentration of this element in brain that could allow a

comparison. In our study, brain Li concentration was low (0.03µg g^{-1} ww), but we do not know if this is indeed low, and by consequence, a harmless concentration for these turtles. Regardless, this element also has an important effect in vertebrate brain, even at low doses (Berridge et al., 1989; O'Donnell and Gould, 2007; Pigatto et al., 2016). It has also been identified as having an interaction and effects on Ca regulation (Khandwala and Van Uum, 2006; Lehmann and Lee, 2013), and therefore, deleterious effects on the skeletal tissue metabolism of vertebrates (Lall and Lewis-McCrea, 2007).

Thallium is accumulated in bone in different species (e.g., hens, dogs, rats, mice, humans, ducks), and kidney usually showed the second highest concentrations and liver the lowest (Cwynar et al., 2014). In our study, kidney had a very low concentration (0.04 µg g^{-1}), while liver and bone had the highest concentrations (5.74 and 16.7 µg g^{-1} ww, respectively). Tl is considered as one of the most toxic heavy metals, known to act as a disruptor on Ca homeostasis. It has also been reported that bird eggs with high Tl concentrations may produce hatchlings with skeletal deformities and alteration in cartilages, among other skeletal problems (Bannon, 2015). DIx had a positive relationship with Tl in bone. We could not find any study on this element and asymmetry, although the Tl concentration in eggs, bone, and liver in these turtles was high compared with the hen experimental work conducted by Cwynar et al. (2014), who fed four groups of hens with different concentrations of Tl (group I: standard feed; II: addition of 2.6 mg kg^{-1}; III: 8 mg kg^{-1}; IV: 16 mg Tl kg^{-1}). Eggs from group I had 0.12 µg g^{-1} and those from group IV 3.29 µg g^{-1} (our egg samples had 1.31 µg g^{-1} in the whole egg), liver from group IV had 1.45 µg g^{-1} (our liver samples had 5.74 µg g^{-1}), and bone from group IV had 9.49 µg g^{-1} (our bone samples had 18.7 µg g^{-1}).

Bone also presented the only negative relationship between DIx and heavy metals with Pb. This element has been found to affect many important physiological functions, such as endocrine alterations involving calcitropic hormones' homeostasis, thus increasing the risk of skeletal disorders (Ibrahim et al., 2012; Rana, 2014). The highest Pb concentration found

in this study was in bone (0.89±0.7 μg g^{-1} ww), where Pb tends to accumulate and be sent back into circulation during the reproductive process and in old individuals, be it in humans and most mammals (ATSDR, 2007b), and in reptiles (Grillitsch and Schiesari, 2010). The positive relationship observed in the present study was perhaps influenced by the fact that our turtles were in the reproductive stage, a stage when Pb tends to mobilize. Compared with other sea turtle species and populations, this concentration is low (García-Fernández et al., 2009; Jerez et al., 2010b; Sakai et al., 2000). In an experiment on carapaces (based on shell strength through biomechanical modeling) from three different species of freshwater turtles, Rivera and Stayton (2013) found that while stress levels increased with increasing asymmetry, variations in stress also increased with increasing asymmetry.

In light of the above discussion and the relationships identified, we suggest that Ridley turtles with a higher degree of asymmetric carapace costal scutes tend to present more heavy metals concentration in different tissues (except for Pb) than individuals with a lower degree of asymmetry (Cortés-Gómez et al., 2014; Páez-Osúna et al., 2010a, b; Zavala-Norzagaray et al., 2014). It would also be very important to determine whether these high metal concentrations are a threat to these turtles' health or whether Ridleys exhibit greater resistance to heavy metals than another species. This is very likely, at least for some elements, since Cd concentrations found in this population are very high compared with other species (Bilandzic et al., 2012; Burger et al., 2010; Cortés-Gómez et al., 2014; da Silva et al., 2014; Frodello and Marchand, 2001; Rana, 2014). As we have said, most of the heavy metals with positive relationships with DIx have interactions with calcium/skeletal metabolisms. More studies are needed to clarify whether the metals with positive relationships with DIx also play an important role in causing asymmetry in this species during embryonic development.

3.5.4.3.2 Eggs

Heavy metals did not have a clear tendency to accumulate in eggs (Tables 4 and 6), thus differing from adult tissues. Indeed, most egg components had negative relationships with

DIx (Li, Cd, Sr, and Pb). Here, it is important to mention that Cd had significant relationships with the three parts of the egg, with a positive relation in albumin and negative relations in yolk and shell. As previously mentioned, this turtle nesting population from *La Escobilla* contains very high Cd concentrations in most tissues compared with other sea turtle species worldwide (Cortés-Gómez et al., 2014), and Ti, Li, and Sr are elements that are not frequently studied. This work provides evidence about their presence in most tissues of the turtles as well as their possible maternal transference, especially in more asymmetric females (since eggs were taken from the same dead turtles as the tissues). Even if lipids are not usually an important depot for metals (Vos et al., 2003), some authors have found a positive relationships between blood of nesting female turtles and yolk of their eggs, such as Pb, Cd and Hg (Aguirre et al., 2006; Godley et al., 1999b; Páez-Osúna et al., 2010a; Sakai et al., 2000). According to these results, more symmetric turtles seem being excreting metals like Li, Sr, Pb and Ca through eggs, better than more asymmetric individuals, especially through yolk and albumin (negative relationships with DIx). This could be explained by the detoxification role that eggs play in some turtle species (Ehsanpour et al., 2014; Guirlet et al., 2008; Nagle et al., 2001).

3.5.4.4 DIx and metalloids

3.5.4.4.1 Tissues

Metalloids did not show any tendency in the relationships for asymmetric carapace costal scutes. However, Al had very high concentrations in brain and bone (100±123 and 87.5±94 μg g^{-1} ww, respectively) as well as three significant relationships with DIx (positive with liver and negative with brain and blood). In many species, the digestive tract is an effective barrier to avoid Al absorption, and in a normal organism, the small amount absorbed is soon excreted in urine (Drüeke, 2002). However, when exposure is very high or when there is kidney dysfunction, Al begins to accumulate in various tissues such as bone, brain, liver, and kidney (Drüeke, 2002; Mahor and Ali, 2015; Scheuhammer, 1987). Al toxicity has never been studied in reptiles, but in other vertebrate species, it has proved to be highly neurotoxic and even causes some bone pathologies (Mahor and Ali, 2015). In

humans, it has been established that Al concentrations in brain tissue should be less than 2 μg g^{-1} to avoid any pathology (Verstraeten et al., 2008). We observed a clear accumulation of this element in many tissues of the turtles. This could be connected with the high Cd concentration in kidney, with the associated renal problems causing ineffective Al detoxification (an effect previously reported by Malluche (2002)), thus leading to Al accumulation in brain and bone tissue.

Finally, As is a very important element due to its highly toxic effect in many species. In humans and some experimental animals, in high and acute doses, this element tends to accumulate in liver>kidney>muscle and brain (ATSDR, 2007a). Accumulation of As depends on many factors, but marine species (e.g., jellyfish, cephalopods, some fish) are known to have a high concentration of this element (Bears et al., 2006; Fujihara et al., 2003; Williams et al., 2006). Accumulation also depends on the organism type, trophic status (carnivorous species like turtles and fish tend to have higher concentrations than herbivorous ones), and exposure concentrations, among others (Kunito et al., 2008; Williams et al., 2006). In human newborns and rats, it has been demonstrated that even if brain tissue tends to accumulate less As than liver, isotopic dimethylarsinic acid As accumulates in brain and is highly toxic (ATSDR, 2007a; Kenyon and Hughes, 2001). We found a positive significant relationship with DIx in brain.

Even if metalloids did not show any pattern in their relationships with DIx, they did have several significant relationships. With the information obtained so far, we cannot conclude their role in terms of asymmetry. However, we can surmise that there is not a single direct influence of these elements on asymmetry, but perhaps a combination of them. A larger study on metalloid concentrations is needed to confirm this, while other studies (cellular, molecular, and biochemical) are needed to better understand the direct impact of these elements on turtle cells.

3.5.4.4.2 Eggs

Selenium was the only element in the three parts of the egg with a negative relationship with DIx in albumin, but positive in yolk and shell; albumin also presented the lowest concentration (0.81 μg g^{-1} ww) compared with yolk and shell (2.8 and 6.6 μg g^{-1}, respectively). It was previously reported that inorganic Se (potentially more toxic) tends to accumulate in yolk, while organic Se accumulates in albumin (Janz et al., 2010). Some studies also mention that reptiles may need more Se than other vertebrates and that the deficiency of this element can lead to embryonic deformities (ATSDR, 2003; Fordyce, 2013; Miller and Hontela, 2011). These results suggest that more asymmetric turtles tend to pass more inorganic and less organic Se to their eggs than more symmetric individuals.

3.5.5. Conclusions

The results of this study provide important evidence on the issue of asymmetry in marine turtles, both from a biological and methodological point of view. First, the statistical tool that we developed simplifies data collection in fieldwork; with only a carapace photograph, we are able to determine an index of asymmetry (specifically, the developmental instability index or DIx) of a single individual, thus avoiding the influence of size. Our results also suggest that individuals with more asymmetric carapaces seem to be more susceptible to accumulate heavy metals. This subject need to be studied in greater depth to validate this hypothesis as well as the use of carapace asymmetry as a biomarker. Nevertheless, metalloids did not show any pattern with DIx, but did have some significant relationships. The results from this study also showed significant relationships between the DIx of adult females and the concentration of metals in their eggs. More studies are needed on this species to assess whether pollutants may be related to more asymmetric hatchlings and whether more asymmetric females produce more asymmetric hatchlings.

References

Abdallah, M.A.M., Abd-Allah, M.A.M., 2011. Bioaccumulation of toxic metals in Loggerhead turtles from Mediterranean Sea coast, Egypt, in: Özhan, E. (Ed.), 10th International Conference on the Mediterranean Coastal, Environment. MEDCOAST 11, Rhodes, Greece, pp. 570-579.

Adel, M., Saravi, H.N., Dadar, M., Niyazi, L., Ley-Quinonez, C.P., 2015. Mercury, lead, and cadmium in tissues of the Caspian Pond Turtle (Mauremys caspica) from the southern basin of Caspian Sea. Environmental Science and Pollution Research.

Aguirre, A.A., Balazs, G.H., Zimmerman, B., Galey, F.D., 1994. Organic contaminants and trace metals in the tissues of green turtles (*Chelonia mydas*) afflicted with fibropapillomas in the Hawaiian Islands. Marine Pollution Bulletin 28, 109-114.

Aguirre, A.A., Gardner, S.C., Marsh, J.C., Delgado, S.G., Limpus, C.J., Nichols, W.J., 2006. Hazards associated with the consumption of sea turtle meat and eggs: A review for health care workers and the peneral Public. Ecohealth 3, 141-153.

Aguirre, A.A., Lutz, P.L., 2004. Marine turtles as sentinels of ecosystem health: Is fibropapillomatosis an indicator? EcoHealth 1, 275-283.

Akaike, H., 1974. A new look at the statistical model identification. IEEE Transactions on Automatic Control 19, 716-723.

Allenbach, D.M., 2010. Fluctuating asymmetry and exogenous stress in fishes: a review. Reviews in Fish Biology and Fisheries 21, 355-376.

Andreani, G., Santoro, M., Cottignoli, S., Fabbri, M., Carpene, E., Isani, G., 2008. Metal distribution and metallothionein in Loggerhead (*Caretta caretta*) and Green (*Chelonia mydas)* sea turtles. Science of the Total Environment 390, 287-294.

ATSDR, 2003. Toxicological profile for selenium. U.S. Departament of Health and Human Services. Agency for Toxic Substances and Disease Registry, Atlanta, Georgia.

ATSDR, 2007a. Toxicological profil for arsenic, Agency for Toxic Substances and Disease Registry. U.S. Departament of Health and Human Services. Public Health Service. Agency for Toxic Substances and Disease Registry, Atlanta, Georgia, p. 500.

ATSDR, 2007b. Toxicological profile for lead. U.S. Departament of Health and Human Services. Public Health Service. Agency for Toxic Substances and Disease Registry, Atlanta, Georgia.

ATSDR, 2012. Toxicological profile for cadmium. U.S. Departament of Health and Human Services. Agency for Toxic Substances and Disease Registry, Georgia, U.S.

Ayres-Fernández, C., Cordero-Rivera, A., 2004. Asymmetries and accessory scutes in *Emys orbicularis* from Northwest Spain. Biologia, Bratislava 59, 85-88.

Băncilă, R.I., Plăiaşu, R., Tudor, M., Samoilă, C., Cogălniceanu, D., 2012. Fluctuating asymmetry in the Eurasian Spur-Thighed tortoise,*Testudo graeca ibera Linneaus*, 1758 (Testudines: *Testudinidae*). Chelonian Conservation and Biology 11, 234-239.

Bannon, D.I., 2015. Chapter 20. Wildlife toxicity assessment for thallium, Wildlife Toxicity Assessments for Chemicals of Military Concern. Elsevier Science.

Barbieri, E., 2009. Concentration of heavy metals in tissues of Green turtles (*Chelonia mydas*) sampled in the Cananéia Estuary, Brazil. Brazilian Journal of Oceanography 57, 243-248.

Bears, H., Richards, J.G., Schulte, P.M., 2006. Arsenic exposure alters hepatic arsenic species composition and stress-mediated gene expression in the common killifish (*Fundulus heteroclitus*). Aquatic Toxicology 77, 257-266.

Beasley, D.A.E., Bonisoli-Alquati, A., Mousseau, T.A., 2013. The use of fluctuating asymmetry as a measure of environmentally induced developmental instability: A meta-analysis. Ecological Indicators 30, 218-226.
Berridge, M.J., Downes, C.P., Hanley, M.R., 1989. Neural and developmental actions of lithium: A unifying hypothesis. Cell 59, 411-419.
Bilandzic, N., Sedak, M., Ethokic, M., Ethuras Gomercic, M., Gomercic, T., Zadravec, M., Benic, M., Prevendar Crnic, A., 2012. Toxic element concentrations in the bottlenose (*Tursiops truncatus*), striped (*Stenella coeruleoalba*) and Risso's (*Grampus griseus*) dolphins stranded in eastern Adriatic Sea. Bulletin of Environmental Contamination and Toxicology 89, 467-473.
Bishop, C.A., Brooks, R.J., Carey, J.H., Ng, P., Norstrom, R.J., Lean, D.R.S., 1991. The case for a cause-effect linkage between environmental contamination and development in eggs of the common snapping turtle (*Chelydra s.serpentina*) from Ontario, Canada. Journal of Toxicology and Environmental Health 33, 521-547.
Bishop, C.A., Pettit, K.E., Kennedy, S.W., Stegeman, J.J., Norstrom, R.J., Brooks, R.J., 1998. Environmental contamination and developmental abnormalities in eggs and hatchlings of the common snapping turtle (*Chelydra serpentina serpentina)* from the Great Lakes-St Lawrence River basin (1989-91). Environmental Pollution 101, 143-156.
Boughammoura, S., Kessabi, K., Chouchene, L., Messaoudi, I., 2013. Effects of cadmium and high temperature on some parameters of calcium metabolism in the killifish (*Aphanius fasciatus*). Biological Trace Element Research 154, 73-80.
Breiman, L., 1999. Random Forest.
Briseño, P., Rodríguez, O., 2014. Derrame de crudo deja daños en ocho playas de Oaxaca. ISTMOMX.
Brotz, L., Cheung, W.W.L., Kleisner, K., Pakhomov, E., Pauly, D., 2012. Increasing jellyfish populations: Trends in large marine ecosystems. Hydrobiologia 690, 3-20.
Budis, H., Kalisinska, E., Lanocha, N., Kosik-Bogacka, D.I., 2013. The concentration of manganese, iron and strontium in bone of red fox *Vulpes vulpes* (L. 1758). Biological Trace Element Research 155, 361-369.
Buică, G., Cogălniceanu, D., 2013. Using digital images in the study of fluctuating asymmetry in the spur-thighed tortoise Testudo graeca. Turkish Journal of Zoology 37, 723-729.
Burger, J., Campbell, K.R., Murray, S., Campbell, T.S., Gaines, K.F., Jeitner, C., Shukla, T., Burke, S., Gochfeld, M., 2007. Metal levels in blood, muscle and liver of water snakes (Nerodia spp.) from New Jersey, Tennessee and South Carolina. Science of the Total Environment 373, 556-563.
Burger, J., Jeitner, C., Schneider, L., Vogt, R., Gochfeld, M., 2010. Arsenic, cadmium, chromium, lead, mercury, and selenium levels in blood of four species of turtles from the Amazon in Brazil. Journal of Toxicology and Environmental Health, Part A 73, 33-40.
Burnham, K.P., Anderson, D.R., 1998. Model selection and inference. A practical information-theoretic approach. Springer-Verlag, New York, US.
Burnham, K.P., Anderson, D.R., 2002. Model selection and multimodel inference: A practical information-theoretic approach. Springer-Verlag, New York.
Carpentier, A.S., Jean, C., Barret, M., Chassagneux, A., Ciccione, S., 2016. Stability of facial scale patterns on green sea turtles *Chelonia mydas* over time: A validation for the use of

a photo-identification method. Journal of Experimental Marine Biology and Ecology 476, 15-21.
Carr, A., 1957. Notes on the zoogeography of the Atlantic sea turtles of the genus *Lepidochelys*. Revista de Biologia Tropical 5, 45-61.
Caurant, F., Bustamante, P., Bordes, M., Miramand, P., 1999. Bioaccumulation of cadmium, copper and zinc in some tissues of three species of marine turtles stranded along the French Atlantic coasts. Marine Pollution Bulletin 38, 1085-1091.
Clauss, M., Paglia, D.E., 2012. Iron storage disorders in captive wild mammals: The comparative evidence. Journal of Zoo and Wildlife Medicine 43, S6-S18.
Cortés-Gómez, A.A., Fuentes-Mascorro, G., Romero, D., 2014. Metals and metalloids in whole blood and tissues of Olive Ridley turtles (L*epidochelys olivacea*) from La Escobilla Beach (Oaxaca, Mexico). Marine Pollution Bulletin 89, 367-375.
Cwynar, P., Kołacz, R., Grudnik, T., 2014. The influence of chronic thallium intoxication on laying hens, including its cumulation
in tissues, organs, and eggs. Polish Journal of Environmental Studies 23, 949-954.
D'Ilio, S., Mattei, D., Blasi, M.F., Alimonti, A., Bogialli, S., 2011. The occurrence of chemical elements and POPs in loggerhead turtles (*Caretta caretta*): an overview. Marine Pollution Bulletin 62, 1606-1615.
da Silva, C.C., Klein, R.D., Barcarolli, I.F., Bianchini, A., 2016. Metal contamination as a possible etiology of fibropapillomatosis in juvenile female green sea turtles *Chelonia mydas* from the southern Atlantic Ocean. Aquatic Toxicology 170, 42-51.
da Silva, C.C., Varela, A.S., Jr., Barcarolli, I.F., Bianchini, A., 2014. Concentrations and distributions of metals in tissues of stranded green sea turtles (Chelonia mydas) from the southern Atlantic coast of Brazil. Science of the Total Environment 466-467, 109-118.
Davis, A.K., Grosse, A.M., 2008. Measuring fluctuating asymmetry in plastron scutes of Yellow-bellied Sliders: the importance of gender, size and body location. The American Midland Naturalist 159, 340-348.
Depledge, M.H., Tyrrell, J., Fleming, L.E., Holgate, S.T., 2013. Are marine environmental pollutants influencing global patterns of human disease? Marine Environmental Research 83, 93-95.
Deraniyagala, P.E.P., 1939. The tetrapod reptiles of Celyon. Celyon Journal Science, Celyon.
Dongen, S.V., 2006. Fluctuating asymmetry and developmental instability in evolutionary biology: past, present and future. Journal of Evolutionary Biology 19, 1727-1743.
Drüeke, T.B., 2002. Intestinal absorption of aluminium in renal failure. Nephrol Dial Transplant 17, 13-16.
Duarte, C.M., 2014. Global change and the future ocean: a grand challenge for marine sciences. Frontiers in Marine Science 1, 63.
Edwards, A.W.F., 1971. Distances between populations on the basis of gene frequencies. Biometrics 27, 873-881.
Eeva, T., Tanhuanpää, S., Rabergh, C., Airaksinen, M., Nikinmaa, M., Lehikoinen, E., 2000. Biomarkers and fluctuating asymmetry as indicators of pollution-induced stress in two hole-nesting passerines. Functional Ecology 14, 235-243.

Ehsanpour, M., Afkhami, M., Khoshnood, R., Reich, K.J., 2014. Determination and maternal transfer of heavy metals (Cd, Cu, Zn, Pb and Hg) in the Hawksbill sea turtle (*Eretmochelys imbricata*) from a nesting colony of Qeshm Island, Iran. Bulletin of Environmental Contamination and Toxicology 92, 667-673.
Ericson, B., Caravanos, J., Chatham-Stephens, K., Landrigan, P., Fuller, R., 2013. Approaches to systematic assessment of environmental exposures posed at hazardous waste sites in the developing world: the Toxic Sites Identification Program. Environmental Monitoring and Assessment 185, 1755-1766.
Faust, D.R., Hooper, M.J., Cobb, G.P., Barnes, M., Shaver, D., Ertolacci, S., Smith, P.N., 2014. Inorganic elements in green sea turtles (*Chelonia mydas*): relationships among external and internal tissues. Environmental Toxicology and Chemistry 33, 2020-2027.
Fordyce, F.M., 2013. Selenium deficiency and toxicity in the environment, in: Selinus, O. (Ed.), Essentials of Medical Geology: Revised Edition. Springer Netherlands, Dordrecht, pp. 375-416.
Frazier, J.G., 1983. Analisis estadistico de la tortuga golfina *Lepidochelys olivacea* (Eschscholtz) de Oaxaca, Mexico. CIencia Pesquera 4, 49-75.
Frias-Espericueta, M.G., Osuna-Lopez, J.I., Ruiz-Telles, A., Quintero-Alvarez, J.M., Lopez-Lopez, G., Izaguirre-Fierro, G., Voltolina, D., 2006. Heavy metals in the tissues of the sea turtle *Lepidochelys olivacea* from a nesting site of the northwest coast of Mexico. Bulletin of Environmental Contamination and Toxicology 77, 179-185.
Frodello, J.P., Marchand, B., 2001. Cadmium, copper, lead, and zinc in five toothed whale species of the Mediterranean Sea. International Journal of Toxicology 20, 339-343.
Fujihara, J., Kunito, T., Kubota, R., Tanabe, S., 2003. Arsenic accumulation in livers of pinnipeds, seabirds and sea turtles: subcellular distribution and interaction between arsenobetaine and glycine betaine. Comparative Biochemistry and Physiology, Part C 136, 287-296.
Garcia-Fernandez, A.J., Gomez-Ramirez, P., Martinez-Lopez, E., Hernandez-Garcia, A., Maria-Mojica, P., Romero, D., Jimenez, P., Castillo, J.J., Bellido, J.J., 2009. Heavy metals in tissues from loggerhead turtles (*Caretta caretta*) from the southwestern Mediterranean (Spain). Ecotoxicology and Environmental Safety 72, 557-563.
Gardner, S.C., Fitzgerald, S.L., Vargas, B.A., Rodriguez, L.M., 2006. Heavy metal accumulation in four species of sea turtles from the Baja California peninsula, Mexico. Biometals 19, 91-99.
Genuer, R., Poggi, J.-M., Tuleau-Malot, C., 2015. VSURF: An R package for variable selection using Random Forests. The R Journal 7, 19-33.
Gibbons, M.J., Richardson, A.J., 2013. Beyond the jellyfish joyride and global oscillations: advancing jellyfish research. Journal of Plankton Research 35, 929-938.
Godley, B.J., Thompson, D.R., Furness, R.W., 1999. Do heavy metal concentrations pose a threat to marine turtles from the Mediterranean sea? Marine Pollution Bulletin 38, 497-502.
González Muñoz, M.J., Meseguer Soler, I., 2009. Elementos ultratrazas ¿Nutrientes o tóxicos? Toxicología 26, 93-103.
Grillitsch, B., Schiesari, L., 2010. The Ecotoxicology of Metals in Reptiles. 337-448.
Guirlet, E., Das, K., Girondot, M., 2008. Maternal transfer of trace elements in leatherback turtles (*Dermochelys coriacea)* of French Guiana. Aquatic Toxicology 88, 267-276.

Hansen, A.M., Bryan, C.E., West, K., Jensen, B.A., 2016. Trace element concentrations in liver of 16 species of cetaceans stranded on Pacific Islands from 1997 through 2013. Archives of Environmental Contamination and Toxicology 70, 75-95.
Helsel, D.R., 2011. Statistics for censored environmental data using Minitab and R. John Wiley & Sons.
Hill, R.L., 1971. Polymorphism of costal and vertebral laminae in the sea turtle *Lepidochelys olivacea*. Mededeling Stichting Natuurbehoud Suriname 2, 3-9.
Ho, G.W.C., Leung, K.M.Y., Lajus, D.L., Ng, J.S.S., Chan, B.K.K., 2009. Fluctuating asymmetry of Amphibalanus (*Balanus*) amphitrite (Cirripedia: *Thoracica*) in association with shore height and metal pollution (English). Hydrobiologia (The Hague) 621, 21-32.
Ibrahim, N.M., Eweis, E.A., El-Beltagi, H.S., Abdel-Mobdy, Y.E., 2012. Effect of lead acetate toxicity on experimental male albino rat. Asian Pacific Tropical Medicine Press, United States, North America.
Ikonomopoulou, M.P., Olszowy, H., Limpus, C., Francis, R., Whittier, J., 2011. Trace element concentrations in nesting flatback turtles (*Natator depressus*) from Curtis Island, Queensland, Australia. Marine Environmental Research 71, 10-16.
Janz, D., DeForest, D., Brooks, M., Chapman, P., Gilron, G., Hoff, D., Hopkins, W., McIntyre, D., Mebane, C., Palace, V., Skorupa, J., Wayland, M., 2010. Selenium toxicity to aquatic organisms, Selenium in the Aquatic Environment. Society of Environmental Toxicology and Chemistry (SETAC), Pansacola, Florida, pp. 141-231.
Jayasinghe, R., Tsuji, L.J., Gough, W.A., Karagatzides, J.D., Perera, D., Nieboer, E., 2004. Determining the background levels of bismuth in tissues of wild game birds: a first step in addressing the environmental consequences of using bismuth shotshells. Environmental Pollution 132, 13-20.
Jerez, S., Motas, M., Canovas, R.A., Talavera, J., Almela, R.M., Bayón del Rio, A., 2010. Accumulation and tissue distribution of heavy metals and essential elements in loggerhead turtles (*Caretta caretta*) from Spanish Mediterranean coastline of Murcia. Chemosphere 78, 256-264.
Kenyon, E.M., Hughes, M.F., 2001. A concise review of the toxicity and carcinogenicity of dimethylarsinic acid. Toxicology 160, 227-236.
Khandwala, H.M., Van Uum, S., 2006. Reversible hypercalcemia and hyperparathyroidism associated with lithium therapy: case report and review of literature. Endocrine Practice: Official Journal Of The American College Of Endocrinology And The American Association Of Clinical Endocrinologists 12, 54-58.
Kunito, T., Kubota, R., Fujihara, J., Agusa, T., Tanabe, S., 2008. Arsenic in marine mammals, seabirds, and sea turtles. Reviews of Environmental Contamination and Toxicology, 31-69.
Labrada-Martagon, V., Rodriguez, P.A., Mendez-Rodriguez, L.C., Zenteno-Savin, T., 2011. Oxidative stress indicators and chemical contaminants in East Pacific green turtles (*Chelonia mydas*) inhabiting two foraging coastal lagoons in the Baja California peninsula. Comparative Biochemistry and Physiology Part C 154, 65-75.
Lall, S.P., Lewis-McCrea, L.M., 2007. Role of nutrients in skeletal metabolism and pathology in fish — An overview. Aquaculture 267, 3-19.

Lazic, M.M., Kaliontzopoulou, A., Carretero, M.A., Crnobrnja-Isailovic, J., 2013. Lizards from urban areas are more asymmetric: using fluctuating asymmetry to evaluate environmental disturbance. PLoS One 8, e84190.
Leamy, L.J., Klingenberg, C.P., 2005. The genetics and evolution of fluctuating asymmetry. Annual Review of Ecology, Evolution, and Systematics, 1-21.
Lehmann, S.W., Lee, J., 2013. Lithium-associated hypercalcemia and hyperparathyroidism in the elderly: what do we know? Journal of Affective Disorders 146, 151-157.
Lens, L., Eggermont, H., 2008. Fluctuating asymmetry as a putative marker of human-induced stress in avian conservation. Bird Conservation International 18.
Lens, L., Van Dongen, S., Kark, S., Matthysen, E., 2002. Fluctuating asymmetry as an indicator of fitness: can we bridge the gap between studies? Biological Reviews of the Cambridge Philosophical Society 77, 27-38.
Levin, M., 2005. Left-right asymmetry in embryonic development: a comprehensive review. Mechanisms of Development 122, 3-25.
Mahieu, S., Millen, N., Gonzalez, M., Contini Mdel, C., Elias, M.M., 2005. Alterations of the renal function and oxidative stress in renal tissue from rats chronically treated with aluminium during the initial phase of hepatic regeneration. Journal of Inorganic Biochemistry 99, 1858-1864.
Mahor, G., Ali, S., 2015. An update on the role of medicinal plants in amelioration of aluminium toxicity. Bioscience Biotechnology Research Communications 8, 175-188.
Malluche, H.H., 2002. Aluminium and bone desease in chronic renal failure. Nephrol Dial Transplant 17, 21-24.
Marco, A., López-Vicente, M., Pérez-Mellado, V., 2004. Arsenic Uptake by Reptile Flexible-Shelled Eggs from Contaminated Nest Substrates and Toxic Effect on Embryos. Bulletin of Environmental Contamination and Toxicology 72.
Marin, S.e., Lăcrimioara, S.e., Cecilia, R., 2011. Evaluation of performance parameters for trace elements analysis in perennial plants using ICP-OES technique. Journal of Plant Development 18, 87-93.
Mast, R.B., Carr, J.L., 1989. Carapacial scute variation in Kemp's Ridley sea turtle (*Lepidochelys kempi*) hatchlings and juveniles, in: Caillouet, C.W., Jr., Landry, A.M., Jr. (Eds.), Proceedings of the First International Symposium on Kemp's Ridley Sea Turtle Biology, Conservation and Management. TAMU-SG-89-105, pp. 202-219.
McPherson, C.A., Lawrence, G.S., Elphick, J.R., Chapman, P.M., 2014. Development of a strontium chronic effects benchmark for aquatic life in freshwater. Environmental Toxicology and Chemistry 33, 2472-2478.
Miller, L.L., Hontela, A., 2011. Species-specific sensitivity to selenium-induced impairment of cortisol secretion in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). Toxicology and Applied Pharmacology 253, 137-144.
Moller, A.P., 2006. A review of developmental instability, parasitism and disease. Infection, genetics and evolution. Infection, Genetics and Evolution 6, 133-140.
Monna, F., Camizuli, E., Revelli, P., Biville, C., Thomas, C., Losno, R., Scheifler, R., Bruguier, O., Baron, S., Chateau, C., Ploquin, A., Alibert, P., 2011. Wild brown trout affected by historical mining in the Cevennes National Park, France. Environmental Science & Technology 45, 6823-6830.

Moreno, H., 2012. Derrama Pemex miles de litros de petróleo al mar en Salina Cruz, La Jornada. http://www.jornada.unam.mx/2012/08/24/estados/033n1est.
Nagle, R.D., Rowe, C.L., Congdon, J.D., 2001. Accumulation and selective maternal transfer of contaminants in the turtle *Trachemys scripta* associated with coal ash deposition. Archives of Environmental Contamination and Toxicology 40, 531–536.
Navarro Sánchez, E.J., 2015. Efecto de la temperatura de incubación y la diferenciación sexual sobre la morfología de crías de tortuga marina *Lepidochelys olivacea*, Ciencias del mar y limnología. Universidad Nacional Autónoma de México, México, D.F., p. 89.
Nunes, B., Capela, R.C., Sergio, T., Caldeira, C., Goncalves, F., Correia, A.T., 2014. Effects of chronic exposure to lead, copper, zinc, and cadmium on biomarkers of the European eel, *Anguilla anguilla*. Environmental Science and Pollution Research 21, 5689-5700.
O'Donnell, K.C., Gould, T.D., 2007. The behavioral actions of lithium in rodent models: leads to develop novel therapeutics. Neurosci Biobehav Rev 31, 932-962.
Páez-Osúna, F., Calderón-Campuzano, M.F., Soto-Jiménez, M.F., Ruelas-Inzunza, J.R., 2010a. Lead in blood and eggs of the sea turtle, *Lepidochelys olivacea*, from the Eastern Pacific: concentration, isotopic composition and maternal transfer. Marine Pollution Bulletin 60, 433-439.
Páez-Osúna, F., Calderón-Campuzano, M.F., Soto-Jiménez, M.F., Ruelas-Inzunza, J.R., 2010b. Trace metals (Cd, Cu, Ni, and Zn) in blood and eggs of the sea turtle *Lepidochelys olivacea* from a nesting colony of Oaxaca, Mexico. Archives of Environmental Contamination and Toxicology 59, 632-641.
Parsons, P.A., 1989. Fluctuating asymmetry: an epigenetic measure of stress. Biology Revision 65, 131-145.
Perrault, J.R., Miller, D.L., Garner, J., Wyneken, J., 2013. Mercury and selenium concentrations in leatherback sea turtles (*Dermochelys coriacea*): Population comparisons, implications for reproductive success, hazard quotients and directions for future research. Science of The Total Environment 463-464, 61-71.
Pigatto, P.D., Dell'Osso, B., Guzzi, G., 2016. Lithium overdosage and related tests. Int J Bipolar Disord 4, 1.
Polak-Juszczak, L., 2011. Impact of strontium on skeletal deformities in Baltic cod (*Gadus morhua callaris L.*). Chemosphere 83, 486-491.
Pors Nielsen, S., 2004. The biological role of strontium. Bone 35, 583-588.
Pritchard, P.C.H., 1966. Sea turtles of shell beach, British Guiana. Copeia: Herpetological Notes 1, 123-125.
R Core Team, 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
Rana, S.V., 2014. Perspectives in endocrine toxicity of heavy metals-a review. Biological Trace Element Research 160, 1-14.
Rheingold, A.L., Hues, S., 1983. Strontium and zinc content in bones as an indicator of diet. Journal of Chemical Education 60, 233-234.
Rivera, G., Stayton, C.T., 2013. Effects of asymmetry on the strength of the chelonian shell: a comparison of three species. Journal of Morphology 274, 901-908.
Rodriguez-Mercado, J.J., Altamirano-Lozano, M.A., 2013. Genetic toxicology of thallium: a review. Drug and Chemical Toxicology 36, 369-383.
Saeki, K., Sakakibaea, H., Sakai, H., Kunito, T., Tanabe, S., 2000. Arsenic accumulation in three species of sea turtles. Biometals 13, 241-250.

Sakai, H., Saeki, K., Ichihashi, H., Suganuma, H., Tanabe, S., Tatsukawa, R., 2000. Species-specific distribution of heavy metals in tissues and organs of Loggerhead turtle (*Caretta caretta*) and Green turtle (*Chelonia mydas*) from Japanese Coastal Waters. Marine Pollution Bulletin 40, 701-709.
Sanchez-Chardi, A., Garcia-Pando, M., Lopez-Fuster, M.J., 2013. Chronic exposure to environmental stressors induces fluctuating asymmetry in shrews inhabiting protected Mediterranean sites. Chemosphere 93, 916-923.
Scheuhammer, A.M., 1987. The chronic toxicity of aluminium, cadmium, mercury, and lead in birds: A review. Environmental Pollution 46, 263-295.
Shannon, C.E., 1948. A mathematical theory of communication. Bell System Technical Journal 27, 379–423.
Shi, H., Magaye, R., Castranova, V., Zhao, J., 2013. Titanium dioxide nanoparticles: a review of current toxicological data. Particle and Fibre Toxicology, 10:15.
Sonne, C., Aspholm, O., Dietz, R., Andersen, S., Berntssen, M.H., Hylland, K., 2009. A study of metal concentrations and metallothionein binding capacity in liver, kidney and brain tissues of three Arctic seal species. Science of the Total Environment 407, 6166-6172.
Storelli, M.M., Barone, G., Storelli, A., Marcotrigiano, G.O., 2008. Total and subcellular distribution of trace elements (Cd, Cu and Zn) in the liver and kidney of green turtles (*Chelonia mydas*) from the Mediterranean Sea. Chemosphere 70, 908-913.
Sun, H.J., Rathinasabapathi, B., Wu, B., Luo, J., Pu, L.P., Ma, L.Q., 2014. Arsenic and selenium toxicity and their interactive effects in humans. Environment International 69, 148-158.
Tarlow, E.M., Blumstein, D.T., 2007. Evaluating methods to quantify anthropogenic stressors on wild animals. Applied Animal Behaviour Science 102, 429-451.
Verstraeten, S.V., Aimo, L., Oteiza, P.I., 2008. Aluminium and lead: molecular mechanisms of brain toxicity. Archives of Toxicology 82, 789-802.
Vos, J.G., Bossart, G.D., Fournie, M., 2003. Toxicology of marine mammals.
Walker, C.H., Sibly, R.M., Hopkin, S.P., Peakall, D.B., 2005. Principles of Ecotoxicology, Third Edition. CRC Press.
Williams, L., Schoof, R.A., Yager, J.W., Goodrich-Mahoney, J.W., 2006. Arsenic bioaccumulation in freshwater Ffshes. Human and Ecological Risk Assessment: An International Journal 12, 904-923.
Wyneken, J., 2001. The anatomy of sea turtles. NMFS, US Department of Commerce, Miami, Florida.
Yalçin-Özdilek, S., Göksel Özdilek, H., Sangün Kemal, M., 2011. Change in physical and chemical composition of Green Turtle (*Chelonia mydas*) eggshells during embryonic development. Chelonian Conservation and Biology 10, 265-270.
Zavala-Norzagaray, A.A., Ley-Quiñónez, C.P., Espinosa-Carreon, T.L., Canizalez-Roman, A., Hart, C.E., Aguirre, A.A., 2014. Trace elements in blood of sea turtles *Lepidochelys olivacea* in the Gulf of California, Mexico. Bulletin of Environmental Contamination and Toxicology 93, 536-541.

4. GENERAL DISCUSSION

In this Doctoral Thesis, a three-year biomonitoring program was performed on inorganic elements on an arribada beach from the Southern Pacific Mexican coast. Biochemistry, oxidative stress and asymmetry were also explored as possible biomarkers related to these inorganic elements in Olive Ridley turtle blood and tissues.

In the first part of this Thesis, the worldwide distribution of these elements for the 7 turtle species was assessed. The meta-analysis undertaken showed some features of contamination: Leatherbacks (*Dermochelys coriacea*) show a distinct pattern of contamination as compared to other species, possibly as a consequence of their pelagic habitat and jellyfish diet. Liver and kidney on one side and bone on the other show a very specific pattern of contaminants. No clear oceanic basin effect was observed, which is not a very good sign for the general state of the planet: contamination is ubiquitous. In this first chapter, much useful information is provided for future researchers on how to homogenize and transform information to be able to compare data from different sources. For instance: 1) identifying inorganic elements of major concern in different ocean basin and turtle populations, which can help researchers to study specific elements and their effect on the turtle health; 2) providing humidity percentages from the most studied tissues to be able to transform wet weight information into dry weight and vice versa; 3) all the formulas to transform straight carapace length into curved carapace length and vice versa for every species. Ultimately, it provides a general overview of the status of these elements, and all the lacking and priority information regarding species, populations and elements needed in the marine turtle toxicology field worldwide.

4.1 Biomonitoring

In the biomonitoring part of this Thesis, the concentration of different inorganic elements was assessed for the population from La Escobilla (Oaxaca, Mexico). Blood of 241 live turtles and 7 different tissues from 58 dead turtles was explored in 8 different arribadas over three years. It was then possible to establish a database for this population, and to examine the tendencies of the most potentially toxic elements over the three years. The tissues where

less studied metals accumulate were also established. One of the most striking results is the very high Cd concentration found since the first monitoring work in liver and kidney (summer 2012). Moreover, this element was still constantly high over the 3 monitoring years, 82.88±36.65 μg g^{-1} in liver and 50.88±110.99 μg g^{-1}in kidney. These Cd concentrations are the highest ever reported worldwide for any sea turtle species. Another interesting finding is the significant differences of Cd in blood between live and dead turtles; dead turtles showed significantly higher concentrations of this metal than live ones. We also found that Al and Tl were significant higher in dead than in live turtles. Some authors have said that blood is an ideal tissue for heavy metal monitoring (Ley-Quiñónez et al., 2011; Ley-Quiñónez et al., 2017; Setim Prioste et al., 2015). However, few significant correlations between blood and tissues of dead turtles were found in this study (Chapter II). Blood is important as an acute–recent exposition indicator to these metals, but it does not seem to represent the accumulation or the availability of these elements far afield from where the samples were taken.

4.2 Biomarkers

Chapter IV is about oxidative stress and cellular protection. Oxidative stress is an unavoidable aspect of aerobic life and is the result of an imbalance between the production of ROS and antioxidant defenses in living organisms (Morcillo et al., 2016). Metals are important inducers of oxidative stress, promoting the formation of ROS. Fortunately, cells have developed mechanisms of antioxidant defense to protect from them. In the present work, ROS production and expression of selected genes involved in cellular protection and antioxidant defenses were explored. Due to the relationship between molecular indicators of oxidative stress and environmental xenobiotics (e.g. heavy metals), superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) are considered indicators of the cell defense mechanisms against ROS, and therefore an important tool as biomarkers of pollution exposure (Morcillo et al., 2016). On the other hand, metallothioneins (MT) are among the most important proteins in the protection of cells against heavy metal toxicity (especially Cd, Cu and Zn) as clearly demonstrated using mouse models under acute and

chronic Cd toxicity (Klaassen et al., 2009). In addition, MTs also have an important role in protecting cells from ROS-mediated injury and reducing oxidative stress (Andreani et al., 2008; Ruttkay-Nedecky et al., 2013). As a result, we decided to evaluate both gene expression and enzyme activity of several molecular indicators and relate them to inorganic elements. Since we failed to find sequences for the genes of interest for *L. olivacea*, gene expressions of *sod, cat*, gr and *mt* were first successfully partially sequenced. Therefore, we assessed these gene expressions in blood from 20 live turtles and from liver and kidney from 20 recently deceased individuals. Enzyme activities for SOD, CAT and GR in the same tissues were also measured. *Mt* gene expression was found to have positive relationships with As and negative relationships with Cu in kidney. In addition, significant relationships between gene expression and enzyme activity were mainly found in liver. Regarding enzyme activity, liver SOD activity was found to have negative relationships in liver with Cd, Cr, Fe, Pb, Ti and Tl concentrations; while CAT activity was found to have positive relationships in the same organ with Al, As, Cd, Se and Zn; and with As, Cd and Sb in kidney. GR activity was found to have positive relationships in liver with Al, As, Cd, Cr, Fe, Sb and Ti; and with Sb in kidney. As was the only element with positive relationships for GR in both gene expression and enzyme activity in liver.

In Chapters V and VI of this Thesis, we work with biochemical analytes. Blood analysis can be used not only to diagnose sea turtle diseases but also to assess the health status of populations. Therefore, establishing baseline blood chemical profiles for healthy, wild populations of endangered sea turtle species has become a high priority (Ley-Quiñónez et al., 2017). Different analytes (ALP, AST, ALT, creatinine, albumin, cholesterol, glucose, proteins, triglycerides, urea and P-nitrophenyl acetate esterase activity) were assessed and related to inorganic elements (As, Cd, Cr, Mn, Ni, Pb, Sr, Ti, Zn and Se). Evidence suggests several associations between metal concentrations and biochemical parameters. This study reinforces the utility of the blood of sea turtles for the simultaneous monitoring of environmental contaminants and clinical parameters. A negative correlation between EA and cortisol was observed. Other relationships were also found between these biomarkers

and important pollutants such as Cd, As and Pb, and elements of growing concern such as Ti and Sr. The correlations detected in the present study between the elements analyzed and biochemical parameters, in addition to the previous high concentrations reported, indicate that these contaminants may have a negative effect on the health of these turtles. With the significant number of turtles sampled (100), these results can be used as a baseline guide for future research and to assess the health of rescue individuals in this area.

In Chapter VII, we tried to develop a new possible biomarker using the asymmetry of the carapace. Developmental instability (DI) is the inability of an individual to withstand perturbations during its development (Băncilă et al., 2012; Beasley et al., 2013; Bishop et al., 1998; Mast and Carr, 1989). DI has been used as an indicator index of individual fitness and is considered an environmental stress biomarker in different species related, among other things, to certain pollutants such as organochloride and metals (Băncilă et al., 2012; Beasley et al., 2013; Ho et al., 2009). DI through fluctuating asymmetry (FA) has become a popular method for measuring phenotypic response to environmental stress (Beasley et al., 2013; Leamy and Klingenberg, 2005). The strongest aspect of FA as a potential biomarker in wildlife is that it is a non-lethal tool. Furthermore, compared with other biomarkers, FA is relatively easy to obtain using measurements or photographs (Beasley et al., 2013; Buică and Cogălniceanu, 2013; Davis and Grosse, 2008; Dongen, 2006).

Carapace scute asymmetry is apparent not only in the relationship between the number of left and right costal scutes, but also in the sizes and shapes of these scutes. To quantify this condition and facilitate comparisons and analyses, a new index to better describe the scutes' heterogeneity within an individual was developed. Two different measures of shape heterogeneity in a single metric were combined: (i) the diversity of the width of scutes was measured by the geometric averaged Shannon H entropy for each side (Shannon, 1948), and (ii) the difference in coastal scute size between both sides (*i.e.*, asymmetry) was measured using Edwards' angular distance (Edwards, 1971). This integrative index gave the expected relative values when tested in different situations, being higher for (a) greater

asymmetry of the scutes or their relative widths, (b) a greater number of scutes on the left or right side, and (c) more diverse widths of scutes on the right or left side. We named it the developmental instability index (DIx).

There are a few studies on the relationship between asymmetry and pollutants in vertebrates, such as shrew bones and heavy metals (Sanchez-Chardi et al., 2013), and fish and diverse stressors (Allenbach, 2010). These experimental studies identified some positive relationships between pollutant concentrations and fluctuating asymmetry. In marine turtles, it was found that asymmetry is a characteristic developed at the embryo stage with different causes (Mast and Carr, 1989; Navarro Sánchez, 2015). With facial scales in *Chelonia mydas*, there seems to be little change in asymmetry during the individual's development in adult life (Carpentier et al., 2016). Through DIx, it was feasible to determine if the load of pollutants was higher in asymmetrical adult individuals than in individuals with less asymmetry in their carapace. If this were the case, asymmetry in marine turtles would be a characteristic effect of counter-selection, related, at least in part to contamination, with higher contamination levels having an adverse effect on fitness. Negative relationships were also found between DIx and some element concentrations in certain egg components. Carapace shape in marine turtles is determined during embryonic life, but metal concentrations in this work were measured many years later. Thus, pollutants present in these adult turtles cannot be considered a relic of their contamination at the embryonic/hatchling stage, but are acquired instead during their juvenile/adult life span. These results suggest that individuals with more asymmetric carapaces seem to be more susceptible to accumulate heavy metals. This subject needs to be studied in greater depth to validate both this hypothesis as well as the use of carapace asymmetry as a biomarker.

Comparing all these possible biomarkers (Table 1), different tendencies are observed. In general, gene expression and enzyme activity seem to be stimulated by these inorganic elements. All these positive relationships could mean that turtles are adapting to the metal-production of ROS and damage through a high transcription of these antioxidants, except

for *cat* in kidney, where we found a negative relationship and the highest Cd concentrations, and for SOD activity, which had negative relationships (using the 3 analyzed tissues together) with 6 different elements. Multiple positive relationships with GR seem to be part of the compensatory effect of GR due to the decrease of SOD and CAT production against the high and chronic exposure to certain xenobiotics, such as Cd.

Table 1. Positive (↑) and negative (↓) relationships in all tissues for all the markers tested in this Thesis.

	Pb	Cd	Li	Sb	Sr	Ti	Tl	Al	As	Cu	Cr	Fe	Ni	Se	Zn
Gene expression															
sod	↑	↑							↑		↑	↑	↑		↑
cat		↑↓					↓				↑↓	↓	↑		
gr	↑	↑							↑		↑	↑	↑		↑
mt									↑						
Enzyme activity															
SOD	↑↓	↓↓				↓	↓				↓	↓			
CAT		↑		↑				↑	↑	↑				↑	↑
GR		↑		↑		↑		↑	↑		↑	↑			
Biochemistry															
Cortisol					↑	↑		↓	↓	↑				↓	
EA	↓↑	↓				↓		↓			↓			↓	↑
AST	↑				↑				↑						↓
ALT					↑					↓					
Album					↑										↓
Creat		↓				↑								↑	↓
Glucose	↑	↓							↓					↑	
Urea					↑	↑			↑					↑	
Asymmetry															
Tissues	↓	↑	↑		↑		↑	↑↓	↑	↓	↑↓				
Eggs	↓	↑↓	↓		↓	↑		↓	↑	↑	↑			↑↓	

Within the biochemistry analytes, esterase activity appears be the most negatively impacted with mostly negative relationships. Esterase activity (EA) is widely studied due to its multifunctional roles in diverse biochemical pathways, such as protection against oxidative damage, lipid peroxidation, contribution to innate immunity, detoxification of reactive molecules, modulation of endoplasmic reticulum stress and regulation of cell proliferation/apoptosis, *inter alia* (Ceron et al., 2014; Franco et al., 2015; Laird et al., 2014;

Li et al., 2006). In humans, it has been reported that some metals could modulate activity of this enzyme (Hernandez et al., 2009; Laird et al., 2014; Li et al., 2006), and according to our results, this EA could be negatively impacted by these inorganic elements. Cortisol also presented several (6) relationships with the elements, half of them were negative and half positive. Elevation of plasma corticosteroids in reptiles, as a result of stress exposure, has been demonstrated in many species including marine turtles (Aguirre et al., 1995; Gregory et al., 1996; Gregory and Schmid, 2001). It has been noted that toxicants, such as metals and metalloids, can be perceived as stressors by the hypothalamic–pituitary–adrenal axis and affect stress response, inducing changes in cortisol levels (Brodeur et al., 1997). In general, the rest of the analytes seem to be more or less influenced by some of the inorganic elements studied. Of special note here is that Cd had only negative relationships (EA, creatinine and glucose), and the inhibition of creatinine in particular could indicate renal damage, which is to be expected with the very high Cd concentrations found in that organ. Another interesting result is the multiple positive relationships of Sr (to cortisol, AST, ALT, albumin and urea); this very under-studied metal may have important stimulation in some of these studied analytes.

Asymmetry is a new development marker and without any similar work on the subject it is difficult to draw conclusions. However, DIx of the carapace was found to be positively related in most of the tissues. This may indicate that more asymmetrical individuals had more accumulated metals. More studies in this area are needed before we can validate the use of DIx as biomarker in Ridley turtles.

In summary, *Lepidochelys olivacea* from La Escobilla beach seem to be very highly contaminated with certain inorganic elements. In particular, the very high Cd concentrations require special attention and follow up studies. Several significant relationships were also detected relating different biomarkers with some elements. These results demonstrated which tissues are preferable in the measurement of different biochemical or molecular markers. This thesis also provided very complete databases for

biochemical and molecular parameters and inorganic elements for future research and for assessing the health of rescue individuals in this area. Additionally, we developed a new tool to assess the asymmetry of the carapace of the individuals using just a single photo, an option which greatly simplifies fieldwork in this area.

Nevertheless, it is important to note that organisms in the wild are typically exposed to multiple natural and anthropogenic stressors in addition to a mixture of xenobiotics. All these factors interact with each other and produce multiple biological effects. In this respect, it is always important to use these biomarkers carefully, and with what little information exists, they cannot be routinely used as biomarkers of the biological impact of environmental pollutants by themselves, but only in comparison with previous results. With these limitations in mind we recommend that future researchers on this topic: 1) undertake more laboratory/control experiments before using these antioxidant or biochemical parameters as biomarkers in wild turtle populations; 2) assess the usefulness of the carapace Dix; more studies on a higher number of turtles are needed, as well as comparisons of the mothers and hatchling asymmetry, and of the concentration of pollutants in target tissues with the DIx of the hatchlings. Could asymmetry be used as a valid biomarker for metals? And do highly polluted turtles produce highly asymmetrical hatchlings? 3) conduct future works trying to assess biomarkers and pollutants on two different populations and/or different species at the very least. Then, comparisons of different relationships and the consistency (or otherwise) of the results could be explored. And, 4) that they try to identify the sources of the most threatening elements in accordance with our results. In order to do this, the analyses of isotopes and the use of satellite tracking would be useful tools.

References

Aguirre, A.A., Balazs, G.H., Spraker, T.R., Gross, T.S., 1995. Adrenal and hematological responses to stress in juvenile green turtles (Chelonia mydas) with and without fibropapillomas. . Physiological Zoology 68, 831-854.

Allenbach, D.M., 2010. Fluctuating asymmetry and exogenous stress in fishes: a review. Reviews in Fish Biology and Fisheries 21, 355-376.

Andreani, G., Santoro, M., Cottignoli, S., Fabbri, M., Carpene, E., Isani, G., 2008. Metal distribution and metallothionein in Loggerhead (*Caretta caretta*) and Green (*Chelonia mydas)* sea turtles. Science of the Total Environment 390, 287-294.
Băncilă, R.I., Plăiaşu, R., Tudor, M., Samoilă, C., Cogălniceanu, D., 2012. Fluctuating asymmetry in the Eurasian Spur-Thighed tortoise,*Testudo graeca ibera Linneaus*, 1758 (Testudines: *Testudinidae*). Chelonian Conservation and Biology 11, 234-239.
Beasley, D.A.E., Bonisoli-Alquati, A., Mousseau, T.A., 2013. The use of fluctuating asymmetry as a measure of environmentally induced developmental instability: A meta-analysis. Ecological Indicators 30, 218-226.
Bishop, C.A., Pettit, K.E., Kennedy, S.W., Stegeman, J.J., Norstrom, R.J., Brooks, R.J., 1998. Environmental contamination and developmental abnormalities in eggs and hatchlings of the common snapping turtle (*Chelydra serpentina serpentina)* from the Great Lakes-St Lawrence River basin (1989-91). Environmental Pollution 101, 143-156.
Brodeur, J.C., Sherwood, G., Rasmussen, J.B., Hontela, A., 1997. Impaired cortisol secretion in yellow perch (*Perca flavescens*) from lakes contaminated by heavy metals: in vivo and in vitro assessment. . Canadian Journal of Fisheries and Aquatic Sciences 54.
Buică, G., Cogălniceanu, D., 2013. Using digital images in the study of fluctuating asymmetry in the spur-thighed tortoise Testudo graeca. Turkish Journal of Zoology 37, 723-729.
Carpentier, A.S., Jean, C., Barret, M., Chassagneux, A., Ciccione, S., 2016. Stability of facial scale patterns on green sea turtles *Chelonia mydas* over time: A validation for the use of a photo-identification method. Journal of Experimental Marine Biology and Ecology 476, 15-21.
Ceron, J.J., Tecles, F., Tvarijonaviciute, A., 2014. Serum paraoxonase 1 (PON1) measurement: an update. BMC Veterinary Research 10:74.
Davis, A.K., Grosse, A.M., 2008. Measuring fluctuating asymmetry in plastron scutes of Yellow-bellied Sliders: the importance of gender, size and body location. The American Midland Naturalist 159, 340-348.
Dongen, S.V., 2006. Fluctuating asymmetry and developmental instability in evolutionary biology: past, present and future. Journal of Evolutionary Biology 19, 1727-1743.
Edwards, A.W.F., 1971. Distances between populations on the basis of gene frequencies. Biometrics 27, 873-881.
Franco, L., Romero, D., García-Navarro, J.A., Teles, M., Tvarijonaviciute, A., 2015. Esterase activity (EA), total oxidant status (TOS) and total antioxidant capacity (TAC) in gills of *Mytilus galloprovincialis* exposed to pollutants: Analytical validation and effects evaluation by single and mixed heavy metal exposure. Marine Pollution Bulletin.
Gregory, L.F., Gross, T.S., Bolten, A.B., Bjorndal, K.A., Guillette, L.J.J., 1996. Plasma corticosterone concentrations associated with acute captivity stress in wild loggerhead sea turtles (*Caretta caretta*). General and Comparative Physiology 104, 312-320.
Gregory, L.F., Schmid, J.R., 2001. Stress responses and sexing of wild Kemp's ridley sea turtles (*Lepidochelys kempii*) in the northeastern Gulf of Mexico. General and Comparative Endocrinology 124, 66-74.
Hernandez, A.F., Gil, F., Leno, E., Lopez, O., Rodrigo, L., Pla, A., 2009. Interaction between human serum esterases and environmental metal compounds. Neurotoxicology 30, 628-635.

Ho, G.W.C., Leung, K.M.Y., Lajus, D.L., Ng, J.S.S., Chan, B.K.K., 2009. Fluctuating asymmetry of Amphibalanus (*Balanus*) amphitrite (Cirripedia: *Thoracica*) in association with shore height and metal pollution (English). Hydrobiologia (The Hague) 621, 21-32.
Klaassen, C.D., Liu, J., Diwan, B.A., 2009. Metallothionein protection of cadmium toxicity. Toxicology and Applied Pharmacology 238, 215-220.
Laird, B.D., Goncharov, A.B., Ayotte, P., Chan, H.M., 2014. Relationship between the esterase paraoxonase-1 (PON1) and metal concentrations in the whole blood of Inuit in Canada. Chemosphere 120C, 479-485.
Leamy, L.J., Klingenberg, C.P., 2005. The genetics and evolution of fluctuating asymmetry. Annual Review of Ecology, Evolution, and Systematics, 1-21.
Ley-Quiñónez, C., Zavala-Norzagaray, A.A., Espinosa-Carreon, T.L., Peckham, H., Marquez-Herrera, C., Campos-Villegas, L., Aguirre, A.A., 2011. Baseline heavy metals and metalloid values in blood of loggerhead turtles (*Caretta caretta*) from Baja California Sur, Mexico. Marine Pollution Bulletin 62, 1979-1983.
Ley-Quiñónez, C.P., Rossi-Lafferriere, N.A., Espinoza-Carreon, T.L., Hart, C.E., Peckham, S.H., Aguirre, A.A., Zavala-Norzagaray, A.A., 2017. Associations between trace elements and clinical health parameters in the North Pacific loggerhead sea turtle (*Caretta caretta*) from Baja California Sur, Mexico. Environmental Science and Pollution Research 24, 9530-9537.
Li, W.-F., Pan, M.-H., Chung, M.-C., Ho, C.-K., Chuang, H.-Y., 2006. Lead Exposure Is Associated with Decreased Serum Paraoxonase 1 (PON1) Activity and Genotypes. Environmental Health Perspectives 114, 1233-1236.
Mast, R.B., Carr, J.L., 1989. Carapacial scute variation in Kemp's Ridley sea turtle (*Lepidochelys kempi*) hatchlings and juveniles, in: Caillouet, C.W., Jr., Landry, A.M., Jr. (Eds.), Proceedings of the First International Symposium on Kemp's Ridley Sea Turtle Biology, Conservation and Management. TAMU-SG-89-105, pp. 202-219.
Morcillo, P., Esteban, M.A., Cuesta, A., 2016. Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. Chemosphere 144, 225-233.
Navarro Sánchez, E.J., 2015. Efecto de la temperatura de incubación y la diferenciación sexual sobre la morfología de crías de tortuga marina *Lepidochelys olivacea*, Ciencias del mar y limnología. Universidad Nacional Autónoma de México, México, D.F., p. 89.
Ruttkay-Nedecky, B., Nejdl, L., Gumulec, J., Zitka, O., Masarik, M., Eckschlager, T., Stiborova, M., Adam, V., Kizek, R., 2013. The role of metallothionein in oxidative stress. Int J Mol Sci 14, 6044-6066.
Sanchez-Chardi, A., Garcia-Pando, M., Lopez-Fuster, M.J., 2013. Chronic exposure to environmental stressors induces fluctuating asymmetry in shrews inhabiting protected Mediterranean sites. Chemosphere 93, 916-923.
Setim Prioste, F.E., de Oliveira Souza, V.C., Ramos Queiroz, M., 2015. Chemical Element Concentrations in the Blood of Green Turtles (*Chelonia Mydas*) Captured at Fernando De Noronha Marine National Park, Brazil. Journal of Environmental & Analytical Toxicology 05.
Shannon, C.E., 1948. A mathematical theory of communication. Bell System Technical Journal 27, 379–423.

5. FINAL CONCLUSIONS

FIRST. Pb, Al, Ti and Sr showed significant decreased in turtle tissue concentrations during the three monitoring years. However, Cd concentrations found in this study are the most alarming non-essential element with the highest concentrations worldwide for any sea turtle species in liver and kidney. Then, it is convenient to keep monitoring this and other pollutants to take decisions about the conservation management of this and other marine species.

SECOND. Liver was the tissue with more positive significant correlations between essential and non-essential elements and the morphological age marker (curve carapace length, CCL). Then, this could be the ideal tissue to study the relationships among the size and the age and inorganic elements.

THIRD. Aluminium was the element with more alterations related to the carcass decomposition; muscle and fat appear to be the tissues with more alterations in the inorganic elements concentrations depending on the decomposition processes. This information may be useful to take into account to avoid bias in future biomonitoring programs regarding this element.

FOURTH. To correlate the concentrations in blood to predict the accumulation of the elements in tissues, does not appear to be accurate in Olive Ridley. Blood still a very useful tool regarding acute expositions, though to assess chronic exposition, tissues still crucial.

FIFTH. Olive Ridley turtles from La Escobilla seems to be adapting to the metals-production of ROS and damage through high transcription of *sod, cat* and *gr* in blood, liver and kidney. Except for *cat* in kidney, where we had a negative relationship to Cd concentrations, this information may be pointing out the high exposition to this metal and the insufficient response of *cat* against this element.

SIXTH. GR activity seems to be part of the compensatory effect due to the decreased of SOD

and CAT production against the high and chronic exposure to certain xenobiotics. This could mean that SOD and CAT activity are not sufficient against the high concentrations of certain reactive oxygen species, including some heavy metals.

SEVENTH. EA appears to be the most sensitive biochemical analyte analyzed affected by metals. We then recommended more studies to validate the use of the EA as a biomarker of exposition to futures inorganic element biomonitoring programs.

EIGHTH. A new morphologic tool to assess the developmental instability (DIx) in the carapace of Ridley turtles was developed. A follow-up of this study is necessary to validate the use of this DIx related with metal accumulation. Our preliminary results showed that individuals with more asymmetric carapaces seem to be more susceptible to accumulate heavy metals.

6. RESUMEN EN ESPAÑOL

En la actualidad se han dejado al descubierto cifras que demuestran una inminente extinción de numerosas especies animales y vegetales en el mundo. De acuerdo con las listas publicadas por la Unión Mundial para la Naturaleza y su Comisión de Supervivencia de Especies, se estima que en los próximos treinta años desaparecerá una quinta parte de las especies que habitan actualmente en nuestro planeta (IUCN, 2012). En una escala global, las especies 7 especies existentes de tortugas marinas están actualmente en la Lista Roja de Especies Amenazadas (IUCN, 2012), siendo catalogadas como vulnerables, en peligro de extinción o en peligro crítico.

A pesar del escaso número de especies, las tortugas marinas se distribuyen por todo el mundo, habitando en casi todos los océanos, ocupando nichos ecológicos únicos y mostrando variaciones intra-específicas en el tamaño de la población, así como en su reproducción y morfología (Fowler, 2005). Estas especies marinas se han adaptado con mucho éxito a su ambiente, siendo miembros importantes de los ecosistemas marinos en todo el mundo.

Entre otras presiones antropogénicas sobre las tortugas marinas, muchas áreas de anidación y alimentación quedan inhabilitadas o deterioradas por la acumulación de desechos químicos, derrames de petróleo o acumulación de otros deshechos antropogénicos. Los residuos industriales, junto con los vertidos accidentales y la acumulación, cada vez mayor de contaminantes ambientales persistentes en el medio marino, condicionan la supervivencia de estas especies (Eckert et al., 2000). Como afirman algunos autores, el incremento de la contaminación ambiental marina, junto a la pérdida de los lugares ideales de anidación son las principales amenazas para la supervivencia de las tortugas marinas en todo el mundo (Storelli et al., 2005). Estos autores también afirman que cuando existen grandes diferencias en las concentraciones de metales dentro de una misma especie, será entonces la localización geográfica de la zona de alimentación de cada población y el tamaño o edad de los animales lo que explique estas diferencias.

Finalmente, y entre otras cosas cabe mencionar también que, por innumerables generaciones, las comunidades costeras han dependido de las tortugas marinas y sus huevos para la obtención de proteínas y otros productos (Eckert et al., 2000). En muchas regiones, incluidas las mayorías de las playas antes mencionadas esta práctica aún continúa, así como la eventual comercialización de los mismos (Morales-González 2008). El consumo de dichos productos puede ocasionar importantes problemas en el ámbito de la salud pública, debido a la presencia de bacterias, parásitos y contaminantes medioambientales (Aguirre et al., 2006).

La tortuga golfina (*Lepidochelis olivacea*) es la especie más abundante de las ocho especies de tortugas marinas existentes. En el pacífico oriental se distribuye desde el sur de Estados Unidos hasta Chile. Sus hábitos reproductivos son espectaculares ya que tienen playas de desoves masivos, donde llegan miles de tortugas al mismo tiempo durante tres o cuatro días, siendo este fenómeno conocido como "arribada". México, Costa Rica y la India son los países con las playas más importante de arribadas (Marquez et al., 1996).

La playa de La Escobilla en Oaxaca, México está considerada como un santuario, ya que es uno de los entornos naturales de mayor concentración de tortuga golfina en el momento del desove. Se estima que una cuarta parte de la población mundial de estas tortugas desova en las playas de Oaxaca, la mayor parte en La Escobilla y en menor número en la playa de Morro Ayuta (Abreu-Grobois and Plotkin, 2008).

Dentro de los contaminantes ambientales se considera que el estudio de la acumulación de metales en fauna marina silvestre es de gran interés debido a los efectos potencialmente tóxicos que estos elementos pueden causar, sobre todo en los animales situados en la parte alta de la cadena alimenticia, así como en las personas que lleguen a consumir sus productos (Cardellicchio et al., 2000; Das et al., 2003). Estos estudios permiten estimar el tipo y grado de exposición a la que estas especies están sometidos, además, estimar los riesgos potenciales para su conservación o supervivencia (Das et al., 2003). Aunque los contaminantes ambientales en las tortugas marinas han sido

evaluados por varios autores en todo el mundo (véase el capítulo I), muy pocos estudios han documentado biomarcadores o efectos sobre la salud derivados de estas exposiciones.

El estrés oxidativo es un aspecto inevitable de la vida aeróbica. Es el resultado de un desequilibrio entre la producción de especies reactivas del oxígeno (ROS) y las defensas antioxidantes en los organismos. Por lo tanto, este estrés oxidativo es producido por la acumulación celular de ROS, moléculas con fuertes propiedades oxidativas (Valdivia et al., 2007), y esto podría ocurrir por muchos factores. Entre ellos, la exposición a una variedad de factores estresantes ambientales, tales como contaminantes, produce una mayor producción de ROS y un potencial antioxidante reducido que conduce al estrés oxidativo (Labrada-Martagon et al., 2011).

Se ha demostrado, en trabajos in vivo e in vitro, que estos ROS tienen el potencial de dañar proteínas, lípidos y ácidos nucleicos, causando daño tisular, disfunción metabólica y muerte celular de la apoptosis (Labrada-Martagon et al., 2011; Morcillo et al., 2016; Valdivia et al., 2007). Debido a la relación entre los indicadores moleculares del estrés oxidativo y los xenobióticos ambientales (por ejemplo, los elementos inorgánicos), la superóxido dismutasa (SOD), la catalasa (CAT) y la glutatión reductasa (GR) se consideran indicadores de los mecanismos de defensa celular contra ROS y como biomarcadores de la exposición a la contaminación (Morcillo et al., 2016).

Por otra parte, las metalotioneínas (MT) están entre las proteínas más importantes en la protección de las células contra la toxicidad algunos elementos inorgánicos (especialmente Cd, Cu y Zn) como se ha demostrado usando modelos de ratón bajo toxicidad aguda y crónica de Cd (Klaassen et al., 2009). Además, las MT también tienen un papel importante en la protección de las células de lesiones mediadas por ROS y la reducción del estrés oxidativo (Andreani et al., 2008; Ruttkay-Nedecky et al., 2013). La mayor parte de la información se basa en determinaciones a nivel de actividad proteínica o enzimática de MTs y enzimas antioxidantes y se ha prestado menos atención a su regulación en la expresión génica (Matovic et al., 2015; Misra and Niyogi,

2009; Regoli and Giuliani, 2014; Venancio et al., 2013). Incluso menos trabajos sobre este tema se han dirigido a poblaciones de vida silvestre, ya que la mayoría de las obras se desarrollan en experimentos controlados por el laboratorio.

En el caso de las tortugas marinas, la literatura sobre el estrés oxidativo es reciente y muy limitada. Existen solo dos publicaciones en tortugas marinas relacionando la actividad enzimática antioxidante y algunos metales, ambas en tortugas verdes (*Chelonia mydas*). Labrada-Martagon et al. (2011) trabajó con tortugas vivas (sangre) y Valdivia et al. (2007) con tortugas de muerte incidental (hígado y riñón). Otros trabajos han correlacionado la actividad enzimática antioxidantes de la sangre de *Eretmochelys imbricata* y organoclorados (Tremblay et al. (2016), así como de *Lepidochelys kempii* y brevetoxina (Perrault et al. (2014). En cuanto a la protección celular y el estrés oxidativo, sólo encontramos un trabajo en dos poblaciones diferentes de tortugas marinas (*C. caretta y C. mydas*) relacionadas las MTs y algunos metales (Andreani et al., 2008). No existen trabajos publicados sobre indicadores moleculares o actividad enzimática (*sod, cat, gr o mt*) en *L. olivacea*.

Por otro lado, la bioquímica sanguínea también se utiliza comúnmente como una ayuda para el diagnóstico para evaluar la salud en animales domésticos y silvestres. Estos parámetros pueden variar dependiendo de las especies, género, edad, dieta, proceso patológico, técnicas de análisis, procesos patológicos y contaminantes techniques (Anderson et al., 2011; Casal et al., 2009; Fazio et al., 2011). Debido a todas estas variaciones no es fácil establecer parámetros normales en tortugas marinas y se recomienda tener una base de datos por especies y por poblaciones (áreas de anidación/alimentación). Esta base de datos, a continuación, proporcionar indicaciones de la salud, las enfermedades, la nutrición e incluso evaluar la calidad del hábitat (Camacho et al., 2013). Toda esta información ayudaría a hacer una mejor evaluación de la salud de las diferentes poblaciones y también proporcionaría una herramienta importante para hacer un mejor diagnóstico y cuidado veterinario en animales de rescate.

Muy pocos estudios han evaluado la bioquímica en tortugas marinas, y menos relacionándolos con contaminantes (Camacho et al., 2013; Keller and McClellan-Green, 2004; Keller et al., 2004; Keller et al., 2006; Labrada-Martagon et al., 2011), dos de estos estudios se han realizado en *L. olivacea* (Santillana, 2013; Santoro and Meneses, 2007). Para fines de conservación de las tortugas marinas, es esencial determinar cómo reaccionan estos reptiles a diferentes tipos de contaminantes antropogénicos (Camacho et al., 2013). Para ello, se necesita una base de datos sobre parámetros clínicos de salud normales para las especies y para la población, además de un monitoreo regular de contaminantes en sus áreas de alimentación.

En el capítulo I de esta Tesis (sección1.2) se muestra el Estado del Arte y un meta análisis sobre los elementos inorgánicos más estudiados alrededor del mundo en las 7 especies de tortugas marinas (Pb, Cd, Hg, Al, As, Cr, Cu, Fe, Mn, Ni, Se and Zn). En este trabajo se ha encontrado que todos estos elementos fueron encontrados por arriba del límite de detección en todas las especies y poblaciones estudiadas. El meta análisis por su parte mostró diferentes comportamientos del nivel de contaminación: por ejemplo, las tortugas Laúd (*Dermochelys coriacea*) tuvo un patrón diferente de contaminantes comparada con las otras especias. El hígado y el riñón por un lado y el hueso por otro, mostraron patrones muy específicos frente a los contaminantes y comparados con los otros tejidos. No se encontró ningún efecto por mar/zona, lo cual significa que la contaminación es ubicua.

La primera parte de los capítulos experimentales (sección 3) contiene tres capítulos (capítulos II al IV). En estos capítulos se describe la concentración de, en una primera parte (sección 3.1) 8, y en su segunda parte 27 elementos inorgánicos (sección 3.2) en tortugas vivas (3.2.1) y muertas (3.2.2). Uno de los resultados más destacados es la alta concentración de Cd encontrada desde el primer trabajo de monitoreo en hígado y riñón (capítulo II). Por otra parte, este elemento ha continuado con muy altas concentraciones a lo largo de los 3 años 82,88 ± 36,65 (hígado) y 150,88 ± 110,99 $\mu g\ g^{-1}$ ww (riñón). Estas concentraciones de cadmio son las más altas reportadas a nivel

mundial para cualquier especie de tortuga marina. Otro hallazgo interesante fueron las diferencias significativas de Cd en sangre entre tortugas vivas y muertas; las tortugas muertas mostraron concentraciones significativamente mayores de este metal que las vivas en sangre (0.14 vs 4.2 μg g^{-1} ww). Tambien Al y Tl presentaron concentraciones mucho más altas en sangre de tortugas muertas comparadas con las vivas.

La segunda parte de esta Tesis contiene los capítulos V al VIII. Estas secciones (3.1 a 3.5) contienen estudios que evalúan los biomarcadores de uso común en diversas especies para determinar el efecto de elementos inorgánicos en la salud de los individuos. Sin embargo, hasta la fecha existen muy pocos trabajos en tortugas marinas y ninguno de ellos en *Lepidochelys olivacea*. Además, durante esta Tesis se desarrolló un nuevo posible biomarcador utilizando la asimetría del caparazón de estas tortugas.

En primer lugar, se estudiaron algunos biomarcadores moleculares (sección 3.3): se determinó la expresión génica y la actividad enzimática de la metalotioneína (MT), superóxido dismutasa (SOD), catalasa (CAT) y glutatión reductasa (GR); así como su relación con varios elementos inorgánicos (Cd, Cr, Cu, Fe, Li, Ni, Pb, Sb, Se, Sr, Ti, T1 y Zn). Primero, los genes de *sod, cat* y *gr* se secuenciaron parcialmente a partir de la sangre, hígado y riñón de *L. olivacea* ya que esta información no existía en la base de datos de genes. Las MTs están entre las proteínas más importantes en la protección de las células contra la toxicidad de metales pesados. La expresión génica de *Mt* tuvo relaciones positivas con As y negativas con Cu en riñón. Además, la expresión génica y las relaciones significativas de la actividad enzimática se encontraron principalmente en el hígado. En cuanto a la actividad enzimática, la actividad hepática de SOD tuvo relaciones negativas en el hígado con Cd, Cr, Fe, Pb, Ti, Tl; mientras que la actividad de CAT tuvo relaciones positivas en el mismo órgano con Al, As, Cd, Se y Zn, y en riñón con As, Cd y Sb. La actividad de GR tuvo relaciones positivas en el hígado con Al, As, Cd, Cr, Fe, Sb y Ti, y en el riñón con Sb. El As fue el único elemento con relaciones positivas de GR en la expresión génica y la actividad enzimática en el hígado.

También se determinaron proteínas de estrés (secciones 3.4.1 y 3.4.2) mediante análisis bioquímico en muestras de suero de dos años diferentes. La actividad de ALT, AST, ALP, albúmina, creatinina, glucosa, urea, colesterol, actividad esterasa y cortisol se determinó durante dos años. Estos parámetros bioquímicos también se relacionaron con algunos elementos inorgánicos (As, Cd, Cr, Mn, Ni, Pb, Sr, Ti, Zn y Se). Se observó una correlación negativa entre la EA y el cortisol, se encontraron también otras relaciones entre estos biomarcadores e importantes contaminantes como Cd, As y Pb, y algunos elementos de creciente interés como Ti y Sr. Este estudio representa la primera evaluación de la posible relación entre elementos inorgánicos y parámetros bioquímicos en *Lepidochelys olivacea*. La evidencia sugiere varias asociaciones entre las concentraciones de metales y los parámetros bioquímicos. Por lo tanto, este estudio refuerza la utilidad de la sangre de las tortugas marinas para el monitoreo simultáneo de contaminantes ambientales y parámetros clínicos. Finalmente, y con el importante número de tortugas muestreadas (n= 100), estos resultados pueden utilizarse como guía de referencia para futuras investigaciones y para evaluar la salud de los individuos de rescate en esta área.

En el último capítulo de esta Tesis (VIII), primero se desarrolló una nueva herramienta para medir la asimetría del caparazón de las tortugas (DIx). En segundo lugar, se relacionó este DIx con concentraciones de 15 elementos inorgánicos (Bi, Cd, Li, Pb, Sb, Sr, Ti, Tl, Al, As, Co, Cr, Cu, Ni y Se) de 17 tortugas muertas (sangre, hígado, riñón, músculo, grasa, hueso, cerebro y huevo). *Lepidochelys olivacea* se caracteriza por una notable variabilidad morfológica en el número y la forma de los escudos de su caparazón, cuyo origen se piensa que se basa en un fondo genético permisivo. La influencia de los contaminantes en la inestabilidad del desarrollo y una de sus consecuencias, la asimetría de los individuos, se ha demostrado en varias especies. Sin embargo, el uso de esta asimetría como biomarcador de la contaminación en individuos adultos nunca ha sido explorado. Por lo tanto, se desarrolló un índice para cuantificar la inestabilidad del desarrollo (Developmental Instability Index –DIx-); tomando en cuenta el número y el tamaño relativo de los escudos costales del caparazón. Después se exploró la relación entre las concentraciones de elementos inorgánicos de DIx en

varios tejidos y componentes de huevo (cascarón, albumina y vitelo) de tortugas encontradas muestras en la suroeste de la costa pacífica mexicana. Se encontraron varias relaciones positivas entre DIx y elementos no esenciales, especialmente en los tejidos; así como relaciones negativas entre DIx y algunos elementos en ciertos componentes del huevo. La forma del caparazón en las tortugas marinas se determina durante la vida embrionaria, pero las concentraciones de metales en este trabajo se midieron muchos años después. Por lo tanto, los contaminantes presentes en estas tortugas adultas no pueden considerarse una reliquia de su contaminación en la etapa embrionaria o de cría, sino más bien adquirida durante su vida juvenil/adulta. Estos resultados sugieren que los individuos con caparazones más asimétricos parecen ser más susceptibles a acumular más metales pesados. Este tema debe ser estudiado en mayor profundidad para validar esta hipótesis, así como el uso de la asimetría del caparazón como un posible biomarcador de contaminación.

En conclusión, las tortugas Golfinas de la playa de *La Escobilla* parecen estar muy contaminada por ciertos elementos inorgánicos, especialmente en lo que se refiere a las concentraciones muy altas de Cd, esto requiere especial atención y seguimiento ya que el Cd es un metal con un alto potencial tóxico. Así mismo, se detectaron varias relaciones significativas relacionando diferentes biomarcadores con algunos elementos. Estos resultados también mostraron los tejidos de elección para medir diferentes marcadores bioquímicos, moleculares o elementos inorgánicos. Esta Tesis también genero completas bases de datos para parámetros bioquímicos, moleculares y elementos inorgánicos para futuras investigaciones y para evaluar la salud de los individuos rescatados en esta área. Sin embargo, también es importante mencionar que los organismos de vida silvestre están típicamente expuestos a múltiples factores estresantes naturales y antropogénicos; adicionalmente, a una mezcla de xenobióticos. Todos estos factores interactúan entre sí y producen múltiples efectos biológicos. Por ello, siempre es importante utilizar cuidadosamente estos biomarcadores y, con la poca información existente, no pueden todavía utilizarse rutinariamente como biomarcadores del impacto biológico de los contaminantes medioambientales por sí solos, sino sólo en comparación con los resultados previos. Sugiero así mismo más

experimentos de laboratorio/controlados antes de usar estos antioxidantes como biomarcadores en poblaciones de tortugas silvestres.

References

Abreu-Grobois, A., Plotkin, P.T., 2008. *Lepidochelys olivacea*, The IUCN Red List of Threatened Species.

Aguirre, A.A., Gardner, S.C., Marsh, J.C., Delgado, S.G., Limpus, C.J., Nichols, W.J., 2006. Hazards associated with the consumption of sea turtle meat and eggs: A review for health care workers and the general public. Ecohealth 3, 141-153.

Anderson, E.T., Minter, L.J., Clarke, E.O., 3rd, Mroch, R.M., 3rd, Beasley, J.F., Harms, C.A., 2011. The Effects of Feeding on Hematological and Plasma Biochemical Profiles in Green (Chelonia mydas) and Kemp's Ridley (Lepidochelys kempii) Sea Turtles. Vet Med Int 2011, 890829.

Andreani, G., Santoro, M., Cottignoli, S., Fabbri, M., Carpene, E., Isani, G., 2008. Metal distribution and metallothionein in Loggerhead (*Caretta caretta*) and Green (*Chelonia mydas)* sea turtles. Science of the Total Environment 390, 287-294.

Camacho, M., Oros, J., Boada, L.D., Zaccaroni, A., Silvi, M., Formigaro, C., Lopez, P., Zumbado, M., Luzardo, O.P., 2013. Potential adverse effects of inorganic pollutants on clinical parameters of Loggerhead sea turtles (*Caretta caretta*): Results from a nesting colony from Cape Verde, West Africa. Marine Environmental Research 92, 15-22.

Cardellicchio, N., Giandomenico, S., Ragone, P., Di Leo, A., 2000. Tissue distribution of metals in striped dolphins (Stenella coeruleoalba) from the Apulian coasts, Southern Italy. Marine Environmental Research 49, 55-66.

Casal, A.B., Camacho, M., Lopez-Jurado, L.F., Juste, C., Oros, J., 2009. Comparative study of hematologic and plasma biochemical variables in Eastern Atlantic juvenile and adult nesting loggerhead sea turtles (Caretta caretta). Vet Clin Pathol 38, 213-218.

Das, K., Debacker, V., Pillet, S., Bouquegneau, J.-M., 2003. Heavy metals in marine mammals. Toxicology of marine mammals 3, 135-167.

Eckert, K.L., Bjorndal, K., Abreu-Grobois, F.A., Donnelly, M., 2000. Técnicas de investigación y manejo para la conservación de las tortugas marinas, in: Marinas, G.E.e.T. (Ed.). UICN/CSE

Fazio, E., Liotta, A., Medica, P., Bruschetta, G., Ferlazzo, A., 2011. Serum and plasma biochemical values of health loggerhead sea turtles (Caretta caretta). Comparative Clinical Pathology 21, 905-909.

Fowler, S.L., 2005. Sharks, rays and chimaeras: the status of the Chondrichthyan fishes: status survey. IUCN.

IUCN, 2012. Red List of Threatened Species, Version 2012.1. International Union for Conservation of Nature, http://www.iucnredlist.org/.

Keller, J., McClellan-Green, P., 2004. Effects of organochlorine compounds on cytochrome P450 aromatase activity in an immortal sea turtle cell line. Marine Environmental Research 58, 347-351.

Keller, J.M., Kucklick, J.R., Stamper, M.A., Harms, C.A., McClellan-Green, P.D., 2004. Associations between organochlorine contaminant concentrations and clinical health parameters in loggerhead sea turtles from North Carolina, USA. Environ Health Perspect. 112, 1074-1079.

Keller, J.M., McClellan-Green, P.D., Kucklick, J.R., Keil, D.E., Peden-Adams, M.M., 2006. Effects of organochlorine contaminants on loggerhead sea turtle immunity: Comparison of a correlative field study and in vitro exposure experiments. Environmental Health Perspectives 114, 70-76.
Klaassen, C.D., Liu, J., Diwan, B.A., 2009. Metallothionein protection of cadmium toxicity. Toxicology and Applied Pharmacology 238, 215-220.
Labrada-Martagon, V., Rodriguez, P.A., Mendez-Rodriguez, L.C., Zenteno-Savin, T., 2011. Oxidative stress indicators and chemical contaminants in East Pacific green turtles (*Chelonia mydas*) inhabiting two foraging coastal lagoons in the Baja California peninsula. Comparative Biochemistry and Physiology Part C 154, 65-75.
Marquez, M.R., Peñaflores, C., Vasconcelos, J.C., 1996. Olive ridley turtles (*Lepidochelys olivacea*) show signs of recovery at La Escobilla, Oaxaca. Marine Turtle Newsletter 73, 5-7.
Matovic, V., Buha, A., Ethukic-Cosic, D., Bulat, Z., 2015. Insight into the oxidative stress induced by lead and/or cadmium in blood, liver and kidneys. Food Chem Toxicol 78, 130-140.
Misra, S., Niyogi, S., 2009. Selenite causes cytotoxicity in rainbow trout (*Oncorhynchus mykiss*) hepatocytes by inducing oxidative stress. Toxicology in Vitro 23, 1249-1258.
Morales-González , W.M., 2008. Análisis del trafico del huevo de Tortuga Golfina (*Lepidochelys olivacea*) en una ciudad del Istmo de Tehuantepec en Oaxaca. , Laboratorio de Investigación en Reproducción Animal. Universidad Autónoma "Benito Juárez" de Oaxaca. México, Oaxaca, Mexico.
Morcillo, P., Esteban, M.A., Cuesta, A., 2016. Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. Chemosphere 144, 225-233.
Perrault, J.R., Schmid, J.R., Walsh, C.J., Yordy, J.E., Tucker, A.D., 2014. Brevetoxin exposure, superoxide dismutase activity and plasma protein electrophoretic profiles in wild-caught Kemp's ridley sea turtles (*Lepidochelys kempii*) in southwest Florida. Harmful Algae 37, 194-202.
Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar Environ Res 93, 106-117.
Ruttkay-Nedecky, B., Nejdl, L., Gumulec, J., Zitka, O., Masarik, M., Eckschlager, T., Stiborova, M., Adam, V., Kizek, R., 2013. The role of metallothionein in oxidative stress. Int J Mol Sci 14, 6044-6066.
Santillana, P.R., 2013. Valores Hematológicos y bioquímicos sanguíneos de tortugas anidantes de Golfina (*Lepidochelys olivacea*) en El Salvador. BIOMA, 11-17.
Santoro, M., Meneses, A., 2007. Haematology and plasma chemistry of breeding olive ridley sea turtles (*Lepidochelys olivacea*). The Veterinary Record 161, 818-819.
Storelli, M.M., Storelli, A., D'Addabbo, R., Marano, C., Bruno, R., Marcotrigiano, G.O., 2005. Trace elements in loggerhead turtles (*Caretta caretta*) from the eastern Mediterranean Sea: overview and evaluation. Environmental Pollution 135, 163-170.
Tremblay, N., Ortiz Arana, A., Gonzalez Jauregui, M., Rendon-von Osten, J., 2016. Relationship between organochlorine pesticides and stress indicators in hawksbill sea turtle (*Eretmochelys imbricata*) nesting at Punta Xen (Campeche), Southern Gulf of Mexico. Ecotoxicology.

Valdivia, P.A., Zenteno-Savin, T., Gardner, S.C., Aguirre, A.A., 2007. Basic oxidative stress metabolites in eastern Pacific green turtles (*Chelonia mydas agassizii*). Comparative Biochemistry and Physiology, Part C 146, 111-117.
Venancio, L.P.R., Silva, M.I.A., da Silva, T.L., Moschetta, V.A.G., de Campos Zuccari, D.A.P., Almeida, E.A., Bonini-Domingos, C.R., 2013. Pollution-induced metabolic responses in hypoxia-tolerant freshwater turtles. Ecotoxicology and Environmental Safety 97, 1-9.

I. ANEXES

I.I Scientific production related to the Doctoral Thesis

I.I.I Scientific articles

Cortés-Gómez, A.A., Fuentes-Mascorro, G., Romero, D., 2014. Metals and metalloids in whole blood and tissues of Olive Ridley turtles (*Lepidochelys olivacea*) from La Escobilla Beach (Oaxaca, Mexico). Marine Pollution Bulletin 89, 367-375.

Cortés-Gómez, Adriana A; Girondot, Marc; Romero, Diego. The current situation of inorganic elements in marine turtles: A general review and meta-analysis. Environmental Pollution 229, 567:585.

Cortés-Gómez, Adriana A.; Girondot, Marc; Romero, Diego. Inorganic elements in blood and tissues of Olive Ridley turtles (*Lepidochelys olivacea*) from La Escobilla Beach (Oaxaca, Mexico). Three years of monitoring. (Prepared to be submitted)

Cortés-Gómez, Adriana. A.; Morcillo, Patricia; Guardiola, Francisco A.; Espinosa, Cristobal; Esteban, María A.; Cuesta, Alberto; Girondot, Marc; Romero, Diego. Molecular oxidative stress markers in Olive Ridley turtles (*Lepidochelys olivacea*) and its relation to metal concentrations in wild populations. (Submitted)

Cortés-Gómez, Adriana A.; Tvarijonaviciute, Asta; Teles, Mariana; Cuenca, Rafaela; Fuentes-Mascorro, Gisela; Romero, Diego. P-nitrophenyl acetate esterase activity and cortisol as biomarkers of metal pollution in blood of Olive Ridley turtles (*Lepidochelys olivacea*). (Submitted)

Cortés-Gómez, Adriana A.; Tvarijonaviciute, Asta; Romero, Diego. Potential effects of inorganic pollutants on biochemical parameters of Olive Ridley sea turtles (*Lepidochelys olivacea*): Results from an *arribada* nesting beach from Mexico. (Prepared to be submitted)

Cortés-Gómez, Adriana A; Romero, Diego; Girondot, Marc. Carapace asymmetry: A possible biomarker for metal accumulation in adult Olive Ridleys marine turtles? (Submitted)

I.I.II Conferences

Cortés-Gómez, Adriana A.; Girondot, Marc; Romero, Diego. Carapace asymmetry: A possible biomarker for metal and metalloids accumulation in adult Olive Ridleys marine turtles? **37th Annual Symposium of Sea Turtle Biology and Conservation.** Las Vegas, USA. April 15 – 20 2017. Poster

Cortés-Gómez, Adriana A.; Morcillo, Patricia; Guardiola, Francisco A.; Esteban, M. Ángeles; Cuesta, Alberto; Girondot, Marc; Fuentes-Mascorro, Gisela; Romero, Diego. Heavy metals and oxidative stress biomarkers in olive ridley (*Lepidochelys olivacea*) turtles from "La Escobilla" beach, Oaxaca, Mexico. **36th Annual Symposium of Sea Turtle Biology and Conservation.** Lima, Peru. February 29 - March 04, 2016 Oral presentation.

Cortés-Gómez, Adriana A.; Tvarijonaviciute, Asta; Cerón, José; Girondot, Marc; Fuentes-Mascorro, Gisela; Romero, Diego. Biochemical parameters and inorganic elements in *Lepidochelys olivacea* from "La Escobilla" beach, Oaxaca, Mexico. **36th Annual Symposium of Sea Turtle Biology and Conservation.** Lima, Peru. February 29 - March 04, 2016. Poster.

Cortés-Gómez, Adriana A. Polluants et biomarqueurs chez la Tortue Olivâtre (*Lepidochelys olivacea),* de la plage "La Escobilla", Oaxaca, Mexique. **Deuxième Colloque National sur les Tortues Marines.** Maison des Océan, Paris, France. September 8-10 2015. Oral presentation.

Cortés-Gómez, Adriana A.; Tvarijonaviciute, Asta; Cerón, José; Fuentes-Mascorro, Gisela; Romero, Diego. Relationship between Paraoxonase-1 and metal concentrations in blood of

Olive Ridley turtles from “La Escobilla” Beach (Oaxaca, Mexico). **35th Annual Symposium of Sea Turtle Biology and Conservation**. Dalaman, Turkey. April 19-24 2015. Oral presentation.

Cortés-Gómez, Adriana A.; Fuentes-Mascorro, Gisela; Romero, Diego. Lead and cadmium in blood, liver and kidney of olive ridley (*Lepidochelys olivacea)* from “La Escobilla” beach (Oaxaca, Mexico). **34th Annual Symposium of Sea Turtle Biology and Conservation**. New Orleans, USA. April 2014 10-17. Oral presentation

Cortés-Gómez, Adriana A.; Romero, Diego; Fuentes-Mascorro, Gisela. Inicio de una base de datos sobre elementos traza en tortugas golfinas *(Lepidochelys olivacea)* del santuario de La Escobilla, Oaxaca. **3ª Reunión Nacional sobre Tortugas Marinas.** Morelia, Mexico. October 2-5 2013. Oral presentation.

I.II Other scientific productions during the Doctoral Thesis

Pasanisi, E**; Cortés-Gómez, AA**; Perez-Lopez, M; Soler, F; Hernandez-Moreno, D; Guerranti, C; Martellini, T; Fuentes-Mascorro, G; Romero, D; Cincinelli, A. Levels of perfluorinated acids (PFCAs) in different tissues of Lepidochelys olivacea sea turtles from the Escobilla beach (Oaxaca, Mexico). Science of the Total Environment 2016; 572: 1059-1065.

Adel, Milad; **Cortés-Gómez, AA**; Dadar, Maryam; Riyahi, Hossein; Girondot, Marc. A comparative study of trace elements in the blood of male and female Caspian Pond Turtles (Mauremys caspica) from the southern basin of the Caspian Sea. Environmental Science and Pollution Research (Accepted, September 2017). DOI: 10.1007/s11356-017-0067-2

I.III Grants obtained

PhD scholarship from the Consejo Nacional de Ciencia y Tecnología (CONACyT), Mexico N° 216671. 2013-2017.

ERASMUS prácticas internacionales. Universidad de Murcia resolución R-382/2015. Junio 2015.

Grant student to attend to the 34th Annual Symposium of Sea Turtle Biology and Conservation. New Orleans, USA. April 2014 10-17.

Grant student to attend to the 35th Annual Symposium of Sea Turtle Biology and Conservation. Dalaman, Turkey. April 19-24 2015

Grant student to attend to the 36th Annual Symposium of Sea Turtle Biology and Conservation. Lima, Peru. February 29 - March 04, 2016.

Grant student to attend to the 37th Annual Symposium of Sea Turtle Biology and Conservation. Las Vegas, USA. April 15 – 20 2017.

I.IV International internships

Laboratoire d'Écologie, Systématique et Évolution, Université Paris-Sud, AgroParisTech, Centre National de la Recherche Scientifique, Université Paris Saclay, 91405 Orsay, France. From September 2015 to August 2017.