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"Microbiological and Parasitological Survey on Mediterranean Loggerhead Sea Turtles"

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“Each wave of the sea has a different light,
just as the beauty of who we love.”
Virginia Woolf

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Chapter 6

List of Abbreviations

AK	Amikacin
AMP	Ampicillin
API	Analytical Profile Index
bp	Base Pairs
C	Chloramphenicol
CAZ	Ceftazidime
CCL	Curved Carapace Lenght
CD	Cefpodoxime-clavulanic acid
ChHV	Chelonid Herpesvirus
CI	Confidence Interval
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamicin
CNS	Coagulase Negative Staphylococci
CPD	Cefpodoxime
CPS	Coagulase Positive Staphylococci
CT	Colistin sulphate
dd	Decimal Degrees
df	Degrees of Freedom
DO	Doxycycline
EPG	Eggs Per Gram
ESBL	Extended Spectrum Beta-Lactamase
FED	Focal Erosive Dermatitis
GPD	Grey Patch Disease
LETD	Lung-Eye-Trachea-Disease
LGRV	Loggerhead Genital-Respiratory Herpesvirus

LOCV	Loggerhead Oro-Cutaneous Herpesvirus
MTRC	Marine Turtle Research Centre
NA	Nalidixic Acid
OPG	Oocysts Per Gram
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Papillary Dermatitis
qPCR	Quantitative Polymerase Chain Reaction
S	Streptomycin
SCUD	Septicaemic Cutaneous Ulcerative Disease
SXT	Trimethoprim-sulfamethoxazole
TE	Tetracylcine

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Abstract

During the last decades, marine ecosystems have been over-exploited, and exposed to multiple stressors, resulting in the deterioration of their health status. Increasing pollution, harmful algal blooms, habitat degradation, emerging and re-emerging diseases in marine species, and many other concerning symptoms have given rise to the urgent need to monitor the fragile status of marine ecosystems. One method to address this complex issue is to identify and monitor sentinel species. Health assessment, exposure to environmental contaminants, mortality documentation and infectious disease surveillance are all complementary aspects of sentinel species monitoring, and could be investigated by wildlife rescue centres.

Sea turtles have already been used as bio-indicator of environmental pollution, due to their characteristics of longevity, trophic level, and habitat use. Nevertheless, sea turtle diseases have not been fully investigated, especially in the wild, and an exhaustive health assessment of sea turtle populations is still not possible.

This study consisted in a microbiological and parasitological survey on Mediterranean loggerhead sea turtles, with the main objective to assess the health status of both the individuals and the population, concurrently addressing the role of sea turtles as carriers of potential zoonotic agents and as sentinels for their ecosystems. Specifically, it focused mainly on loggerhead sea turtles in the Tyrrhenian Sea, examining both diseased and healthy animals, admitted and rehabilitated in a rescue centre, as well as unhatched eggs from loggerhead hatched nests.

The survey outlined the bacterial and parasitic communities of wild sea turtles in the Mediterranean, providing additional information to assess the health of individual sea turtles, based on which it is possible to recognize

deviations and signals of emerging threats to individuals, populations and ecosystems alike. The vast majority of the detected microorganisms are regarded as opportunistic pathogens, yet they should be taken into account when planning sea turtle conservation efforts. Moreover, some of the microorganisms detected in sea turtles are potential zoonotic agents, raising health concerns for other marine animals, as well as for humans that, for occupational or recreational activities, would come into contact with sea turtles. The various microorganisms appeared to be interconnected with each other in determining the health status of sea turtles, as well as with the ecosystem, being influenced by environmental factors. In conclusion, this study strengthened the link between turtle health and ecosystem health and consequently the role of sea turtles as sentinels of the ecosystem, integrating within the wider concept of One Health.

I - Monitoring Ecosystem

I - 1 Ecosystem Health and Sentinel Species

The concept of ecosystem health, especially in the marine environment, is relatively new. The requirements for an ecosystem to be defined healthy are many: to possess stable species abundance and diversity (at least not declining); not to be affected by environmental deterioration, nor unsustainable exploitation; not to experience frequent pollution events; not to register high frequencies of emerging or re-emerging diseases, nor intoxication; not to undergo mass mortality events (in particular those of "keystone" species) [Aguirre et al., 2002]. Over the last decades, marine ecosystems have endured multiple stressors that have affected their health, mainly caused by anthropogenic activities [Galgani et al., 2014; Waltzek et al., 2012]. Loss of breeding and nursery habitats, spread of persistent chemical pollutants (e.g. polychlorinated biphenyls and dioxins), high frequency of harmful algal blooms, and increased occurrence of emerging and re-emerging diseases (e.g. dolphin brucellosis, seal and porpoises Morbillivirus, sea otter toxoplasmosis, coral reef aspergillosis) are just few symptoms of the health deterioration we are observing nowadays in marine ecosystems [Aguirre and Tabor, 2004]. Recently, an urgent need for investigations and monitoring programs has risen, in order to evaluate the present and future status of these fragile ecosystems [Aguirre et al., 2002]. Two methods are the most frequently used to assess ecosystem health: one is to measure multiple environmental parameters [Hall and Kerr, 1991;

Halpern et al., 2012]; the other is to identify and monitor sentinel species [Hilty and Merenlender, 2000; Carignan and Villard, 2002].

Concerning sentinel species monitoring, not every species is suited to represent the proverbial "canary in the mineshaft", as specific characteristics are required. In particular, marine mammals are the most commonly adopted, which can be ascribed to their long life, high trophic level, tendency to bio-accumulate anthropogenic toxins, and the attention they attract from the public [Simeone et al., 2015; Aguirre et al., 2002]. Moreover, they frequently inhabit the same near-shore ecosystems utilized by humans and are susceptible to several of the same noxious agents (pathogens, toxins and chemical compounds), therefore acquiring also public health significance [Simeone et al., 2015]. Also sea turtles, which will be the subject of this dissertation, possess similar characteristics, and have already been adopted as sentinel species in different contexts (e.g. antibiotic-resistance, marine litter, fibropapillomatosis epidemiology) [Aguirre and Lutz, 2004; Al-Bahry et al., 2011; Camacho et al., 2014; Camedda et al., 2014; Galgani et al., 2014]. Additionally, sea turtles possess a dual nature, which will be better explored later: on one hand, they exhibit different degrees of site fidelity towards coastal habitats, allowing to collect reliable information about specific locations; on the other hand, they are migratory species, integrating environmental conditions over large coastal or ocean areas [Stewart et al., 2008]. Sometimes, monitoring just one sentinel species cannot assess the health status of an ecosystem. But instead an investigation on various species, representing different taxa, trophic levels, or ecological processes, might be necessary to address cumulative impacts of multiple environmental

variables [Tabor and Aguirre, 2004]. Sentinel species monitoring is composed of different, integrative aspects: health assessment, anthropogenic contaminant exposure, mass mortality documentation, and infectious disease surveillance [Aguirre et al., 2002].

Health assessment

Since the '40s, health has been no longer considered as lack of disease, but as a state of complete physical, mental and social well being [WHO, 1948]. In wildlife, specifically, it has been defined as the result of interacting biological, social, and environmental determinants that promote and maintain the organism's capacity to cope with change over time [Stephen, 2014]. Many efforts have been done to characterize and understand marine animal health. Indeed, normal values have to be determined in order to comprehend what is physiological, and to discriminate it from what is pathological. This is already being pursued for bottlenose dolphins (*Tursiops* spp.), Hawaiian monk seals (*Neomonachus schauinslandi*), bowhead whales (*Balaena mysticetus*), Northern fur seals (*Callorhinus ursinus*), other marine mammals and sea turtles [Tabor and Aguirre, 2004]. The health of the *B. mysticetus* population, for example, was related to ecosystem health and the offshore and coastal industrial (i.e., oil) activities in the Bering Sea [Rosa et al., 2000].

Anthropogenic contaminant exposure

Determining the levels of exposure to environmental contaminants in sentinel species is a useful tool to assess the degree of pollution of an ecosystem. The accumulation of heavy metals in sea birds [Tabor and Aguirre, 2004; Ishii et al., 2013] and the presence of antibiotic-resistant bacteria in sea turtles and other marine animals have been used for this purpose [Miranda and Zemelman, 2001; Al-Bahry et al., 2012]. Additionally, the early detection of toxic diseases in wild animals could prevent them from posing a significant risk to human populations: sea otters, and other animals feeding on bio-accumulating organisms (e.g. shellfish), are excellent sentinels to determine present and future hazards to human health [OIE, 2010; Jessup et al., 2004; Stewart et al., 2008].

Mass mortality documentation

Strandings could provide information about the health status of populations. Indeed, pathogens responsible for epizootics in marine animals are isolated in stranded animals first (e.g. Phocine Distemper Virus, Phocine Herpesvirus), as diseased animals strand more easily [OIE, 2010]. Nevertheless, caution should be exerted when inferring data from stranded animals to a population, because many sampling criteria (sex, life stage, geographic and temporal distribution) might be highly altered [Aguirre et al., 2002]. Mortality events of marine vertebrates could also be used to evaluate the status of marine ecosystems: in the north Atlantic, the increasing frequency of marine mammal mass mortality has been

suggested as an effect of human activities, particularly heavy pollution along coastal areas [Harvell et al., 1999; Aguirre et al., 2002].

Infectious disease surveillance

In the past, wildlife diseases have been neglected, unless they represented a direct threat to agriculture or human health [Daszak et al., 2000]. Nowadays, wildlife diseases have been recognized to adversely influence environmental health. On one hand, they could cause a loss of biodiversity, an effect that is particularly detrimental when it involves endangered species (e.g. chytridiomycosis in amphibians, white nose syndrome in bats, fibropapillomatosis in sea turtles,) [Jones, 2004; Belant and Deese, 2010; Aguirre and Lutz, 2004]. On the other hand, wildlife diseases can negatively affect human health, due to the increasingly frequent interactions between wildlife and humans [Belant and Deese, 2010; Daszak et al., 2001]. Indeed, many of the human emerging diseases have been connected to wildlife reservoir species [Jones, 2004]. These are the main reasons why surveillance of wildlife diseases is becoming increasingly important, and its results represent valuable information to four areas of public responsibility: public health; domestic animal health; wildlife conservation; environmental management [OIE, 2010; Ryser-Degiorgis, 2013]. During the past years, there has been an increase in the reports of marine animal diseases [Harvell et al., 1999; Marcogliese, 2008]. Several causative factors have been suggested, including: habitat alteration; invasive species; anthropogenic activities; and climate change [Marcogliese, 2008]. Actually, the emergence of disease is simplistically a

change in the balance between the host and the pathogen [Daszak et al., 2000]. Humans have been responsible for so countless alterations, that probably they caused the increasing wildlife susceptibility to pathogens [Belant and Deese, 2010]. The scarce baseline and epidemiological information on normal disease levels in the ocean hampers an appropriate and exhaustive evaluation of marine animal diseases [Harvell et al., 1999]. Indeed, it is necessary to determine what is endemic to marine environments: for example, studies have been conducted to determine if faecal pathogens harboured by seabirds derived from anthropogenic source, and more importantly, if they could be transmitted to other coastal animals [Bogomolni et al., 2008]. Monitoring marine animal diseases could advance the present knowledge on the ecology of infectious diseases, allowing not only a better assessment of ocean health, but also a better prediction of risks for human health, as it will be discussed shortly [Stewart et al., 2008; Bogomolni et al., 2008].

I - 2 Zoonoses

Only recently wildlife has begun to be regarded as vector and reservoir of zoonoses [OIE, 2010; Daszak et al., 2000]. Actually, around 60% of human infectious diseases are zoonotic, including more than 20 viral and more than 60 bacterial families, yet the majority of them could be considered as opportunistic infections [Cutler et al., 2010; Taylor et al., 2001 Woolhouse and Gowtage-Sequeria, 2005]. Wildlife has been identified as the source of almost three-quarters of zoonoses that emerged over the past two decades (e.g. influenza, severe acute respiratory

syndrome), giving rise to concerns about the threat it could pose to human health, not to mention to the economic stability [Taylor et al., 2001; Cutler et al., 2010; Mazet et al., 2009; Bengis et al., 2004; Zinsstag et al., 2007; OIE, 2010; Belant and Deese, 2010]. Two types of zoonoses are described: 1) diseases of animal origin, rarely transmitted to humans, but maintained by human-to-human transmission (e.g. Human Immunodeficiency Virus; Ebola Virus); 2) diseases of animal origin, frequently transmitted to humans (directly or by vectors), but seldom maintained by human-to-human transmission (e.g. *Erlichia*; *Leptospira*; Nipah Virus; West Nile Virus) [Bengis et al., 2004; Temmam et al., 2014]. Sometimes, a domestic animal species can act as the link between wildlife circulation and human circulation [Temmam et al., 2014]. That is why a complete understanding of the epidemiology of zoonotic pathogens in wild animals, as well as in humans and domestic animals is required to effectively carry out a public health programme [OIE, 2010]. Several authors tried to determine the factors that lead to the increasing interactions among human, domestic and wildlife populations, agreeing on the most relevant ones: growth of human population; rapid urbanisation; international travel and commerce; waste management; consumption of wildlife; changes in land use (e.g. farming, hunting, agriculture, fishery, recreational activities) and in ecosystems (e.g. biodiversity loss, forest encroachment, habitat degradation and fragmentation) [Acevedo-Whitehouse and Duffus, 2009; Bogomolni et al., 2008; Mazet et al., 2009; OIE, 2010; Temmam et al., 2014].

Zoonotic agents have been reported to spill over from terrestrial to marine species, and back, suggesting the failure of the land-sea interface as a barrier to disease transmission [Aguirre et al., 2006; Zavala-Norzagaray et

al., 2015; Harvell et al., 1999], and outlining marine animals not only as victims but also as vectors, as they could transfer pathogens to different locations in the ocean and terrestrial environments. Some potentially pathogenic microorganisms are already present in marine environments; some others are introduced by human activities (e.g. sewage discharges, agricultural run-offs) [Nogales et al., 2011; Harvell et al., 1999]. Most of the new marine diseases are not caused by new pathogens, but rather by known ones infecting newly recognized hosts (e.g. Canine Distemper Virus in phocids, Morbillivirus in cetaceans) [Harvell et al., 1999]. All pathogens found in common among marine mammals, sea birds and sharks, are recognized by the American Biological Safety Association as human pathogens, yet very little is known about their impact on animal and human health [Bogomolni et al., 2008]. These pathogens can be found in association with marine animals, phytoplankton, zooplankton, sediments and detritus [Stewart et al., 2008]. Pathogen transmission could occur through different routes: inhalation, contact or ingestion of water, exposure to marine aerosols, consumption of contaminated food resources (e.g. fish, shellfish, marine mammal products), handling of infected animals or carcasses (e.g. bites, wounds, stranding events) [Bogomolni et al., 2008; Harvell et al., 1999; Higgins, 2000; Stewart et al., 2008; Thompson et al., 2005; Waltzek et al., 2012]. Therefore, marine animal researchers, rehabilitators, trainers, veterinarians and volunteers, as well as subsistence hunters (e.g. whalers, sealers) have an increased risk of zoonoses acquisition through occupational exposure [Waltzek et al., 2012]. On the contrary, the role of lands used for recreational activities is controversial: some authors suggest them as a possible mean of infection [Bogomolni et

al., 2008; Staff et al., 2012], whereas others consider them relatively safe for the general public [Cutler et al., 2010; Waltzek et al., 2012]. Bacteria, viruses, fungi and protozoa that can infect humans have been detected in a range of marine sentinel species, including pinnipeds, cetaceans and sea otters [Stewart et al., 2008]. Concerning sea turtles, their role as reservoir of zoonotic agents has been addressed by several studies. Whilst different bacterial species (e.g. *Campylobacter*, *Edwardisella*, Escherichia, Citrobacter, *Klebsiella*, *Mycobacterium*, *Salmonella*, *Vibrio*) are considered to be potentially pathogenic to humans, viruses and fungi were not significantly linked to human disease [Alfaro et al., 2006; Fichi et al., 2016; Flint, 2013; Ahsan et al., 2017; Aguirre et al., 2006; Warwick et al., 2013; Ariel, 2011]. The exposure to sea turtle zoonoses might be increasing in the next years due to the expanding interest in ecotourism and conservation efforts, yet the interaction with free-living animals is considered of minor concern for human health [Ives et al., 2017; Warwick et al., 2013]. On the contrary, interactions with captive sea turtles, and the consumption of turtle products (i.e. meat and eggs), which is still common in some indigenous populations, raise important health concerns for the public [Warwick et al., 2013; Aguirre et al., 2006; Zavala-Norzagaray et al., 2015]. Severe dehydration, diarrhoea, vomiting, and even death have been reported as effects of the consumption of sea turtle products, caused by the presence not only of bacteria (e.g. *Salmonella* Chester, *Vibrio mimicus*), but also of a still unknown toxin (i.e. chelonitoxism) [O'Grady and Krause, 1999; Campos et al., 1996; Aguirre et al., 2006]. Nevertheless, as the prevalence of sea turtle acquired zoonoses is not known, and cases related to sea turtle consumption are likely underestimated, because of the

illegality of the practice [Warwick et al., 2013; Aguirre et al., 2006], biosecurity is always recommended when dealing with sea turtles in both field and captive situations [Jones et al., 2016].

I - 3 Antibiotic Resistance

Antibiotic resistance could, in some respects, be regarded as a zoonotic infection, since resistant bacteria from any source could spread their resistance to other bacteria, be they environmental or animal pathogens, and cross this way the species barrier [Bogomolni et al., 2008; Maravić et al., 2015]. Since the '80s, when the World Health Organisation highlighted the importance of antibiotic resistance on a worldwide scale, resistant bacteria have continued to increase, overwhelming the development of new antibiotics [Ahsan et al., 2017], and turning into a global problem from the medical, economical and ecological points of view [WHO, 2012; Johnson et al., 1998; Foti et al., 2009]. The rapid development of the phenomenon was mainly related to the presence of antimicrobials in the environment, result of a widespread overuse of disinfectants and pharmaceutical products in agriculture, human and veterinary practices [Kümmerer, 2009ab; Al-Bahry et al., 2011]. Impressively, some studies revealed that resistance levels decrease slowly even after the elimination of the selective pressure exerted by antimicrobials [Bogomolni et al., 2008]. The extensive employment of antibiotics in human medicine, veterinary medicine and animal productions, as well as agricultural runoff and coastal development, led to an increment of multi-drug resistant bacteria [Al-Bahry et al., 2012; Zieger et al., 2009], which are now commonly detected

among all pathogenic and commensal bacteria that inhabit humans and domestic animals, as well as wild animals and almost every environmental sample [Baquero et al., 1998; Al-Bahry et al., 2009; Al-Bahry et al., 2012; Zieger et al., 2009]. The presence of antibiotic resistance in commensal and environmental bacteria is of major concern, because they can serve as reservoir for resistance genes, and spread them to other bacteria, including pathogenic species [Nogales et al., 2011; Bogomolni et al., 2008]. Enterobacteriaceae, in particular, play a significant role in this transfer to and from other species, including human pathogens [Maravić et al., 2015]. Moreover, Berger-Bächi [2002] reported increasing numbers and intensifying severity of infections caused by resistant bacteria. The rise in antimicrobial resistance and its possible implications for animal and human health have led to an enhanced surveillance of microbial susceptibility [Kelly et al., 2006]. Different studies have been conducted to identify reservoir of antibiotic resistance in both environments and wildlife populations, including marine ones [Maravić et al., 2015; Foti et al., 2009; Miranda and Zemelman, 2001; Johnson et al., 1998]. Indeed, the detection of resistant bacteria from marine animals suggests their role in the dissemination of resistant strains, as well as the high anthropogenic impact endured by the ecosystems they inhabit [Ahsan et al., 2017; Al-Bahry et al., 2012; Foti et al., 2009; Maravić et al., 2015]. Several studies tried to investigate how wild marine animals, which have never been submitted to antibiotic therapy, could acquire antibiotic resistant bacteria. Although the sources of contamination might be different (e.g. sewage discharges; polluted effluents; agricultural runoff; animal manure; fish farms), the outcome is almost always the same: antibiotic resistant bacteria get to the

marine environment, where they contaminate marine habitats and are transmitted to marine animals [Al-Bahry et al., 2011; Miranda and Zemelman, 2001; Ahsan et al., 2017; Cabello, 2006; Chelossi et al., 2003; Nogales et al., 2011; Zieger et al., 2009; Foti et al., 2009]. Antibiotic resistant bacteria have been detected in many marine animals, from fish to mammals, including sea turtles and sea birds [Miranda and Zemelman, 2001; Johnson et al., 1998; Al-Bahry et al., 2009; Steele et al., 2005]. Interestingly, also thresher (*Alopias vulpinus*) and mako sharks (*Isurus paucus*) exhibited multi-drug resistant bacteria, although they do not forage in coastal environments, which are the most commonly contaminated [Bogomolni et al., 2008]. Even a Cuvier's beaked whale *(Ziphius cavirostris*) was found to host antibiotic resistance bacteria, despite being a deep water species, suggesting that terrestrial sources of these resistance genes may similarly have deep water sinks. As a matter of fact, high pressure may enhance the antibiotic resistance development [Hind and Attwell, 1996; Ferguson et al., 2006]. Also migratory species that were exposed to polluted effluents on their migratory routes might contain antibiotic resistant bacteria, as well as antibiotic residues and heavy metals [Miranda and Zemelman, 2001; Al-Bahry et al., 2011; Al-Bahry et al., 2009; Al-Bahry et al., 2012; Foti et al., 2009]. Actually, these species might play a very important role in the dissemination of antibiotic resistance in the marine environment [Bogomolni et al., 2008]. In particular, sea turtles, due to their life history (e.g. longevity, habitat fidelity, migratory nature), have been proposed as indicator of pollution in marine habitats, both coastal feeding ground and migratory routes [Ahsan et al., 2017; Al-Bahry et al., 2009; Al-Bahry et al., 2011; Al-Bahry et al.,

2012; Foti et al., 2009; Zieger et al., 2009]. Knowledge of the antibiotic resistance of marine animal bacteria might have practical applications in the rehabilitation facilities [Johnson et al., 1998]. On the other hand, marine animals treated with antibiotics during rehabilitation, usually yield resistant bacteria, thus playing a crucial role in spreading resistance genes in their natural environment, once they are re-introduced [Johnson et al., 1998; Ahsan et al., 2017]. In this context, antibiotics should always be used rationally, in order to prevent the selection of antibiotic resistant bacteria.

I - 4 Rehabilitation Centres

All aspects presented in the previous paragraphs might be collectively investigated by wildlife rehabilitation centres. Wildlife rehabilitation has been defined as the treatment and temporary care of injured, diseased and displaced indigenous animals, and the subsequent release of healthy animals to appropriate habitats in the wild [Miller, 2012]. Nevertheless, that is not the only objective of wildlife rehabilitation centres, which are management tools carrying out at least three different tasks: conservation, research, and education [Ullmann and Stachowitsch, 2015].

Conservation

The main purpose of rehabilitation centres is the reintroduction of healthy animals in the wild [Tribe and Brown, 2000; Molina-López et al., 2017]. The rehabilitation should be as quick and efficient as possible, in order to

avoid complications related to captivity (e.g. stress, infectious diseases, behavioural disorders) [Tribe and Brown, 2000; Orós et al., 2016]. On one hand the reintroduction of individuals in the wild could pose genetic and infectious risks to the resident population: released animals could breed and hybridize, or act as carriers for microorganisms which were not present in that geographical area, resulting in a population less fit for its environment [Tribe and Brown, 2000; Karesh, 1995]. On the other hand, reintroduction plays a significant role in conserving, stabilizing or augmenting a population. Moreover, data recorded in the centres could provide valuable information about the menaces in the wild that should be addressed by conservation programs [Karesh, 1995; Molina-López et al., 2017].

Research

Rehabilitation centres grant a large amount of scientific data, applicable to different research topics [Ullmann and Stachowitsch, 2015]: population census and distribution data could help assessing the status of a wild population and the impact of releasing animals in a specific area [Karesh, 1995]; the analysis of admission causes could disclose new threats to wild populations [Molina-López et al., 2017]; the medical management could lead to the development of new rehabilitation techniques and the improvement of the animal welfare [Tribe and Brown, 2000]; the access to a controlled setting could give the opportunity to better evaluate specific animal characteristics [Page-Karjian et al., 2015]; the analysis by disposition rate (i.e. euthanasia, mortality, release) could allow

comparative studies between centres [Orós et al., 2016]. Moreover, rehabilitation centres have been suggested to play a role in monitoring the health of ecosystems [Sleeman, 2008]. Indeed, collected data might be analysed to infer the situation in the wild, with few adjustments to take into account, as datasets might be biased toward a specific age class, spatial or temporal distribution [Camacho et al., 2016]. In that respect, rehabilitation centres could represent a valuable asset for the surveillance of pathogens in the wild, since diseased animals are more likely to be recovered and, vice versa, recovered individuals are more likely to suffer from disease. Additionally, all recovered animals should be submitted to a thorough health assessment, to rapidly reach a diagnosis and establish a therapy, but also to prevent the transmission of potential zoonotic agents to human handlers as well as to avoid the introduction of diseases in wild populations [Tribe and Brown, 2000; Camacho et al., 2016].

Education

The most beneficial role of rehabilitation centres is probably the public education, which increases the perceived value of the environment, promotes the understanding of environmental issues, and induces positive attitudes and pro-environmental behaviours [Martin et al., 2015; Tribe and Brown, 2000]. Public education could be achieved through direct involvement of the community in practical activities, or through awareness campaigns about wildlife problems and efforts taken to solve them. Education should target both professional and non-professional figures, but more importantly, they should educate the youth. Therefore, rehabilitation

centres could serve as a bridge between science and everyday life, involving people in the conservation cause and cultivating new generations of environmental stewards [Martin et al., 2015; Ullmann and Stachowitsch, 2015].

Sea Turtle Rehabilitation Centres

Concerning sea turtles, the Regional Activity Centre for Specially Protected Areas (established in Tunis in 1985 by the United Nations Environment Programme, in order to assist Mediterranean countries in implementing the Protocol concerning Specially Protected Areas and Biological Diversity in the Mediterranean) acknowledged the importance of rehabilitation centres during the '80s [RAC/SPA, 2004]. Rehabilitation centres are recognized to play a significant role in the conservation of wild populations, by reducing mortality of sub-adult and adult turtles, which are the most commonly affected by the two major threats at sea (i.e. fishery bycatch, boat collision) [Orós et al., 2016; Ullmann and Stachowitsch, 2015]. Indeed, modelling studies have indicated that large juvenile turtles have a high reproductive value [Wallace et al., 2008] and that population stability is more affected by the survival of older turtles than by that of eggs and hatchlings [Mazaris et al., 2005; Mazaris et al., 2006; Heppel et al., 2002]. Moreover, sea turtle rehabilitation centres keep data on each recovered turtle, providing information on both mortality factors and spatio-temporal distribution [Casale et al., 2010]. In this marine context, public education should target, above all, fishermen, as they are the main operators who can reduce the post-release mortality of bycaught turtles

[Casale et al., 2007]. Considering that the mortality rate of bycaught sea turtles is high [Casale, 2011], and that each adult is the only surviving individual out of 500-1000 hatchlings, not to mention their life history (slow growth, long period before reproduction), evidently every rescued turtle is important [Ullmann and Stachowitsch, 2015].

II - Loggerhead Sea Turtles

In the following paragraphs a general description of loggerhead sea turtles (*Caretta caretta*) will be presented, as well as their main infectious agents, focusing on bacteria, viruses and parasites. A broad overview of loggerhead sea turtles is fundamental to study and understand all aspects of their diseases [Alfaro et al., 2006].

II - 1 Biology

Loggerhead sea turtles are distributed throughout the subtropical and temperate waters across neritic and oceanic habitats in the Pacific, Indian and Atlantic Oceans and the Mediterranean Sea [Abdelrhman et al., 2016; Plotkin, 2002; Miller, 1997]. The Mediterranean Sea is among the world's richest places, in terms of biodiversity. Indeed, it has been acknowledged as a Global Biodiversity Hotspot (Myers et al. 2000) [Cuttelod et al., 2008]. Between 4% and 18% of the global marine species dwell in the Mediterranean. This is particularly remarkable, considered that the Mediterranean represents just 0.82% in surface and 0.32% in volume of the world ocean areas [Bianchi and Morri, 2000]. Among the three species

commonly found in Mediterranean waters, *Caretta caretta* is the most abundant one [Margaritoulis et al., 2003; Bentivegna, 2002; Bentivegna et al., 2001; Aznar et al., 1998; Camedda et al., 2014]. This species has evolved a local subpopulation, relatively isolated from the Atlantic ones, as proved by genetic markers [Margaritoulis et al., 2003; Casale et al., 2009a]. The western and eastern Mediterranean basins are both used, providing nesting beaches (mainly situated in the eastern basin, e.g. Greece, Turkey, Cyprus, Libya, Tunisia, Israel, Syria, Lebanon, Egypt) [Margaritoulis et al., 2003; Bentivegna, 2002; Mingozzi et al., 2007; Casale and Margaritoulis, 2010], foraging grounds (e.g. Gulf of Gabes, Turkey, Egypt, Adriatic Sea, Ionian Sea, Strait of Sicily, Tyrrhenian Sea, Spain) [Margaritoulis et al., 2003; Bentivegna et al., 2001; Casale et al., 2008; Lazar and Tvrtković, 2001; Tomás et al., 2002; Casale et al., 2012b; Gómez de Segura et al., 2003], wintering areas (e.g. Gulf of Gabes; Adriatic Sea) [Margaritoulis et al., 2003; Lazar and Tvrtković, 2001; Camiñas, 2004], and migratory pathways (e.g. Strait of Sicily, North African coast) [Stokes et al., 2015; Broderick et al., 2007; Casale et al., 2012a]. Loggerhead sea turtles, as the majority of sea turtles, are long living, slow growing and late maturing, all characteristics that lead to the risk of dying before reproducing [Heppel et al., 2002; Bolten, 2002]. The life span has been imagined in the range of 50-75 years, as longevity records are limited [Wyneken et al., 2006]. Growth rates are highly variable, as well as age at sexual maturation [Heppel et al., 2002], even within the same population, and their estimation requires approaches such as capture–mark–recapture, skeletochronology or length-frequency analyses. On average, loggerheads in the Mediterranean appear to take four

years to grow to 30 cm of Curved Carapace Length (CCL) [Casale et al., 2009b], and between 16 and 29.3 years to reach sexual maturity [Casale et al., 2009a; Casale et al., 2011]. Only mature adults show external sexual dimorphism, notably an elongated tail and longer claws in males [Wyneken et al., 2006; Casale et al., 2014]. The reproductive cycle is strongly dependent on environmental conditions, aiding both survival of the parents and offspring and allowing the maximal reproductive effort [Miller, 1997]. Male and females usually reproduce every few years, depending on the energy reserves that they have accumulated since the last reproduction (capital breeders) [Hamann et al., 2002; Miller, 1997]. Courtship and mating occur in the month or two preceding oviposition, with each male mating with several females, and vice versa [Miller, 1997; Hamann et al., 2002; Heppel et al., 2002]. Females show a stereotyped nesting behaviour, which usually occurs at night, and consists in the following phases: emerging from the surf, ascending the beach, excavating the body pit, digging the egg chamber, oviposition, covering the egg chamber and the body pit, and returning to the sea [Miller, 1997]. The egg incubation lasts 6-13 weeks, depending on the incubation temperature, which also determines the sex of the hatchlings. In order to compensate for the low survivorship of hatchlings, sea turtles reproduce many times in a lifetime, laying several clutches of large quantities of eggs in each nesting year [Heppel et al., 2002; Miller, 1997]. Nevertheless, the number of breeding seasons, as well as the number of clutches and eggs, is variable even within the same species [Miller, 1997]. In the Mediterranean, most nesting occurs between May and September, and is characterized by short incubation duration and probably female-biased sex ratios in hatchling

production [Godley et al., 2001; Margaritoulis et al., 2003]. Sea turtles, perhaps especially loggerheads, are characterized by a complex life cycle [Bolten, 2002]. Hatchlings usually emerge during night, and immediately orient themselves toward the ocean, mainly thanks to visual cues, crawling down the beach [Miller, 1997; Heppel et al., 2002; Lohmann et al., 1997]. Subsequently, thanks to wave direction and magnetic orientation, hatchlings rapidly swim out to the open ocean, where they can develop safe from competition and predation [Bolten, 2002; Lohmann et al., 1997; Mansfield and Putman, 2013]. During this stage, movements and distribution are influenced mainly by meteorological and oceanographic factors (e.g. winds and currents), although post-hatchlings can actively move using magnetic orientation [Putman et al., 2012; Putman et al., 2015]. When they reach bigger size, after a variable period of time, juvenile loggerheads start recruiting to neritic habitats, where they continue their development, probably at a higher growth rate [Bolten, 2002; Heppel et al., 2002]. It has been suggested that the shift from the oceanic stage to the neritic stage is not a clean change, but rather a transitional period, during which turtles keep using both habitats, and which sometimes continues also during their adult life [Bolten, 2002; Jones and Seminoff, 2013; Casale et al., 2008; Tomas et al., 2001; Laurent et al., 1998; Mansfield and Putman, 2013]. Adult loggerhead turtles may temporarily leave their habitat during the reproductive or overwintering migrations, usually through oceanic migration corridors towards courtship, mating, internesting, or nesting areas and warmer foraging grounds [Bolten, 2002; Heppel et al., 2002]. Therefore, loggerhead sea turtles use several different habitats during their life (i.e. terrestrial, oceanic and

neritic), fulfilling important functions in all of them. Elucidating the ecological importance of sea turtles is hampered by the decline of sea turtle populations, but probably the most studied role is the one as consumers [Bjorndal and Jackson, 2002; Heithaus, 2013]. Loggerhead sea turtles are primarily carnivorous, probably the most generalist sea turtles, feeding in various habitats, such as phanerogam beds, gorges and caves, rocky, muddy and sandy bottoms [Casale et al., 2008]. The impacts of turtles on their prey populations are still largely unknown, although many prey items have been identified in most of the age classes [Heithaus, 2013; Bjorndal and Jackson, 2002; Casale et al., 2008]. Oceanic post-hatchlings mainly feed upon epipelagic organisms (e.g. hydrozoans, algae, jellyfish, larval crustaceans, fish eggs) [Jones and Seminoff, 2013; Bjorndal, 1997]; whereas juveniles and adults appear to feed at progressively higher trophic levels [Delgado et al., 2011], consuming both pelagic (e.g. tunicates, crustacean, molluscs, jellyfish, insects, ray-finned fish) and benthic organisms (e.g. flatworms, bivalves, polychaetes, molluscs, echinoderms, crustaceans, ascidians, anemones, snails, fish) [Lazar et al., 2008; Jones and Seminoff, 2013; Casale et al., 2008]. Sponges, as well as algae and plants, though frequently recovered, were completely undigested. On the other hand, the consumption of dead organisms, such as fish discarded by commercial fishery, seems to be a local response typical of the Mediterranean and few other regions, which supports the opportunistic feeding strategy of loggerhead sea turtles [Tomas et al., 2001; Casale et al., 2008; Bentivegna et al., 2001; Jones and Seminoff, 2013]. Besides acting as consumers, sea turtles interact with other marine species in several ways: they serve as preys for other animals; as hosts for parasites and

pathogens; as substrate and transport for epibionts; as resource for cleaning organisms. Moreover, they play important roles for their ecosystems: they transfer nutrients and energy (from rich foraging grounds to poor nesting beaches); they modify the physical structure of the habitat (digging trenches on the seabed in search for prey or digging up seedlings on the beach, preventing the encroachment of vegetation); they maintain high biological activity in marine sediments (bioturbation that impact benthic communities and nutrient dynamics) [Bjorndal and Jackson, 2002; Bjorndal, 1997; Heithaus, 2013]. Loggerhead sea turtles, with their generalist diet, were supposed to have lower site fidelity, but instead, this differing trophic status is reflected by their wider home range [Broderick et al., 2007]. Loggerhead turtles show fidelity to feeding, nesting and wintering habitats, to which they return after successive migrations, guided by biological compasses, oceanic currents, waterborne chemicals, windborne information, bathymetric features and water temperatures [Broderick et al., 2007; Musick and Limpus, 1997; Plotkin, 2002; Lohmann et al., 1997; Casale et al., 2014; Avens et al., 2003; Ullmann and Stachowitsch, 2015]. Sea turtle lives consist in two types of regular or seasonal migrations, which have evolved to compensate for environmental variability and unpredictability: breeding migrations and wintering migrations [Lohmann et al., 1997; Plotkin, 2002]. During the reproductive period, both female and male sea turtles migrate asynchronously from foraging areas to breeding areas [Plotkin, 2002]. At the end of the mating period, males return to the foraging areas, yet few males are reported to be resident in the breeding area throughout the year [Plotkin, 2002; Miller, 1997 Lohmann et al., 1997]. Females, differently, disperse to nesting sites,

most of the time represented by their natal beaches [Miller, 1997; Heppel et al., 2002]. Between ovipositions, females reside in the interesting habitat, near the nesting beaches [Musick and Limpus, 1997]. After the last oviposition, females return to their own specific feeding areas [Plotkin, 2002; Lohmann et al., 1997; Miller, 1997]. Concerning wintering migrations, in temperate areas loggerhead sea turtles have been suggested to leave their shallow feeding grounds for deeper offshore waters during winter, to subsequently return when temperatures rise [Avens et al., 2003; Broderick et al., 2007; Casale and Simone, 2017]. Actually, in the Mediterranean, changes in seawater temperature have not been documented to induce seasonal migrations, with the exception of the northernmost regions, which could explain the seasonal movement described between the western and the eastern basins [Bentivegna, 2002; Casale et al., 2012c; Luschi et al., 2013; Zbinden et al., 2011].

The Mediterranean loggerhead subpopulation is considered Least Concern under current Red List criteria of the International Union for Conservation of Nature. Nevertheless, this status should be considered conservation-dependent, as the population would decrease without intense conservation programs [Casale, 2015]. Indeed, loggerhead sea turtles face numerous perils during their life, starting from the loss of nesting beaches (due to beach armouring, nourishment, mining, cleaning, tourism, and lighting), and continuing with a vastness of direct and indirect threats to which juveniles and adults are exposed: hunting (e.g. food and souvenir trade), bycatch (e.g. long lines, trawlers), boat collision, plastic ingestion and entanglement, habitat pollution, and natural causes (e.g. predation, diseases) [Bjorndal and Jackson, 2002; Bolten, 2002; Casale, 2011; Flint,

2013; Flower et al., 2015; Heppel et al., 2002; Lutcavage et al., 1997; Pritchard, 1997; Wyneken et al., 2006].

II - 2 Diseases

Generally speaking, pathophysiology and pathogenesis of sea turtle diseases have not been fully investigated [Herbst and Jacobson, 2002]. More information is needed on the effects, the prevalence, the routes of transmission, and the promoting factors of diseases in sea turtle populations [Flint, 2013]. Despite some concepts are available on captive sea turtles, there is to consider that the health problems encountered in them, as well as their characteristics (e.g. clinical manifestations, severity), might be different from those encountered in wild populations [Herbst and Jacobson, 2002]. Indeed, healthy hosts usually do not develop disease from infectious agents, due to host-pathogen coevolution, whereas stress and immunosuppression, promoted during captivity, might result in the development of disease [George, 1997; Herbst and Jacobson, 2002]. Determination of parameters and range of conditions in healthy animals within a population is fundamental to recognize any deviation from normality, in order to perform a proper health assessment and to evaluate the potential role of pathogens and infectious diseases in sea turtle population ecology [Herbst and Jacobson, 2002; Flint, 2013]. This will include: agents to which the population is exposed; extent of exposure; prevalence and severity of infections [Herbst and Jacobson, 2002; Flint, 2013]. Data collected from both live and dead sea turtles could help increasing the knowledge of diseases affecting sea turtle populations

[Herbst and Jacobson, 2002; Flint, 2013]. For example, in a recent review on loggerhead sea turtles recovered in a rehabilitation centre of Gran Canaria (Spain), the authors identified juvenile loggerheads as the ones with a significant higher risk of infectious diseases. Additionally, they reported that infectious diseases were the cause of recovery in 5.5% of cases, that these were more prevalent during spring and summer, and caused the highest mortality rate among admitted turtles [Orós et al., 2016]. Such are the kind of data and information that will help to assess the present and future health of sea turtles.

II - 2.1 Bacteria

The incidence of bacterial infections in wild sea turtles is relatively rare, thanks to their tough integument and competent immune systems. On the contrary, bacterial infections are more common in captive sea turtles, yet they can be remarkably reduced through appropriate management. [George, 1997; Higgins, 2002]. Bacteria mainly gain entrance either through injury of the dermal tissues or aspiration of seawater, causing respectively abscesses or pneumonias [George, 1997]. Many others are already present in the body compartments of sea turtle as normal bacterial flora [Higgins, 2002; Glazebrook and Campbell, 1990a; Santoro et al., 2006b]. Either case, bacteria could eventually get to the bloodstream and disseminate throughout the entire body, resulting in multifocal abscesses or septicaemia [George, 1997; Ogden et al., 1981]. Numerous bacteria have been isolated from sea turtles, healthy and diseased alike, including: *Aeromonas* spp.; *Bacteroides* spp.; *Clostridium* spp.; *Edwardsiella* spp.;

Enterobacter spp.; *Escherichia coli*; *Citrobacter* spp.; *Flavobacterium* spp.; *Mycobacterium* spp., *Morganella* spp. *Proteus* spp.; *Providencia* spp., *Pseudomonas* spp.; *Salmonella* spp.; *Shewanella* spp.; *Streptococcus* spp.; *Vibrio* spp. [George, 1997; Glazebrook and Campbell, 1990b; Glazebrook et al., 1993; Higgins, 2002; Flint, 2013; Ahsan et al., 2017; Chuen-Im et al., 2010; Flint et al., 2009; Zavala-Norzagaray et al., 2015]. Usually, more species are cultured from a single lesions, making more difficult to understand whether one species is responsible and the other contaminants, or if they function synergistically [George, 1997]. Nevertheless, bacterial pathogenic nature should be cautiously interpreted, because most of them are opportunistic agents, naturally present in seawater or as part of the sea turtle bacterial flora, becoming pathogenic when the animal health is compromised for any reason (e.g. stress; injuries, challenging environmental conditions) [Ahsan et al., 2017; Glazebrook and Campbell, 1990ab; Glazebrook et al., 1993; Higgins, 2002; Flint, 2013; Flint et al., 2009; Foti et al., 2008; Zavala-Norzagaray et al., 2015; Wyneken et al., 2006; Alfaro et al., 2006]. Indeed, knowledge of the normal composition of bacterial flora of wild sea turtle, which is still limited, could be useful to better interpret the results of any bacteriological culture, as well as the role of bacteria as pathogenic agents [Santoro et al., 2006b]. Generally, the most serious bacterial infections are caused by Gram-negative microorganisms, whereas Gram-positive play a less significant role [Glazebrook and Campbell, 1990a; Innis et al., 2014]. Sign of primary or secondary bacterial infections are mostly non-specific (e.g. lethargy, debilitation, inappetence). [Higgins, 2002; Wyneken et al., 2006].

The main bacterial infections, for the most part in common with other sea turtle species, will be shortly introduced in the next paragraphs.

Ulcerative Stomatitis – Obstructive Rhinitis – Bronchopneumonia complex

A group of bacterial diseases, jointly referred to as US-OR-BP complex, has been described in captive sea turtles, causing high mortality among hatchlings and juveniles. The individual manifestations can be present separately or in combination [George, 1997]. Ulcerative stomatitis has been described as the most frequent bacterial infection in captive sea turtles [Glazebrook et al., 1993], as well as the most frequent consequence of the ingestion of fishing hooks [Orós et al., 2004]. Usually, the first sign is the inflammation and the presence of a caseous plug in the oropharyngeal cavity, resulting in anorexia, listlessness, and eventually dyspnoea [Glazebrook and Campbell, 1990a Glazebrook et al., 1993]. Obstructive rhinitis can occur in association with ulcerative stomatitis, yet not as frequently. Bronchopneumonia represents a further complication, when the caseous material involves the lower respiratory tract. In this case, the turtle might float on one side, unable to maintain neutral buoyancy [Glazebrook and Campbell, 1990b; Glazebrook et al., 1993]. The oral mucosa might exhibit inflamed or ulcerated areas, and caseous material might be present in the nares, oropharyngeal cavity, trachea and bronchi. The bacteria most commonly isolated from affected turtles are: *Aeromonas hydrophila*, *Flavobacterium*, *Pseudomonas*, and *Vibrio alginolyticus* George, 1997; Glazebrook et al., 1993; Orós et al., 2004].

Dermal Infections

Skin lesions are very common in sea turtles, especially on protruding areas such as neck, tail and flippers [Glazebrookand Campbell, 1990ab; Higgins, 2002]. Indeed, skin lesions due to trauma are the most common, and easily become infected. Different dermal syndromes have been described, including: focal erosive dermatitis (FED), focal dermal granulosis, septicaemic cutaneous ulcerative disease (SCUD), and papillary dermatitis (PD) [Leong et al., 1989; Orós et al., 2005]. Lesions might be characterized by discoloration of the dermis, superficial or deep ulceration (FED, SCUD), or by proliferative manifestations (PD). Dermal ulceration is the most alarming sign, as it provides an easy way for bacteria to reach the bloodstream and lead to lethal septicaemia. Despite being different syndromes, the bacterial genera isolated from the lesions might be the same, including: *Aeromonas*; *Citrobacter*; *Proteus*; *Pseudomonas*; *Staphylococcus*; *Vibrio* [George, 1997; Leong et al., 1989; Orós et al., 2005; Glazebrook and Campbell, 1990a].

Gastrointestinal Infections

Several gastrointestinal diseases have been associated with bacterial infections, either as the result of the ingestion of fishing devices, or as multisystemic septicaemic lesions. Different types of esophagitis (e.g. ulcerative, fibrinous), gastritis (e.g. necropurulent, fibrinous), enteritis (e.g. fibrinous, catarrhal, necrotizing, necropurulent, granulomatous), and hepatitis (e.g. fibrinous, necrotizing, granulomatous) have been described

[Torrent et al., 2002; Orós et al., 2004; Flint et al., 2009; Orós et al., 2005]. Several bacteria have been isolated from these lesions: *Aerococcus viridans*, *Enterococcus faecalis*, *Aeromonas*, *Bacillus*, *Citrobacter*, *Escherichia coli*, *Pasteurella*, *Proteus*, *Pseudomonas*, *Serratia*, *Staphylococcus*, *Streptococcus*, and *Vibrio* [Torrent et al., 2002; Fichi et al., 2016; Orós et al., 2004; Orós et al., 2005].

Respiratory Infections

Respiratory infections are not common as dermal or gastrointestinal infections, but are more often fatal and highly contagious. The most common signs are the loss of balance, with the turtle floating on one side, and the dyspnoea [Higgins, 2002; Glazebrook et al., 1993]. Few cases of tuberculosis and granulomatous pneumonia have been reported to be caused by *Mycobacterium avium* and *Mycobacterium marinum*, probably due to airborne and waterborne transmission [Leong et al., 1989; Nardini et al., 2014; George, 1997; Glazebrook and Campbell, 1990a]. Limited attempts have been made in live turtles to isolate the agents responsible for the respiratory infection, because of the intrusive nature of collecting samples from the lungs [Higgins, 2002]. Nevertheless, from dead turtles with sign of bronchopneumonia and granulomatous pneumonia, the following bacteria have been cultured from the lesions: *Aeromonas*, *Bacillus*, *Burkholderia*, *Citrobacter*, *Pasteurella*, *Proteus*, *Pseudomonas*, *Staphylococcus*, *Streptococcus* and *Vibrio* [Orós et al., 2005].

Ocular and Salt Gland Infections

Ocular disorders, associated to traumas or hypovitaminosis, take several forms: from keratitis to conjunctivitis, from blepharitis to corneal ulceration [Glazebrook and Campbell, 1990a; Higgins, 2002; Isler et al., 2014; Orós et al., 2005]. Infections develop easily, involving the primary and accessory tear glands, the eyelids and the eyeballs [Isler et al., 2014]. Signs vary from yellow deposits on the eyelids or the cornea, exophthalmos, enophthalmos, strabismus, blepharospams, chemosis, discharge, to complete erosion of tissues [Glazebrook and Campbell, 1990a; Higgins, 2002; Isler et al., 2014]. Opportunistic pathogens are responsible for invading the damaged tissues, including: *Achromobacter, Aeromonas*, *Burkholderia*, *Flavobacterium*, *Pseudomonas* and *Staphylococcus* [Glazebrook and Campbell, 1990a; Isler et al., 2014; Orós et al., 2005].

There have been very few reports of salt gland adenitis. Most of them described exudate, abscesses or caseous necrotic debris within the parenchyma of the glands, from which several bacteria were isolated, in pure or mixed cultures: *Aerococcus*, *Aeromonas*, *Citrobacter*, *Pseudomonas*, *Staphylococcus*, and *Vibrio* [Orós et al., 2005; Orós et al., 2011; Glazebrook and Campbell, 1990a].

Brain Infections

Few cases of bacterial meningitis have been described, subsequently to trauma or septicaemia [Fichi et al., 2016; Stacy et al., 2010a]. Only one

case of multifocal bacterial encephalitis was reported in a loggerhead sea turtle stranded in Florida. During handling, the animal exhibited hyperflexion of the neck and spastic movements of the flippers. Haemorrhages and necrosis were detected in the brain and the meninges, whereas caseous nodules were between the cerebellum and the brain stem. Bacterial cultures lead to the isolation of *Corynebacterium* [George, 1997].

Renal Infections

Bacterial renal diseases included chronic interstitial nephritis, granulomatous nephritis, and perinephric abscesses. These lesions, consequential to traumatic injuries or to a septicaemic status, have been associated to: *Aeromonas*, *Citrobacter*, *Escherichia coli*, *Proteus*, *Staphylococcus*, and *Vibrio* [Orós et al., 2005].

Bone Infections

Infections of the skeletal system are uncommon [Glazebrook and Campbell, 1990a]. Nevertheless, bacterial osteomyelitis and osteoarthritis have been reported, mainly caused by *Mycobacterium chelonae* and *Enterococcus faecalis* [Flint et al., 2009; Innis et al., 2014; Paré et al., 2006]

II - 2.2 Viruses

In the marine environment, viruses are 10 times more abundant than bacteria, accounting for up to the 94% of nucleic acid containing particles [Alavandi and Poornima, 2012]. Viruses infect from bacteria to marine mammals, including also sea turtles, eventually causing cellular damage, and facilitating the entrance of other pathogens (e.g. bacteria and fungi) [Alavandi and Poornima, 2012; Alfaro et al., 2006]. The etiology of viral diseases can be attributed to various viral families. In particular, two are the best documented in sea turtles (i.e. Herpesviridae and Papillomaviridae), but others (i.e. Iridoviridae, Reoviridae, Retroviridae and Togaviridae), described in terrestrial chelonians, are suspected to infect also sea turtles [Alfaro et al., 2006]. Additionally, the fish pathogens Betanodaviridae have been recently reported in sea turtles, suggesting their role as carriers for this family [Fichi et al., 2016].

Concerning Papillomaviridae, they have been documented to cause generalized proliferative dermatitis, characterized by white cutaneous lesions on the head and the limbs of the sea turtle. Despite the infection appeared to be not lethal, these viruses could become more significant in immunosuppressed animals, and transmit easily through direct contact, representing a risk for sea turtles in rehabilitation centres [Manire et al., 2008b].

Regarding Herpesviridae, a variable host range characterizes them, yet they are well adapted to their hosts, as a consequence of prolonged co-evolution [Alfaro et al., 2006; Alfaro-Núñez et al., 2014]. Herpesvirus infection usually exhibit acute signs, due to the short replication cycle and

cell-associated viraemia, but subsequently they could remain quiescent for the rest of the animal life, due to the ability to establish latent infection [Alfaro-Núñez et al., 2014; Ariel, 2011; Page-Karjian et al., 2015]. Transmission usually occurs following primary infection or reactivation of latent infections, induced by stressful factors (e.g. disease, malnutrition, temperature change) [Page-Karjian et al., 2015]. Herpesviridae are classified in three subfamilies (i.e. alpha-, beta-, and gammaherpesvirinae). There are currently six alphaherpesvirinae known to infect chelonian, named Chelonid Herpesvirus (ChHV) from 1 to 6. In particular, three of them (i.e. ChHV1, ChHV 5, ChHV 6), specific to sea turtles, represent an important health concern, as they have been associated with three distinct contagious syndromes: Grey Patch Disease (GPD); Lung-Eye-Trachea-Disease (LETD); fibropapillomatosis [Stacy et al., 2008; Jones et al., 2016; Alfaro et al., 2006].

Grey Patch Disease

ChHV1 has been described in association with GPD [Jones et al., 2016; Ariel, 2011], a cutaneous disease, first documented by Rebell in captive-reared green turtles post-hatchlings [Rebell et al., 1975; George, 1997]. GPD has been reported to affect 8-weeks-old to 1-year-old sea turtles [Ritchie, 2006; Alfaro et al., 2006]. Transmission has been suggested to be vertical or water-borne [Ariel, 2011]. Lesions occur mostly on the anterior part of the body (i.e. head, neck, and front flippers), in two different forms: spontaneously resolving small circular papules or lethal rapidly spreading areas of grey patches with superficial necrosis [George, 1997; Higgins,

2002; Ariel, 2011; Ritchie, 2006; Origgi, 2006; Alfaro et al., 2006]. Stressing factors, such as poor water quality, high temperatures and overcrowding, have been considered precipitating factors in the development and severity of lesions [Ariel, 2011; Higgins, 2002; Ritchie, 2006; Alfaro et al., 2006]. Additionally, age seems to play a role in the susceptibility and severity of lesions: hatchlings are more susceptible to the lethal form, whereas the majority of juveniles develop the classic lesions but subsequently survive, probably developing also some kind of immunity [Higgins, 2002; George, 1997; Origgi, 2006].

Lung-Eye-Trachea Disease

ChHV6 has been associated with the LETD [Jones et al., 2016]. The disease has been described to affect juvenile turtles (1-2 years old), and to be influenced by both infectious and environmental factors [Origgi, 2006; Ritchie, 2006]. ChHV6 has been successfully isolated and propagated in cell culture, allowing more in-depth studies [Origgi, 2006]. Indeed, the virus has been documented to survive in seawater for about 1-2 weeks [Alfaro-Núñez et al., 2014; Page-Karjian et al., 2015], which is probably true for other herpesviruses as well, representing a successful mechanism for horizontal transmission. Disease usually localizes to the eye, oropharynx, lungs, and trachea, and is characterized by ulceration and accumulation of caseous debris [Alfaro et al., 2006; Origgi, 2006; Ariel, 2011; Ritchie, 2006]. The turtles exhibit respiratory signs, buoyancy abnormalities, keratitis, conjunctivitis, tracheitis and pneumonia,

eventually dying within several weeks or remaining chronically infected for months [Origgi, 2006; Ritchie, 2006].

Fibropapillomatosis

Fibropapillomatosis is probably the most studied infectious disease of sea turtles. The disease was first described in 1938 in a green turtle in Florida, but since the '80s it has reached epizootic proportions, affecting all sea turtles species in all major oceans [Aguirre et al., 1994; Aguirre et al., 2002; Aguirre and Lutz, 2004; Flint et al., 2009; Wyneken et al., 2006; Flint, 2013]. Currently, fibropapillomatosis has a circumtropical distribution, with prevalence varying according to the location [Aguirre et al., 2002; Alfaro-Núñez et al., 2014; Alfaro-Núñez and Gilbert, 2014]. Two theories have been proposed to explain the sudden increase of fibropapillomatosis: one is an environmental change that increased the susceptibility of sea turtles; the other is a virulent mutant form of the virus [Jones, 2004]. Two etiological hypotheses have been advanced for fibropapillomatosis. The first regarded tumours as hyperplastic cellular reactions, probably to scar tissue. The second recognized the existence of one or more etiologic agents, causing neoplastic lesions [Aguirre and Lutz, 2004]. Even within the second etiological hypothesis, controversial suggestion have been put forward, with some authors pointing at enveloped virus (i.e. Herpesvirus and Retrovirus), while others at non-enveloped virus (i.e. Papillomavirus and Polyomavirus), as responsible for the disease [Aguirre and Lutz, 2004]. Presently, the strongest evidence indicates the involvement of a Herpesvirus, specifically ChHV5, although

Koch's postulates have not been fulfilled due to an inability to culture it [Page-Karjian et al., 2015; Herbst et al., 1995; Work et al., 2009; Work et al., 2003]. Four variants of ChHV5 (namely A, B, C, and D) have been described in Florida. Interestingly, different sea turtle species, dwelling in the same geographical location, shared the same variant, suggesting a strong geographical influence on the transmission of disease [Ene et al., 2005; Jones et al., 2016]. ChHV5 is not the sole responsible for manifestation of disease, as it requires additional environmental or immune related co-factors [Alfaro-Núñez et al., 2014; Alfaro-Núñez et al., 2016; Ariel, 2011; Jones et al., 2016; George, 1997]. Parasites (i.e. spirorchid ova, marine leeches), bacteria, chemical pollutants, excessive solar radiation, water temperatures, bio-toxins (*Prorocentrum* species and products of *Lyngbya majuscula* algal blooms), might all be contributing factors, as well as impaired immune system and genetic predisposition [Aguirre et al., 1994; Dailey and Morris, 1995; Landsberg et al., 1999; Flint, 2013; Aguirre and Lutz, 2004; Ariel, 2011; Flint et al., 2009; George, 1997]. Indeed, juvenile turtles are the most affected [Ariel, 2011; George, 1997], becoming infected following recruitment to near-shore environments, especially those with high human density, agricultural runoff, pollutants and bio-toxin producing algae [Aguirre et al., 2002; George, 1997; Flint, 2013; Aguirre et al., 1994; Aguirre and Lutz, 2004; Alfaro-Núñez et al., 2014; Jones et al., 2016; Page-Karjian et al., 2015]. Transmission occurs horizontally by direct or indirect contact with infected epidermal cells or other means (e.g. saliva, mucus, blood, urine, semen), whereas the possibility of vertical transmission has been excluded [Alfaro-Núñez et al., 2014]. It has been suggested that viral particles could survive

in salt water for short periods [Page-Karjian et al., 2015; Alfaro-Núñez et al., 2014]. Infected turtles may either manifest symptoms or remain latently infected, depending on a long and balanced interaction between the virus and its host [Alfaro-Núñez et al., 2014]. Viral DNA has been found at higher level in tumours than in healthy tissue samples. Low viral loads have been found also in clinically healthy animals, suggesting a way to identify turtles that will eventually develop tumours [Quackenbush et al., 2001; Duarte et al., 2012; Fichi et al., 2016; Page-Karjian et al., 2015]. Fibropapillomatosis is a debilitating neoplastic disease, characterized by multiple epithelial fibropapillomas and internal fibromas [Aguirre and Lutz, 2004; Alfaro-Núñez et al., 2014; Alfaro-Núñez and Gilbert, 2014]. Tumours may appear small or big (from 0.1 to 30 cm), smooth or rough, flat or nodular, pigmented or not. They usually localize on soft tissues of the axillary and inguinal regions, eyes, flippers, carapace and plastron, as well as on internal organs (i.e. lungs, heart, liver, spleen, kidneys, gastrointestinal tract, gonads) [Aguirre and Lutz, 2004; Aguirre et al., 2002; Ariel, 2011; Duarte et al., 2012; Flint et al., 2009; Flint, 2013; Ritchie, 2006]. Severity tends to increase, as turtles become larger [Aguirre et al., 1994; Jones et al., 2016; George, 1997; Wyneken et al., 2006]. The tumours are benign and do not usually cause death directly, but they provoke space-occupying effects, such as hampering vision, swimming, diving, feeding, and may ultimately prove fatal [George, 1997; Flint et al., 2009; Flint, 2013; Jones, 2004; Page-Karjian et al., 2015; Jones et al., 2016; Ritchie, 2006; Wyneken et al., 2006]. Additionally, the disease results in suppression of the immune system, whereas the lesions, once ulcerated, are responsible for secondary bacterial infections and

consequent bacteraemia [Jones, 2004; Ritchie, 2006; Wyneken et al., 2006]. Only few reports have described spontaneous regression [George, 1997; Flint, 2013].

Other Herpesviruses

Two additional alphaherpesviruses have been identified in loggerhead sea turtles: the Loggerhead Oro-Cutaneous Herpesvirus (LOCV) and the Loggerhead Genital-Respiratory Herpesvirus (LGRV).
LOCV has been described to cause oropharyngeal ulceration and cutaneous lesions, similar to those associated with GPD (to which it seems closely related).
LGRV has been described to infect genital mucosa, muco-cutaneous junctions and respiratory mucosa, causing ulcerative lesions. The characteristics of infections are similar to the LETD, and the two viruses appeared genetically similar, despite no genital lesion has been described for the LETD. Infection has been suggested to occur through sexual or vector (marine leeches) transmission [Stacy et al., 2008].

II - 2.3 Parasites

Parasites are integral components of marine ecosystems. Their study could provide valuable data on the general ecosystem functioning (e.g. biodiversity, food web stability), as well as helpful information on their hosts (e.g. species migrations, phylogenetic history) [Santoro and Mattiucci, 2009].

Wild sea turtles have a natural burden of parasites: both ecto- and endo-parasites have been reported, including helminths, protozoa, arthropods and annelids [Greiner, 2013; Wyneken et al., 2006]. Many sites of a sea turtle can be affected by parasites, indicating their adaptation for a very long time [Greiner, 2013], but their pathogenicity should be assessed with caution, as it depends on a complex balance involving hosts, parasites and environment [George, 1997]. In the wild, some helminths are considered part of the normal flora. Actually, in healthy sea turtles parasites rarely cause problems [Wyneken et al., 2006], especially adult organisms, which usually have little or no effect, while the immature stages are the ones causing greater damages [George, 1997]. Nevertheless, the host may exhibit signs of illness a considerable amount of time after infestation, when its immune system is compromised by additional factors, whether it be a trauma, a concurrent disease, or a stressful phase of the life cycle (i.e. migration, nesting) [Wyneken et al., 2006; Jacobson, 2007]. Sea turtles usually act as definitive host, yet in a few cases, like for Trypanorhynchs and *Anisakis* spp., they may serve as intermediate or paratenic host [Greiner, 2013]. According to their host-specificity, parasites are classified either as specialists or as generalists: a specialist species is defined as one recovered only from one sea turtle species; on the contrary a generalist species is a parasite found in two or more turtle species (generalist in sea turtles) or in other vertebrate species [Greiner, 2013; Santoro et al., 2006a]. Aznar et al. [1998] suggested that sea turtles are so distinct from other marine vertebrates, that they could exchange parasites only with other sea turtle species, despite regular contacts with parasites from other marine hosts (with the exception of the accidental occurrence of immature

helminths). Additionally, host-specificity can narrow so much that some parasites found in a sea turtle species could not survive, or develop, in another one [Santoro and Mattiucci, 2009]. Indeed, the composition of parasite communities in sea turtles is influenced by several geographical, biological and ecological factors. This is especially true for helminth communities, which are shaped by distinctive sea turtle traits, such as lifespan and life cycle, population distribution and density, site fidelity and migrations, habitat use and diet [Santoro and Mattiucci, 2009; Gračan et al., 2012]. Therefore, it is not surprising that geographically widespread generalist feeders, such as loggerhead sea turtles, are exposed to numerous potential intermediate hosts, resulting in a richer helminth community, dominated by generalists [Santoro et al., 2006a; Aznar et al., 1998]. On the other hand, specialized herbivores, such as green turtles, host a helminth community mainly composed by digenean trematodes, characterized by higher degrees of specificity [Santoro et al., 2006a; Gračan et al., 2012].

Focusing on the loggerhead sea turtle, the most abundant sea turtle species in the Mediterranean, several parasitological studies have been conducted, reporting strong dissimilarities in the helminth communities among turtles from different locations [Santoro et al., 2010a]. Over 300 loggerhead sea turtles were examined across the Mediterranean, resulting in a relative depauperate helminth community: ten species of digenetic flukes, four species of nematodes, two larval acanthocephalans, and one post larval tapeworm were reported [Greiner, 2013]; twelve of these species were defined to be specialists of sea turtles [Santoro et al., 2010a]. In the eastern Atlantic, at the entrance of the Mediterranean, Valente et al. [2009] reported a richer parasite fauna in loggerheads off Madeira compared to

loggerheads from the western Mediterranean, but a lower abundance of helminth species and lower infection levels in individual sea turtles. On the contrary, along the Mediterranean coasts of Spain, Aznar et al. [1998] noted higher infection levels, as well as a helminth community characterized by greater abundance, and composed mainly by two species of digenetic trematodes (i.e. *Enodiotrema megachondrus* and *Calycodes anthos*), plus other occasional species like *Anisakis* spp.. In Italian waters, the same composition, yet with variable abundance and infection levels, was found around Sicily and on the Ionian side of Calabria; whereas off the coasts of Campania sea turtles exhibited the most diverse helminth community (11 species), composed by reduced numbers of *E. megachondrus* and *C. anthos*, but greater amounts of nematodes specific for sea turtles [Santoro et al., 2010a]. Similarly, low prevalence of *E. megachondrus* and *C. anthos* and greater diversity of species were documented in the Adriatic Sea, where Gračan et al. [2012] described a helminth community composed by five digenetic trematodes and three nematodes dominated mainly by *Orchidasma amphiorchis*, *Pachypsolus irroratus* and *Anisakis* spp.. The similarities in helminth communities of geographically distinct sea turtle aggregations could be a result of the analogies among the ecosystems, but more likely it is due to the long-distance movements of sea turtles, which promote population mixing [Santoro et al., 2010a]. On the contrary, the differences in the patterns among helminth communities in Mediterranean loggerhead sea turtles seem to support the hypothesis that parasite communities reflect the ontogenetic shift that juvenile loggerheads undergo from oceanic to neritic habitats. Indeed, smaller turtles, in their oceanic stage, feed mostly on

epipelagic preys; however, their lower food intake coupled with the oligotrophic condition of oceanic habitat limits the availability and encounter with intermediate hosts. On the other hand, when larger turtles recruit to neritic habitat, their diet shifts towards both pelagic and benthic prey, resulting in an elevated risk of helminth acquisition, especially during the transitional period between the two stages [Gračan et al., 2012; Santoro et al., 2010a; Valente et al., 2009].

Regarding the detection and the identification of parasites in sea turtles, the vast majority of parasitological surveys in the literature, partly due to the endangered status of sea turtles, make use of stranded carcasses or animals deceased in rescue centres. Either case, the primary means of detecting parasites is by external and internal examination to collect adult parasites. Another way, especially used for the diagnosis of helminthiasis, is the detection of eggs by the faecal examination method, whether it be by flotation or sedimentation technique. Generally, the identification of helminths requires thorough anatomical examination. The adults are classified according to several distinct morphological characteristics (e.g. cephalic region, reproductive organs, male terminal region). On the other hand, the eggs are identified mainly on the basis of their size and morphology [Greiner, 2013]. The following paragraphs will better explore the most common parasites recovered from loggerhead sea turtles.

Trematoda

Trematodes are the most disparate and copious parasites in sea turtles [Greiner, 2013], more than in any other reptile [Jacobson, 2007]. They are

characterized by complex life cycles, most of which still unknown, that include from one to three intermediate host species in order to be completed [Greiner, 2013]. The aspidogastrid fluke *Lophotaspis vallei* is the only non-digenean trematode, exclusively reported in loggerhead sea turtles [Santoro and Mattiucci, 2009; Greiner, 2013]; a single gastropod has been suggested as its intermediate host [Wharton, 1939]. All trematodes infect sea turtles through the ingestion of cercariae-rich intermediate hosts [George, 1997; Orós et al., 2016], represented in the majority of cases by molluscs [Santoro and Mattiucci, 2009]. The precise identification of all intermediate host species is complicated by the wide range of habitats used by sea turtles, and the high diversity of potential intermediate hosts in each habitat [Stacy et al., 2010b].

Flukes may affect several organs in sea turtles, but generally they are restricted to a primary site for development; as regards gastrointestinal flukes, they dwell preferentially the upper intestine, where they find a nutrient-rich environment [Greiner, 2013]. Beginning from the oesophagus, down to the lower intestine, gastrointestinal fluke species are distributed as follows [Santoro and Mattiucci, 2009; Greiner, 2013; Wyneken et al., 2006]:

- Oesophagus: *Diaschistorchis pandus*, *Lophotaspis vallei*, and *Pachypsolus irroratus*.
- Stomach: *Calycodes anthos*, *Diaschistorchis pandus*, *Enodiotrema megachondrus*, *Lophotaspis vallei*, and *Pachypsolus irroratus*.
- Upper intestine: *Calycodes anthos*, *Cymatocarpus undulatus*, *Enodiotrema carettae*, *Enodiotrema megachondrus*, *Lophotaspis*

vallei, *Orchidasma amphiorchis*, *Rhytidodes gelatinosus*, and *Styphlotrema solitaria*.

- Middle intestine: *Pleurogonius trigonocephala*, and *Pyelosomum renicapite*.
- Lower intestine: *Pyelosomum renicapite*, and *Pyelosomum chelonei*.
- Liver and gall bladder: *Calycodes anthos*.

The prevalence and the intensity infection of gastrointestinal flukes in the Mediterranean, with the only exception of *R. gelatinosus*, were much lower (less than a half) than the one determined overseas (Florida) [Greiner, 2013]. In particular, *C. anthos* and *E. megachondrus* were recovered only in small juvenile turtles of the Adriatic Sea, supporting the hypothesis that these parasites have a predominantly pelagic cycle [Gračan et al., 2012; Santoro et al., 2010a]. Similarly, *O. amphiorchirs* abundance showed negative correlation with increasing host size. On the other hand, *R. gelatinosus* showed correlation with sex, exhibiting higher abundance in juvenile males than in juvenile females [Gračan et al., 2012]. Usually, gastrointestinal flukes cause minor irritation and damage [Wyneken et al., 2006; Jacobson, 2007], yet they can induce clinical disease in case of heavy infestation or debilitated hosts [George, 1997]. The chronic inflammation may result in diarrhoea, starvation, fluid imbalance, and dehydration [Wolke et al., 1982].

Just one trematode species, *Plesiochorus cymbiformis*, has been found living in the urinary bladder of sea turtles, yet no related pathology has been described [Greiner, 2013].

An entirely different matter is represented by blood flukes, included mainly in the family Spirorchiidae, which are considered the most harmful parasites in sea turtles [George, 1997; Jacobson, 2007]. Spirorchiids are vascular system generalists: the adults reside in heart and major blood vessels (e.g. aorta, mesenteric arteries, hepatic vessels), through which they disseminate eggs all over the host's body [Greiner, 2013; George, 1997; Jacobson, 2007; Wolke et al., 1982; Stacy et al., 2010a]. In loggerhead sea turtles, three genera (i.e. *Hapalotrema*, *Neospirorchis* and *Carettacola*) have been identified and reported to affect up to 33% of the Atlantic population [George, 1997; Wolke et al., 1982; Stacy et al., 2010a]. On the contrary, only three records of *Hapalotrema* involved Mediterranean loggerhead sea turtles to date [Santoro et al., 2017]. Spirorchid infestation has been described as a chronic debilitating disease of sub-adult loggerhead sea turtles [George, 1997]. It has been suggested that they become infected when recruiting into neritic habitats, and that infection levels decrease with age, either because of an increased immunity of the host or a reduced fecundity of the parasite [Work et al., 2005]. The severity depends on the site and intensity of infection: adults and eggs in the vascular system may cause irritation and occlusion, resulting in endocarditis, vasculitis, haemorrhages, thrombosis and ischemia [Jacobson, 2007; Wolke et al., 1982]; on the other hand, the disseminated eggs induce granulomatous reaction wherever they lodge [Wyneken et al., 2006; George, 1997; Raidal et al., 1998]. Important sequelae are secondary infection: reports of pneumonitis, enteritis and cystitis are not uncommon [Wolke et al., 1982; Stacy et al., 2010a]. Moreover, the role of spirorchids as vectors, in particular for diseases like fibropapillomatosis, is being

investigated [George, 1997]. It's because of their pathological significance that ante-mortem diagnosis has been implemented via enzyme-linked immunosorbent assays [George, 1997; Orós et al., 2016].

Nematoda

Nematodes are another important component of helminth community in sea turtles, yet relatively few species have been documented, compared to the high diversity of trematodes [Santoro and Mattiucci, 2009; Greiner, 2013]. The majority of records refers to ascarid-like nematodes (Ascaridomorpha), specifically: *Sulcascaris sulcata*, *Anisaki*s spp., *Cucullanus carettae*, *Kathlania leptura*, and *Tonaudia tonaudia* [Greiner, 2013; Greiner, 2013]. Limited reports involve other nematodes, such as *Echinocephalus* spp. and *Angiostoma carettae*. Sea turtles serve as definitive hosts for all nematodes, with the exception of *Anisakis* spp., whose definitive host is represented by cetaceans and pinnipeds. In this case, sea turtles act as paratenic hosts, but their source of infection in the wild is still to be ascertained [Santoro et al., 2010b; Glazebrook and Campbell 1990F]. Generally, molluscs serve as intermediate hosts [Wyneken et al., 2006; George, 1997], but larval anisakids and kathlanids have been documented to develop also in crustaceans and fishes [Santoro and Mattiucci, 2009]. Loggerhead sea turtle is the most important host for *S. sulcata* [Santoro et al., 2010a; Piccolo and Manfredi, 2001], which is the nematode most frequently recovered in this species [Gračan et al., 2012; Piccolo and Manfredi, 2001]. Its intermediate hosts (benthic gastropods and bivalves) are particularly associated to coastal habitats, justifying the

limited distribution of the parasite in shallow coastal regions, predominantly in the eastern Mediterranean and along the coasts of Campania (south-western Italy) [Gračan et al., 2012; Santoro et al., 2010a].

Adult ascarid-like nematodes reside in the stomach and upper intestine of loggerhead sea turtles, as well as larval forms of *Echinocephalus* spp. [Santoro and Mattiucci, 2009; Wyneken et al., 2006; Jacobson, 2007], whereas *Anisakis* larvae can migrate to the liver, spleen, lungs and coelomic cavity [Santoro et al., 2010b]. The few cases of *Angiostoma carettae* regarded exclusively the upper respiratory tract [Manire et al., 2008a]. Usually, nematodes have little pathological significance in sea turtles [George, 1997] and clinical signs are non-specific [Jacobson, 2007; Santoro et al., 2010a]. Merely mild cases of gastritis and enteritis were ascribed to adult nematodes [Greiner, 2013; George, 1997; Orós et al., 2004]; on the contrary, the migrating larvae are responsible for greater damages, including: ulcers, focal necrosis, and granulomatous perihepatitis [Jacobson, 2007; Santoro et al., 2010b; Orós et al., 2004]. As for *Anisakis* spp., genetic markers have been successfully utilised for the molecular identification of the larvae, allowing this nematode to be proposed as a biological tag for the origin and migratory routes of its hosts [Mattiucci et al., 2007].

Cestoda

Cestodes in sea turtles are scarcely documented, despite larval forms are known to infect sea turtles [Jacobson, 2007]. Exclusively the order of

Trypanorhyncha has been detected, albeit with low prevalence, in loggerhead sea turtles [Greiner, 2013; Sey, 1977]. Sea turtles serve as intermediate host for trypanorhynchs, as they use elasmobranchs as definitive host. Little is known on the pathogenicity of these parasites; nevertheless elevated number of cysts have been found distributed in the coelomic cavity, either associated with the mesenteries or attached to other organs (i.e. lungs, stomach, liver and intestine) [Greiner, 2013; Jacobson, 2007].

Protozoa

Amoebae and flagellates, as well as two coccidian species, are listed among protozoan parasites of sea turtles, though only two species have been described in loggerhead sea turtles: *Entamoeba invadens* and *Eimeria caretta* [Greiner, 2013; Wyneken et al., 2006; George, 1997]. On the contrary, there have been no cases of haemoparasitic protozoan in sea turtles [Santoro and Mattiucci, 2009]. All protozoa in sea turtles are characterized by direct life cycle, developing in the intestinal epithelial cells or lumen [Santoro and Mattiucci, 2009; Greiner, 2013], where they might induce enterohepatitis [Jacobson, 2007]. Despite being a fresh-water species, *E. invadens* have been associated with death in captive sea turtles, presumably infested by the ingestion of contaminated food [George, 1997; Jacobson, 2007]. In contrast, *E. caretta* has not been associated with intestinal pathology, though its oocysts have been habitually detected in stool samples of loggerhead sea turtles [Wyneken et al., 2006; George, 1997; McArthur, 2004].

Arthropoda

Arthropods are not common parasites of sea turtles. Only two species have been documented to infest adult sea turtles: an haematophagous midge that feeds on nesting females, and a mite species discovered in the cloacal wall of green sea turtles, frequently inducing cloacitis [Greiner, 2013]. On the contrary, more species, mainly larvae of Diptera and Coleoptera, are known to infest nests of sea turtles, as will be discussed later. However, their dangerousness is controversial: in some cases larvae have been reported to harm healthy hatchlings, reducing the hatching success up to 30%; in other cases larvae have been reported to feed on weakened or dead hatchlings, representing no threat to the reproductive success of sea turtles [Broderick and Hancock, 1997], and actually reducing the risk of infection by removing decaying material [Spadola et al., 2016].

Annelida

Two species of leeches are documented to infest sea turtles, but only *Ozobranchus margoi*, due to its wider distribution, has been recovered from loggerhead sea turtles [George, 1997; Piccolo and Manfredi, 2001]. Marine leeches can complete their life cycle on the sea turtle host, rapidly establishing severe infestation [Jacobson, 2007]. Adult leeches, as their eggs, usually reside on the soft tissues between carapace and plastron (i.e. axillary and inguinal areas), but sometimes they can be recovered on the skin around eyes, mouth and cloaca. Light infestations have no health impact on sea turtles, but animals with heavy burden of leeches can present

with anaemia, macerated dermal tissue, and debilitation [George, 1997; Jacobson, 2007; Piccolo and Manfredi, 2001]. Additionally, *Ozobranchus* species are being investigated for their role as vectors for other pathogens; in particular, their association with fibropapillomatosis [George, 1997; Jacobson, 2007].

Epibionts

The term epibiont should indicate an organism that has neither beneficial nor detrimental effect on its host; therefore, sea turtle epibionts should not be discussed in this paragraph. Nevertheless, they deserve at least to be mentioned, because large numbers of these organisms could increase the stress of the sea turtle host, or just negatively affect it by increasing the surface drag [George, 1997]. Moreover, embedding barnacles, such as the genera *Stephanolepas* and *Stomatolepas*, can cause local tissue damage [Jacobson, 2007], exposing sea turtles to the risk of secondary bacterial or fungal infections [George, 1997]. Controversial roles have been attributed to *Planes minutus*: previously considered responsible for cloacitis and distress in sea turtle hosts, this crab species is now believed to play a cleaning role [Spadola et al., 2016].

III – Objectives

This study consisted in a microbiological and parasitological survey on Mediterranean loggerhead sea turtles. The leading objective was the assessment of the health status of both the individuals and the population.

In pursuing this objective, this study concurrently aimed at outlining the microbiological framework of *Caretta caretta*, in order to provide useful information to assess the commensal or pathogenic role of the different microorganisms detected in this species, as well as the influence of the environment on them. A better understanding of these aspects would be fundamental to address possible emerging threats to this endangered species, and to apply proper conservation measures. Additionally, this survey aimed at eventually disclosing the important role of loggerhead sea turtles both as carriers of potential zoonotic agents and as sentinels for the ecosystem.

To accomplish this, loggerhead sea turtles, mostly coming from the Tyrrhenian Sea, were examined. Specifically, this study focused on: i) diseased turtles, admitted at the Marine Turtle Research Centre (Stazione Zoologica Anton Dohrn of Naples, Italy); ii) healthy turtles, after being rehabilitated and declared free of disease; iii) unhatched loggerhead sea turtle eggs, excavated from already hatched nests.

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Chapter 1

Prevalence, antimicrobial resistance and environment-related modifications of bacteria isolated from loggerhead sea turtles (*Caretta caretta*) in the western Mediterranean

1.1 Introduction

Scientific information on marine ecosystem health is still lacking and research is crucial to further develop the knowledge needed to support robust natural resource management [Aguirre and Tabor, 2004; Cuttelod et al., 2008]. One way of trying to get a handle on marine ecosystem health assessment is by surveying sentinel species. In this case, specific marine wildlife species (e.g. pinnipeds, cetaceans, marine birds) can serve as sentinels for the quality of health of marine ecosystems [Aguirre and Tabor, 2004; Aguirre et al., 2002; Burger and Gochfeld, 2004]. Recently, mortality events involving vertebrates at various trophic levels have been used as a measure of the health of the Gulf of Mexico and Atlantic Ocean [Aguirre et al., 2002]. Indeed, the health of marine animals is seen to reflect the health of their ecosystems, and ultimately of humans [Wilcox and Aguirre, 2004].

Sea turtles are valid sentinels of specific habitats since they are long lived, and different studies reported their high levels of fidelity to migratory routes, foraging areas and wintering sites [Avens et al., 2003; Aguirre and Lutz, 2004; Broderick et al., 2007]. In particular, sea turtles can serve as excellent sentinels of ecosystem health in near shore environments [Aguirre et al., 2002]. Recent evidences indicated that sea turtles may be susceptible to many environmental stressors, such as high temperatures, pollutants, infectious agents, and marine biotoxins. Noxious effects include compromised physiology, impaired immune function, and an increase in disease prevalence. The high profile of sea turtles, the extensive sampling programs, and stranding networks offer an effective platform to monitor

the health of sea turtle populations and, consequently, the health of their ecosystems [Aguirre et al., 2002]. Sea turtles have already been proposed as bio-indicators for the detection of coastal exposure to polluted effluents, in the context of antimicrobial resistant bacteria [Al-Bahry et al., 2009], as well as sentinels of environmentally challenged habitats, in the context of fibropapillomatosis epidemiology [Aguirre and Lutz, 2004]. Specifically in the Mediterranean, loggerhead sea turtles have been proposed as indicator species by the European Union Marine Strategy Framework Directive (Commission's Decision 2010/477/EU), to monitor the impact of marine litter [Galgani et al., 2014]. Thus, the study of sea turtle populations is important not only because they have been proposed as sentinel indicators of the health of their marine environments, but also because many of these marine environments are shared by human populations [Flint et al., 2010].

In terms of biology, evolution and conservation, sea turtles are quite well studied animals. However, regarding pathology and infectious diseases, most of the information about pathogens and parasites in sea turtles has mainly been recorded as anecdotal findings [Alfaro et al., 2006]. Numerous species of bacteria and fungi have been identified in sea turtles; however, their pathogenic nature should be cautiously interpreted as many species may be present without causing significant pathology [Flint, 2013]; other species, on the contrary, can play a very important role in sea turtles diseases, both as primary pathogens or as secondary invaders when the host’s immune system has been compromised. These species are involved in localized infections but also in epizootics characterized by bacteraemia and septicaemia. Most of the bacteria that have been reported in sea turtles are non-specific pathogens and previously they have been found in fish,

crustaceans and other marine animals. Moreover, the association of sea turtles with bacteria has raised concerns about the harmful potential of such infections to humans [Alfaro et al., 2006]; the prevalence of sea turtle-associated human disease is not known, but human contact with wild-caught and captive-housed sea turtles (and their products) represents a recognized potential threat to health from a variety of pathogenic sources of biological and contaminant toxin origin [Warwick et al., 2013].

Many studies have been conducted on the carcasses of these animals, found stranded along the coasts, or deceased in the Rehabilitation Centres, and subsequently submitted to necropsy [Flint et al., 2010; Glazebrook and Campbell, 1990a; Glazebrook and Campbell, 1990b; Glazebrook et al., 1993; Orós et al., 2004; Orós et al., 2005; Fichi et al., 2016]. In contrast, fewer data are available from live animals [Kelly et al., 2006; Foti et al., 2009; Isler et al., 2014]. Thus, this study was aimed at performing a microbiological survey on live loggerhead sea turtles coming from the western Mediterranean, in order to assess the health status of this population and their ecosystem, concurrently identifying potential and emerging pathogens for animal and human health. Specifically, this study focused on oral and cloacal prevalence of various bacterial species as well as on the influences of distinct ecological factors on these species.

1.2 Materials & Methods

1.2.1 Sampling

During the period January 2015-December 2016, a total of 35 loggerhead sea turtles (*Caretta caretta*), housed at the Marine Turtle Research Centre (MTRC) of the Stazione Zoologica Anton Dohrn in Naples, was examined. All the recovered sea turtles came from near shore environments of the middle-eastern Tyrrhenian sea, part of the western Mediterranean basin; the animals were classified according to i) their estimated life stage; ii) the area where they were recovered; iii) the season when they were recovered; iv) the cause of their recovery and v) whether they had ingested plastics or not (Table 1.1). As regards life stage, sea turtles were classified into two categories, according to their CCL, as also described by Casale et al. [2011; 2014]; precisely, animals with CCL<64cm were classified as juveniles [number of animals (n)=18], whereas animals with CCL≥64cm were classified as adults (n=17). This threshold is in contrast with the one suggested by Casale et al. [2014], but it has been established to include one female (CCL=64cm), housed at the MTRC, identified as sexually mature through ultrasonography exam. With reference to the area of recovery, the study area (Figure 1.1) covered almost the entire coasts of Campania and Lazio [from 42.08859444, 11.78683809 decimal degrees (dd) to 39.99883033, 15.42704847 dd]. A subgroup of animals (n=22) was divided into two classes, corresponding to two areas (A and B) differentiated on the basis of hydromorphological and habitat characteristics. In particular, group A (n=9) was composed of sea turtles

coming from the Gulf of Naples (from 40.79099815, 14.03899699 dd to 40.56901928, 14.32464881 dd), a large bay with a sandy-rocky bottom and great depths; group B (n=13) was composed of sea turtles coming from the Gulf of Salerno (from 40.56901928, 14.32464881 dd to 40.25301356, 14.90345959 dd), a wide and deep bay that is more exposed to the inflow of external Tyrrhenian waters [Bentivegna et al., 2001]. The other animals were recovered along the coasts north of the Gulf of Naples (area N; n=10) or south of the Gulf of Salerno (area S; n=3), but they could not be assigned to a specific habitat, as these coasts are characterized by a succession of shallow sandy stretches and deep rocky bottoms, including also islands. As regards the season, two groups were formed, according to sea temperatures: animals that were recovered during the warm period (n=14), from June to October, characterized by water temperatures higher than 20°C; and animals that were recovered during the cool period, from November to May (n=21), characterized by water temperatures lower than 20°C. Concerning the causes of recovery, animals were divided into 4 classes: class 1 (n=17) included bottom trawling, boat strikes and net entrapment, as acute traumas due to interaction with fishing gears; class 2 (n=6) included ingestion of hooks and/or lines, as traumatic interactions with a component of chronicity; class 3 (n=5) included intestinal stasis provoked by natural causes; class 4 (n=7) included all other non-traumatic causes, such as general debilitation, buoyancy disorders, etc. One last classification was made in order to discern between animals that ingested plastics, including in these group the animals that ingested lines (n=17), and those who did not ingest plastics (n=18), through examination of their faeces. The animals were sampled at their arrival at the MTRC, just before

settling them in tanks. For each turtle, two oral and two cloacal swabs were collected using sterile cotton-tipped swabs. One oral swab and one cloacal swab were inoculated into Phosphate Buffered Saline (PBS, Oxoid); the remaining swabs were inoculated into transport medium Cary Blair (Oxoid). Animal handling procedures were performed according to the authorization by the Ministry of Environment and Protection of Land and Sea (Protocol n.0042848/PNM 09/08/2013 and Protocol n.0024471/PNM 22/11/2016).

Fig. 1.1 Areas of recovery of 35 loggerhead sea turtles subject of study.
The larger white marks designate the northern and southern limits, along the coast, of the represented area of study. The smaller, black-centred marks delimit the two areas characterized by distinct habitats. N=North of the Gulf of Naples; A=Gulf of Naples; B=Gulf of Salerno; S=South of the Gulf of Salerno.

Tab. 1.1 Classification of 35 loggerhead sea turtles subject of study.

Animal	Estimated Life Stage	Area of recovery	Season of recovery	Cause of recovery	Plastic ingestion
1	Juvenile	N	I	1	No
2	Juvenile	N	I	4	No
3	Juvenile	N	II	1	Yes
4	Juvenile	N	II	1	No
5	Juvenile	N	II	2	Yes
6	Juvenile	N	II	4	No
7	Juvenile	A	I	2	Yes
8	Juvenile	A	I	3	No
9	Juvenile	A	I	3	No
10	Juvenile	A	II	3	Yes
11	Juvenile	A	II	4	No
12	Juvenile	B	I	1	Yes
13	Juvenile	B	I	1	No
14	Juvenile	B	I	1	No
15	Juvenile	B	I	1	No
16	Juvenile	B	I	2	Yes
17	Juvenile	B	II	1	Yes
18	Juvenile	S	II	1	No
19	Adult	N	I	4	No
20	Adult	N	II	2	Yes
21	Adult	N	II	3	Yes
22	Adult	N	II	4	Yes
23	Adult	A	I	1	No
24	Adult	A	II	1	Yes
25	Adult	A	II	4	Yes
26	Adult	A	II	4	No
27	Adult	B	I	1	Yes

28	Adult	B	I	1	Yes
29	Adult	B	I	1	Yes
30	Adult	B	I	1	No
31	Adult	B	I	1	No
32	Adult	B	I	1	No
33	Adult	B	I	3	No
34	Adult	S	I	2	Yes
35	Adult	S	I	2	Yes

Area of recovery: N=North of the Gulf of Naples; A=Gulf of Naples; B=Gulf of Salerno; S=South of the Gulf of Salerno.

Season of recovery: I=Cool period (Nov-May); II=Warm period (Jun-Oct).

Cause of recovery: 1=Acute fishery interaction; 2=Chronic fishery interaction; 3=Intestinal stasis; 4=Other causes.

1.2.2 Isolation

Samples inoculated in PBS were transferred into Buffered Peptone Water (Oxoid), *Campylobacter*-Selective Enrichment Broth and Rappaport-Vassiliadis Broth (Oxoid), whereas swabs inoculated in Cary Blair medium were transferred in Alkaline Saline Peptone Water.

Samples inoculated into Buffered Peptone Water were incubated at 37°C for 24h and then plated onto *Pseudomonas* Cetrimide Agar (Oxoid), n. 3 MacConkey Agar (Oxoid) and Baird-Parker Agar (Oxoid).

Samples inoculated into *Campylobacter*-Selective Enrichment Broth were incubated in microaerobic atmosphere (oxygen level of 8-9% and carbon dioxide level below 8%) provided by CampyGen (Oxoid) at 42°C for 48h and then plated onto *Campylobacter* blood-free selective agar (Oxoid).

Rappaport-Vassiliadis Broth were incubated at 42°C for 24h and then plated onto Brilliant Green Agar (Oxoid).

Samples inoculated into Alkaline Saline Peptone Water were incubated at 37° for 18-24h and then placed into *Aeromonas* medium base (Oxoid) and Thiosulfate-Citrate-Bile salts-Sucrose Cholera medium (Oxoid).

The *Pseudomonas* Cetrimide Agar, n. 3 MacConkey Agar, Baird-Parker Agar, Thiosulfate-Citrate-Bile salts-Sucrose Cholera medium, Brilliant Green Agar plates were incubated at 37°C for 24–48h, whereas *Aeromonas* medium base plates were incubated at 30°C for 24h; *Campylobacter* blood-free selective agar plates were incubated microaerobically at 42°C for 48h.

1.2.3 Identification

All isolated strains were primarily identified, selecting 2-3 colonies from each plate, on the basis of their colonial morphology, Gram characteristics, growth requirements, pigments production, tube coagulase test, and standard conventional biochemical and phenotypic tests. The isolates were confirmed using Analytical Profile Index systems (API, bioMérieux). *Staphylococcus* spp. isolates, which resulted positive to the tube coagulase test, were submitted to the rapid serum agglutination test with the monospecific antisera for *S. aureus* (Biorad); in case of negative result they were identified using API Staph system (bioMérieux).

1.2.4 Antimicrobial Susceptibility Testing

All isolates were submitted to antimicrobial susceptibility testing using the disk diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI) documents [CLSI, 2012]. The antimicrobials tested were: Amikacin (AK; 30µg; Oxoid); Ampicillin (AMP; 10µg; Oxoid); Ceftazidime (CAZ; 30µg; Oxoid); Chloramphenicol (C; 30µg; Oxoid); Ciprofloxacin (CIP; 5µg; Oxoid); Colistin sulphate (CT; 10µg; Oxoid); Doxycycline (DO; 30µg; Oxoid); Gentamicin (CN; 10µg; Oxoid); Nalidixic Acid (NA; 30µg; Oxoid); Streptomycin (S; 10µg; Oxoid); Tetracylcine (TE; 30µg; Oxoid); Trimethoprim-sulfamethoxazole (SXT; 1.25/23.75µg; Oxoid). The inhibition zones were measured and scored as susceptible, intermediate and resistant, according to the CLSI documents [CLSI, 2014]. When an antimicrobial molecule for a specific agent was not present in the CLSI documents, a similar antimicrobial molecule of the same class was used: specifically, Colistin breakpoints were used to evaluate CT sensibility. In order to evaluate the presence of Extended Spectrum Beta-Lactamase (ESBL) producing bacteria, all strains belonging to the family Enterobacteriaceae were also submitted to the Combination Disk diffusion test, using Cefpodoxime (CPD; 10µg; Oxoid) and Cefpodoxime/clavulanic acid (CD; 10/1µg; Oxoid), and to the ETEST® ESBL (ESBL CT/CTL 16/1; bioMérieux).

1.2.5 Statistical Analysis

Isolation results were analysed 1) to test differences between proportions of Gram-negative and Gram-positive isolates, and between proportions of each bacterial species from oral and cloacal swabs; 2) to explore possible tendencies of bacterial families with classification factors (i.e. life stage; season; cause; plastic) 3) to test differences between proportions of bacterial families in the two habitats represented by areas A and B; 4) to explore possible tendencies of the sum of species isolated from each swab, with each factor; 5) to evaluate possible associations among bacterial species, taking into account the most pathogenic ones (i.e. *P. aeruginosa*; *S. aureus*; *A. hydrophila*; *V. parahaemolyticus* and *V. vulnificus*) as dependent variable, one species at a time.

Chi-square analysis was used to test differences between Gram-negative and Gram-positive isolates, as well as between oral and cloacal swabs, except when numbers were too small to appropriately do so; in these cases, the Fisher Exact test was used. The Logistic Regression and Chi-square analyses were performed, as appropriate, to explore possible tendencies of each bacterial family with each classification factor and to test differences between the two areas, as well as to evaluate possible associations among bacterial species; whereas t-test and ANOVA analyses were performed, as appropriate, to explore possible tendencies of the sum of species with factors. Statistical analyses were performed with MedCalc and statistical significance was set at $p<0.05$.

1.3 Results

1.3.1 Bacterial Isolation

Table 1.2 illustrates the prevalence of bacterial isolates in oral and cloacal swabs collected from 35 live *C. caretta* of the western Mediterranean. Microbial cultures resulted in a mixed growth of opportunistic organisms, both Gram-negative and Gram-positive species. Gram-negative bacteria were predominant, they were isolated from all the collected samples [100%; 95% confidence interval (CI): 94.9-100%]; on the other hand, Gram-positive bacteria were isolated from 59/70 samples (84.3%; 95% CI: 73.6-91.9%), with higher prevalence from cloacal swabs (88.6%; 95% CI: 73.3-96.8%) than oral swabs (80%; 95% CI: 63.1-91.6%), yet not significant. The prevalence of Gram-negative and Gram-positive bacteria was significantly different both in the overall swabs [χ^2=11.938; degrees of freedom (df)=1; $p<0.001$] and in the oral swabs (χ^2=7.7778; df=1; $p<0.05$); on the contrary, the difference was not significant in cloacal swabs. Different bacterial families were simultaneously recovered from each swab; among Gram-negative isolates, Enterobacteriaceae were detected from 26/35 (74.3%; 95% CI: 56.7-87.5%) oral swabs, and from 31/35 (88.6%; 95% CI: 73.3-96.8%) cloacal swabs; among them, the most represented species was *Citrobacter* spp., isolated from 18/35 (51.4%; 95% CI: 34-68.6%) oral swabs and from 25/35 (71.4%; 95% CI: 53.7-85.4%) cloacal swabs. Aeromonadaceae were detected from 6/35 (17.1%; 95% CI: 6.6-33.6%) oral swabs and from 2/35 (5.7%; 95% CI: 0.7-19.2%) cloacal swabs; all isolates were identified as *Aeromonas hydrophila*.

Pseudomonadaceae were detected from 30/35 (85.7%; 95% CI: 69.7-95.2%) oral swabs and from 31/35 (88.6%; 95% CI: 73.3-96.8%) cloacal swabs; whereas Vibrionaceae were detected from 19/35 (54.3%; 95% CI: 36.6-71.2%) oral swabs and 21/35 (60.0%; 95% CI: 42.1-76.1%) cloacal swabs. With respect to Gram-positive isolates, Staphylococcaceae were detected from 28/35 (80%; 95% CI: 63.1-91.6%) oral swabs and from 31/35 (88.6%; 95% CI: 73.3-96.8%) cloacal swabs; in particular, coagulase negative staphylococci (CNS) were isolated from 27/35 (77.1%; 95% CI: 59.9-89.6%) oral swabs and from 27/35 (77.1%; 95% CI: 59.9-89.6%) cloacal swabs; whereas coagulase positive staphylococci (CPS) were isolated from 1/35 (2.9%; 95% CI: 0.1-14.9%) oral swab and from 4/35 (11.4%; 95% CI: 3.2-26.7%) cloacal swabs, and all of them were identified as *S. aureus*. In contrast, *Salmonella* spp. and *Campylobacter* spp. were never recovered.

Only *Shewanella putrefaciens group* isolates were significantly different between oral and cloacal swabs (χ^2=5.8514; df=1 $p<0.05$), whereas no significative difference (χ^2=5.3846; df=1; p=0.054) was detected for *Escherichia coli* isolates, though with a similar pattern, characterized by higher prevalence in cloacal swabs than in oral swabs (Table 1.2).

Tab. 1.2 Prevalence of bacterial isolates from oral and cloacal swabs collected from 35 loggerhead sea turtles.

Bacterial Class	**Total (%)**	**Oral Swabs (%)**	**Cloacal Swabs (%)**	**p values**
Gram +	84.3	80	88.6	0.51297
Gram -	100	100	100	1
Bacterial Family				
Staphylococcaceae	84.3	80	88.6	0.51297
Pseudomonadaceae	87.1	85.7	88.6	1
Aeromonadaceae	11.4	17.1	5.7	0.25946
Vibrionaceae	57.1	54.3	60	0.62906
Enterobacteriaceae	81.4	74.3	88.6	0.21816
Bacterial species				
CPS[a]	7.1	2.9	11.4	0.35648
CNS	77.1	77.1	77.1	1
Pseudomonas aeruginosa	10.0	5.7	14.3	0.42826
Pseudomonas spp.[b]	84.3	85.7	82.9	0.7426
Aeromonas hydrophila	11.4	17.1	5.7	0.25946
Vibrio alginolyticus	50.0	48.6	51.4	0.81107
Vibrio parahaemolyticus	5.7	2.9	8.6	0.61389
Vibrio vulnificus	2.9	2.9	2.9	1
Shewanella putrefaciens group	27.1	14.3	40.0	0.015565
Citrobacter spp.	61.4	51.4	71.4	0.085647
Enterobacter spp.	15.7	8.6	22.9	0.18753
Escherichia coli	7.1	0.0	14.3	0.053645
Hafnia alvei	4.3	2.9	5.7	1
Klebsiella oxytoca	11.4	11.4	11.4	1
Morganella morganii	18.6	17.1	20.0	0.75857
Proteus spp.	17.1	17.1	17.1	1
Other coliform bacteria	8.6	11.4	5.7	0.67329

[a] identified as *S. aureus*

[b] including *P. putida*, *P. fluorescens* and *P. stutzeri*; with the exception of *P. aeruginosa*

1.3.2 Antimicrobial Susceptibility Testing

A prominent proportion of bacterial strains exhibited simultaneous resistance to at least two antimicrobial drugs (Figure 1.2). The most frequently detected resistances were to Ampicillin (100% of tested strains), Tetracycline (71.4%) and Streptomycin (70%). A moderate amount of bacterial strains showed resistance to Doxycylcine (60%), Ceftazidime (40%), Chloramphenicol (36.8%), Ciprofloxacin (35.7%), Nalidixic Acid (31.6%) and Gentamicin (30%). Lower amounts of bacterial strains showed resistance to Colistin sulphate (20%), Trimethoprim-sulfamethoxazole (20%) and Amikacin (7.1%). Strains scored as intermediate resistant were not included in the calculation of the percentage of antimicrobials resistance, previously reported.

The isolates that showed resistance to the greatest number of antimicrobials were *Morganella morganii* (resistance from 0% to 90% of antimicrobials tested, mean 42.9%), followed by *Citrobacter* spp. (12.5%-81.8%, mean 42.5%), *Staphylococcus aureus* (0%-66.7%, mean 41.7%) and *Pseudomonas aeruginosa* (0-60%, mean 35%). Two strains of *Citrobacter freundii* were found to produce ESBL; both strains showed resistance to all the antimicrobials tested, with the exception of Amikacin and Doxycycline.

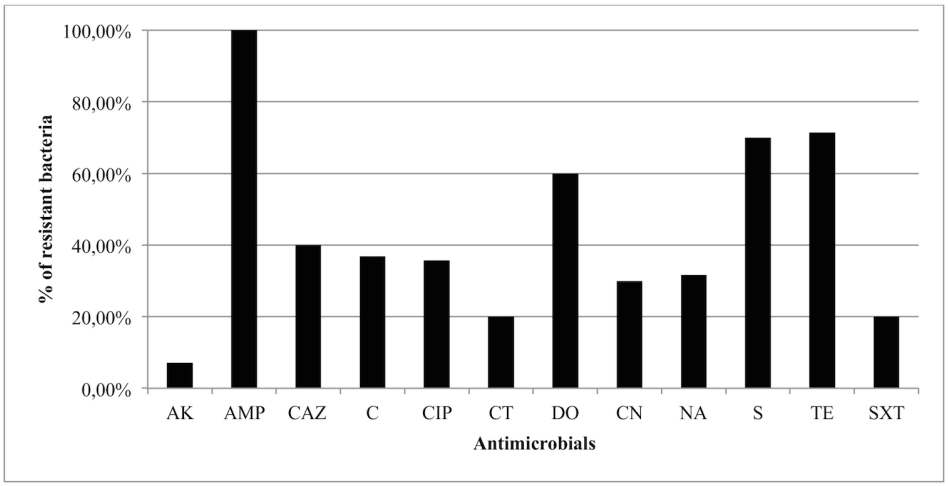

Fig. 1.2 Antimicrobial resistance of bacterial isolates from 35 loggerhead sea turtles.

1.3.3 Influence Of Ecological Factors On Bacterial Species

Several associations were found between classification factors and bacterial families. Specifically, area of recovery and plastic ingestion were the most influencing factors, followed by season and life stages; whereas no significant association was detected using cause of recovery as independent variable. As regards oral swabs, the prevalence of Enterobacteriaceae was found significantly different (χ^2=7.1077; df=1 $p<0.05$) between the two areas, higher in area A (100%) than in area B (46.2%); Significant association was detected between plastic ingestion and the bacterial family Aeromonadaceae (χ^2=9.156; df=1; $p<0.01$), with higher prevalence in oral swabs of sea turtles that did not ingest plastics (33.3%) than in those that ingested plastic (0%). Opposite association, in cloacal swabs, was detected for the Vibrionaceae family (χ^2=7.210; df=1; $p<0.01$), in this case with higher prevalence in sea turtles that ingested

plastics (82.4%) than in those that did not (38.9%); additionally, in cloacal swabs, the Vibrionaceae family was isolated with significant higher prevalence (χ^2=6.945; df=1; p<0.01) during the warm period (85.7%) than during the cool period (42.9%). As regards life stage, a significant difference (χ^2=6.327; df=1; p<0.05) was found between the prevalence of Enterobacteriaceae in juvenile sea turtles (100%) and adult sea turtles (76.5%).

Concerning the sum of bacterial species, both oral and cloacal swabs presented significant differences (respectively t=2.736 df=20; p<0.05 and t=2.360; df=20; p<0.05) between the two areas of recovery; in both cases the number of species was higher in area A (95% CI for the mean in oral swabs=3.9129-6.0871; and in cloacal swabs=4.2286-7.1047), compared to area B (95% CI for the mean in oral swabs=2.5837-4.1855; and in cloacal swabs=3.4628-4.8449). Additionally, cloacal swabs presented a significant (t=3.163; df=33; p<0.01) higher number of species during the warm period (95% CI for the mean=4.6913-6.3087) compared to the cool period (95% CI for the mean=3.4633-4.6319).

No significant association was detected between the most pathogenic species and the other bacterial species isolated in this study.

1.4 Discussion

In the present study, different species of bacteria were identified, with some of them previously recovered from ill sea turtles. The higher prevalence of Gram-negative bacteria is not surprising because Gram-negative bacteria are common isolates in healthy reptiles as also reported

by Alfaro et al. [2006]. Gram-positive bacteria are also common inhabitants, especially of the skin of reptiles, but they are not considered pathogenic; however, coagulase-positive staphylococci are usually pathogenic, and the production of coagulase and pathogenicity has a 95% correlation [Paré et al., 2006]. Among Gram-negative bacteria, Enterobacteriaceae, *Pseudomonas* spp., *Aeromonas* spp., *Vibrio* spp. and *Shewanella putrefaciens group* were included, in line with George [1997] who reports them among the most commonly cultured pathogens from diseased sea turtles, and supported by Fichi et al. [2016] and Foti et al. [2008], who detected similar bacterial species in recent studies conducted in the Central Mediterranean, along the coasts of Italy. In particular, *E. coli* is not unusually cultured from cloacal swabs, as these organisms are a normal component of the bacterial flora of the reptilian intestinal tract [Paré et al., 2006]. Similarly, *S. putrefaciens group* is commonly isolated from the cloaca of loggerhead sea turtles, as confirmed by Kelly et al. [2006].

Those species of bacteria found in turtles are commonly isolated both in localized and systemic infections [Alfaro et al., 2006]. In a study conducted by Flint et al. [2010], microbial infections were considered responsible for an independent disease syndrome (i.e. bacteriosis) in 0.7% of cases and causing death in 5.2% of cases; they noted infections in all systems except the reproductive, and at different degrees of severities. Glazebrook and Campbell [1990ab] reported the ulcerative stomatitis as the most important spontaneous bacterial disease of farmed, oceanarium-reared, and wild turtles in Australia; those authors reported this disease as a part of a complex which included obstructive rhinitis and pneumonia,

and they isolated from the lesions *V. alginolyticus A. hydrophila, Pseudomonas* spp. and *Flavobacterium* spp. [Glazebrook et al., 1993]. A wide range of bacteria, including some of the species reported in the present results, were isolated from different lesions, during post-mortem examination: specifically, *Citrobacter* spp., *Escherichia coli, Proteus* spp., *Pseudomonas* spp., *A. hydrophila, V. alginolyticus,* and *Staphylococcus* spp. were found in association with stomatitis, esophagitis, gastritis, enteritis, hepatitis [Orós et al., 2004], pneumonia, nephritis and traumatic skin lesions [Orós et al., 2005]. *Pseudomonas* spp. and *Staphylococcus* spp. were also isolated in loggerhead sea turtles with keratoconjunctivitis and keratitis, in particular *P. aeruginosa* was suggested to cause ulcerative blepharitis [Isler et al., 2014].

As regards *Campylobacter* spp., Johnson-Delaney [2006] pointed out pet turtles as possible reservoirs of these bacteria, but it was never isolated from sea turtles, consistently with the present results. Concerning *Salmonella* spp., the present results are consistent with those of other authors [Orós et al., 2004; Fichi et al., 2016; Foti et al., 2008]. Sea turtles had not been shown to be a reservoir of *Salmollella* spp. [Johnson-Delaney, 2006]; on the contrary, George [1997] includes *Salmonella* spp. among the most commonly cultured bacteria from diseased turtles, and *Salmonella* isolates has been sporadically observed in diseased sea turtles, as reported in other studies [Keymer et al., 1968; Wiles and Rand, 1987; Raidal et al., 1998; Zizzo et al., 2003].

Many of the bacteria isolated from sea turtles are non-specific pathogens; indeed they have been described in other marine animals, including fish and crustaceans [Alfaro et al., 2006; Higgins, 2000]. In particular, both *A.*

sobria and *A. hydrophila* were previously isolated from sea turtles without a particular pathogenic role [Fichi et al., 2016]. Actually, *A. hydrophila* has long been recognized as an opportunistic pathogen of reptiles [Orós et al., 2005] yet the whole genus *Aeromonas* is a causative agent of fish diseases [Alfaro et al., 2006] and it has been described in association with fatal septicaemia and ulcerative dermatitis in marine mammals [Dunn et al., 2001]. Similarly, *V. alginolyticus* is regarded as normal inhabitant of seawater [Orós et al., 2005] and *Vibrio* spp. have been frequently detected in sea turtles; nevertheless, the genus *Vibrio* is commonly suspected in mortality of crustaceans, teleost fish and shellfish, particularly when adverse environmental conditions, nutritional deprivations or overcrowding are expected [Alfaro et al., 2006]. On the other hand, in marine mammals the most common form of *Vibrio* disease involves the contamination of wounds, thought deaths, presumably due to a *Vibrio* septicaemia, have been also reported. Additionally, *Staphylococcus aureus* and *Pseudomonas aeruginosa* have been prominently isolated from pneumonia, as well as pulmonary and cutaneous abscesses in cetaceans [Dunn et al., 2001].

Many potential zoonotic pathogens have been isolated from diseased reptiles; even if the majority is probably not considered a health risk to healthy human adults, they could pose potential disease risks for the immunocompromised, infants and young children, and the elderly. In particular, *Aeromonas* spp., *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., and *Proteus* spp. have been implicated in enterocolitis, diarrhoea, and genitourinary infections in humans; whereas *Pseudomonas* spp. have been isolated from purulent infections. Potential transmission of these bacteria

may occur from contact with the organism or the water environment the reptiles live in; from scratches or bite wounds; from inhalation or ingestion. Actually, *Morganella morganii* has been reported as one of the most common pathogens identified in the wound cultures associated with snakebites [Dipineto et al., 2014]. For this reason the possible public health significance of a reptile shedding bacteria in the environment should be considered [Johnson-Delaney, 2006].

Concerning antimicrobial resistance, the present results showed similar percentages of resistance among bacterial species isolated from sea turtles to those reported in southern Italy by Foti et al. [2009]. Specifically, the most remarkable analogies were found for Ceftazidime, Chloramphenicol, Nalidixic Acid and Trimethoprim-sulfamethoxazole and, to a lesser extent, for Ciprofloxacin, Gentamicin and Tetracylcine. On the contrary, higher proportion of resistance to Ampicillin and Streptomycin, and lower proportion of resistance to Amikacin and Colistin sulphate were described in the present study. Nevertheless, in both studies Ampicillin was among the antimicrobials with highest percentage of resistance, whereas Amikacin was the antimicrobial with the lowest. One additional similarity is in the isolates that showed resistance to the greater number of antibiotics: Foti et al. identified them in *C. freundii* and *P. aeruginosa,* as in this study. On the other hand, the present results showed conspicuous levels of antibiotic resistance for *Morganella morganii*; this finding was not validated by Foti et al., but was reported in South Carolina by Piñera-Pasquino [2006]. Given that the development of multiple-resistance to antimicrobials is believed to be the result of massive use and release of disinfectants and pharmaceutical products related to agriculture, medical

and veterinary practices [Al-Bahry et al., 2012], the affinities between the present results and those reported by Foti et al. [2009] may be explained by similar selective pressures induced by the over use of antimicrobial agents. Indeed, these data induce deep apprehension on the dissemination of this phenomenon in the marine environment and on the mechanisms that led wild *C. caretta* to acquire resistant bacteria [Foti et al., 2009]. Even in this context, sea turtles have been used as bio-indicators for pollution in the coastal marine habitats: antibiotic-resistant bacteria isolated from green turtles and loggerheads are causing deep concerns regarding the dissemination of resistance to antibiotics in marine wildlife [Al-Bahry et al., 2012].

With reference to the influence of ecological factors, the results reported in this study showed interesting tendencies. Since oral cavity flora is a reflection of environmental bacteria [Paré et al., 2006], the higher prevalence of Enterobacteriaceae in oral cavities of sea turtles recovered in area A is likely representative of the higher prevalence of Enterobacteriaceae in the Gulf of Naples. One possible conclusion is that the Gulf of Salerno represents a healthier ecosystem; actually, members of the Enterobacteriaceae family, such as total and faecal coliforms, might be used as indicators for marine water quality assessment, but their specificity has been repeatedly questioned [Stewart et al., 2008; Thompson et al., 2005]. Another speculation is to attribute this finding to the Sarno River: on one hand this area is characterized by intense urban development, especially along the coast, reaching urban densities three times higher than the regional average and more than twice higher than the national average [De Pippo et al., 2006]. On the other hand, pollution of this river, mainly

due to high amounts of heavy metals and organic wastes from heavily cultivated and industrial areas, has increased the contamination of seawater and has changed the aquatic ecosystem of the Gulf of Naples [Arienzo et al, 2001]. The highest risk is derived from direct sewage discharges, which are a source of human faecal bacteria [Nogales et al., 2011]; in this area, total and faecal coliforms were found nearly two to three times higher than the allowable limits [Arienzo et al., 2001]. Although this is not necessarily a public health concern, the family Enterobacteriaceae include also pathogenic bacteria implicated in opportunistic infections, and previous data reported numerous and constant cases of infectious diseases by oral-faecal transmission in this area [De Pippo et al., 2006; Motta et al., 2008]. With respect to the sum of species, a recent study [Thiele et al., 2017] reported little or no effect by the river pollutants on the planktonic bacterial community of the Gulf of Naples, but this does not diminish their possible effect on other compartments of the marine ecosystem, such as larger organisms (e.g. sea turtles), that bear a longer memory of environmental impacts. Actually, microbial diversity is usually high in chronically perturbed marine environments, whatever the cause might be [Nogales et al., 2011].

As regards plastic ingestion, the present findings were partially confirmed by literature, as microplastic surfaces were described as a distinct microbial habitat where different bacterial species may concentrate and disseminate, in particular Vibrios, and in general *Gammaproteobacteria* [McCormick et al., 2014; Amaral-Zettler et al., 2015; Keswani et al., 2016]. In detail, *Gammaproteobacteria* mainly occur during the early colonisation stages (in the first 9h), later replaced by *Alphaproteobacteria*

(from 24h) and *Bacteriodetes* [Oberbeckmann et al., 2015], communities that precede and facilitate the eventual colonization by benthic diatoms [Carson et al., 2013]. It is likely that the normal flora of sea turtles might be altered by the passage of plastic debris through the digestive tract; indeed, the impact of ingestion could be important in restructuring microbial communities [Amaral-Zettler et al., 2015]: after passing through the digestive tract of an organism, these indigestible particles may still have members of the intestinal microbial community attached to them, especially Vibrios that survive the digestive tract environment, which could then be ingested by another organism [Amaral-Zettler et al., 2015; Oberbeckmann et al., 2015].

Regarding season of recovery, changes in the climate system can cause alterations in the abundance and distribution of pathogens. In particular, the Vibrionaceae family is well known for its seasonality and preference for warm waters [Vezzulli et al., 2015; Vezzulli et al., 2016]. Nevertheless, it is an important finding, as recent evidences of increasing Vibrio-related infections indicate a potential health risk, both for humans and other animals [Vezzulli et al., 2010; Baker-Austin et al., 2013].

Concerning life stages, the higher prevalence of Enterobacteriaceae in younger animals has been reported also in other species [Van Dongen et al., 2013; Masouka et al., 2017]. It is likely that older sea turtles have more diverse microflora in the gastrointestinal tract and Enterobacteriaceae may be outcompeted by other microorganisms. Another speculation is that changes in the immune system affect the prevalence of Enterobacteriaceae, as suggested by the lower values of packed cell volume and lymphocytes found in younger turtles [Rousselet et al., 2013; Kakizoe et al., 2007].

The evidence of the associations between bacterial families and ecological factors strengthen the role of the environment in shaping the bacterial communities of the sea turtles, and, vice versa, the role of sea turtles as a mirror of the environment they come from; indeed the higher prevalence of specific bacteria in one area, instead of another, could be seen as a wake-up call for the organisms who share the same ecosystem.

In conclusion, this study detected a wide range of bacteria, mainly opportunistic pathogens for sea turtles, yet they could pose a health risk in case of immunosuppression. None of these bacteria is an emerging pathogen, as they were previously isolated in many disparate studies conducted on loggerhead and other sea turtles species, coming from a great variety of environments (i.e. Australia, Italy, Spain, Turkey, United States of America). Nevertheless, some of these microorganisms are agents of zoonoses, responsible for diseases in other marine animals, but also humans who share the environment with sea turtles. This study has the advantage of a conspicuous number of live animals subjected to sampling; the present results highlight the role of sea turtles as carriers of potential zoonotic agents, and raise further important issues about the relation between ecology and microbiology in sea turtles, some of which still to be addressed. Indeed, the association between bacteria and ecological factors underline the role of sea turtles as sentinels of marine ecosystems.

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Chapter 2

Aeromonas Induced Polyostotic Osteomyelitis in a Juvenile Loggerhead Sea Turtle

2.1 Introduction

Wild sea turtles are rarely affected by bacterial infections; contrarily, sea turtles in captivity are more vulnerable to different opportunistic bacteria, which could influence several anatomical regions. Nevertheless, bacterial bone infections have been seldom reported.

2.1.1 Aeromonas

Aeromonas species are Gram-negative, rod-shaped, motile or non-motile, facultative anaerobic, non-spore forming bacteria. The genus consists of psychrophiles and mesophiles, as they grow at temperatures going from 0°C to 45°C, though the optimum range is from 22°C to 35°C. *Aeromonas* species resist pH ranges from 4.5 to 9, with optimum between 5.5 and 9, and optimum sodium chloride concentrations from 0% to 4% [Igbinosa et al., 2012]. Hazen et al. [1978] reported *A. hydrophila* in marine systems, when it was still not considered a marine bacterium. Nowadays, *Aeromonas* species are regarded as ubiquitous microorganisms, globally found in a broad range of habitats, especially in aquatic environments [Chen et al., 2015; Gómez-Garcés et al., 2011; Lee et al., 2003], from rivers, lakes, ponds, seawater, surface and ground water, to chlorinated and non-chlorinated water, drinking water, waste water, and sewage [Igbinosa et al., 2012; Janda and Abbott, 2010; Karam et al., 1983]. In addition, *Aeromonas* species have been isolated from food samples, whether coming from the aquatic environment (e.g. fish, shellfish) or not (e.g. meats, vegetables, dairy products) [Karam et al., 1983; Doganis et al., 2016;

Altwegg and Geiss, 1989]; still, only few food borne outbreaks have been documented [Igbinosa et al., 2012]. *Aeromonas* species have sometimes been considered as normal gastrointestinal flora of freshwater fish and some other ectotherms [Thornley et al., 1997], but they can be found also in the intestinal tract of humans and endothermic animals [Igbinosa et al., 2012], suggesting that some of them can be a reservoir for the introduction and exchange of these bacteria in the environmental microbial world [Janda and Abbott, 2010].

Aeromonas species show different virulence and pathogenicity, but *A. hydrophila*, *A. caviae* and *A. veronii biovar sobria* are reported as the ones producing the majority of infections [Chen et al., 2015]. *Aeromonas* infections are regarded mostly as opportunistic diseases [Altwegg and Geiss, 1989], yet immunocompromised and healthy hosts can be involved likewise [Karam et al., 1983]. Infection has been associated mostly with water-related traumas [Doganis et al., 2016; Gunasekaran et al., 2009; Mani et al., 1995], such as accidents during swimming or diving, near-drowning experiences, water contact with open wounds, but also injuries caused by aquatic animals [Mani et al., 1995; Brook, 2009; Johson-Delaney, 2006]. More recently, some motile *Aeromonas* species have been investigated as food- and waterborne pathogens [Igbinosa et al., 2012; Chen et al., 2015]. Already in the 70's *Aeromonas* species have been regarded as agents of disease both in humans and other animals. Ectothermic animals were used to be considered the most severely afflicted, especially under stress conditions [Janda and Abbott, 2010; Altwegg and Geiss, 1989; Thornley et al., 1997; Shotts et al., 1972; Holmes et al., 1996], but *Aeromonas* spp. can be commonly isolated from

clinically healthy reptiles [Rosenthal, 2006]. Fish, amphibians and reptiles are the most commonly documented animals affected by *Aeromonas* infections [Igbinosa et al., 2012]. Such conditions include: septicaemia, ulcerative/haemorrhagic diseases and furunculosis in fish [Chen et al., 2015]; red leg disease and dermatosepticaemia in frogs [Janda and Abbott, 2010; Wright, 2006]; ulcerative stomatitis in snakes and lizards, which can evolve in pneumonia and eventually septicaemia [Jacobson, 2007]; ocular infections in lizards and crocodiles [Jacobson, 2007]; skin infections and septicaemia in tortoises and turtles [Rosenthal, 2006; Jacobson, 2007]. Moreover, *Aeromonas* species have been associated with infections in mammals – such as septic arthritis and seminal vesciculitis in bovines, septicaemia in dogs and infectious processes in seals [Janda and Abbott, 2010] – including humans, in which *Aeromonas* causes both gastrointestinal and extraintestinal diseases, in healthy hosts as well as immunocompromised or susceptible individuals [Igbinosa et al., 2012; Doganis et al., 2016]. The most frequently reported infections include gastrointestinal infections, form diarrhea to peritonitis, and soft tissue infections, from cellulitis to necrotizing fascitis; less common are respiratory and urinary tract infections. Reported sequelae of infections are hepatitis, cholangitis, meningitis, endocarditis, ocular infections, osteomyelitis, and lethal septicaemia [Igbinosa et al., 2012; Chen et al., 2015; Gómez-Garcés et al., 2011; Lee et al., 2003; Janda and Abbott, 2010; Karam et al., 1983; Doganis et al., 2016; Thornley et al., 1997; Gunasekaran et al., 2009; Mani et al., 1995; Brook, 2009; Shotts et al., 1972; Janda and Abbott, 1998]. The interest in the pathogenic nature of

Aeromonas species has increased over time, and surely the genus has now to be regarded as a potential threat to animal and human health alike.

2.1.2 Osteomyelitis

Localized or generalized inflammation and destruction of bone from pyogenic infectious etiologic agents is classified as osteomyelitis [Fitzgerald and Vera, 2006]. Generally the disease might lead to local inflammation and stiffening of soft tissues, reduced motility, osteolysis, septicaemia, and death [Fitzgerald and Vera, 2006].

Osteomyelitis is not uncommon in reptiles and it is quite dissimilar from the disease in mammals. In reptiles, slowly progressive lytic processes rather than proliferative ones characterize the disease. There is often gross enlargment and distortion of the local anatomy with loss of corticomedullary definition and the secondary periosteal and cortical response to infection is much less prominent in reptiles than it is in mammals. Minimal secondary new bone production is present in the early and intermediate stages, but a persistent lytic defect in the bone is often seen after resolution of osteomyelitis in reptiles. In mammals, this represents a strong evidence for active infection, sequestration, or neoplasia. On the contrary, in reptiles, this can be a focus of inflammation or non-ossified fibrous tissue without contained infection or sequestration. Soft tissue calcification can also be a chronic change as a result of infection [Silverman, 2006; Wilkinson et al., 2004]. *Dermochelys coriacea* is the only extant reptile reported to show similar patterns in the pathobiology of septic arthritis and contiguous osteomyelitis to those in

human newborns [Ogden et al., 1981]. In mammals diagnosis is based on a combination of supportive clinical, laboratory, and imaging findings. In turtles, radiography, computed tomography and scintigraphy are all valuable techniques to investigate skeletal abnormalities [Fitzgerald and Vera, 2006; Wilkinson et al., 2004; Solano et al., 2008; Smith et al., 2000].

The present study reports the case of *A. hydrophila* osteomyelitis with extensive involvement of cranial and caudal flippers in a sub-adult loggerhead sea turtle (*Caretta caretta*).

2.2 Case Presentation

2.2.1 Clinical Findings And Maintenance

A sub-adult loggerhead sea turtle (CCL 53,5 cm; Weight 17,7 kg) coming from the Adriatic Sea, along the eastern coast of Italy, was admitted to the MTRC of the Stazione Zoologica Anton Dohrn of Naples on the 23rd December 2015. The turtle was depressed, debilitated, dehydrated, and presented with severe inappetence, limited active movements of the flippers and pain to manipulation. An extended (about 13 cm) longitudinal paramedial fracture was evident on the right side of the plastron. It was decided to keep the turtle in dry dock and to monitor it for the first 24 hours, before transferring it in a tank at 22°C. The turtle had to be sustained by force-feeding through an esophagostomy tube, implanted at the University of Bari, where the turtle was primarily treated after the rescue. This method was continued at the MTRC, using about 200 g of a

mash of fishmeal, until the 19th January 2016, when the esophagostomy tube was removed, and the turtle started being hand-feeded with fresh fish. On the 8th February 2016 the turtle started feeding on its own, receiving a daily feed ration of approximately 200 g, corresponding to 1% of the animal weight.

2.2.2 Diagnostic Imaging

The turtle was submitted to a preliminary radiographic exam at the University of Bari, where it was suspected of having a left coxo-femoral luxation. On the 4th January 2016 radiographic exams were repeated at the Interdepartmental Centre of Veterinary Radiology of the University of Naples Federico II. The radiographic images showed a lytic left femur head and a fracture to the right acromion (Figure 2.1 A-B). As the turtle kept floating, and did not regain the motility of the flippers, on the 19th February 2016 it was submitted to a computed tomography evaluation at the same Centre. Computed tomography scans confirmed the bone lesions already assessed radiographically but showed also other lytic bone lesions to the left scapula, the left acromion, the left humerus head, the right choracoid, the left acetabulum, the right pubis and the right ischium (Figure 2.2 A-B). On computed tomography images, all the bone lesions showed permeative lytic features, complicated by pathologic fractures at the level of the left scapula and acromion and the right acromion and ischium. On the basis of the radiographic and computed tomography findings, a suspect of infectious polyostotic osteomyelitis was made. In order to characterize the ethiology, an ultrasonographic exam, through a

left prefemural window, followed by an echo-assisted fine needle aspiration from the left femur head, were performed (Figure 2.3 A-B). The collected samples were used for bacterial examination.

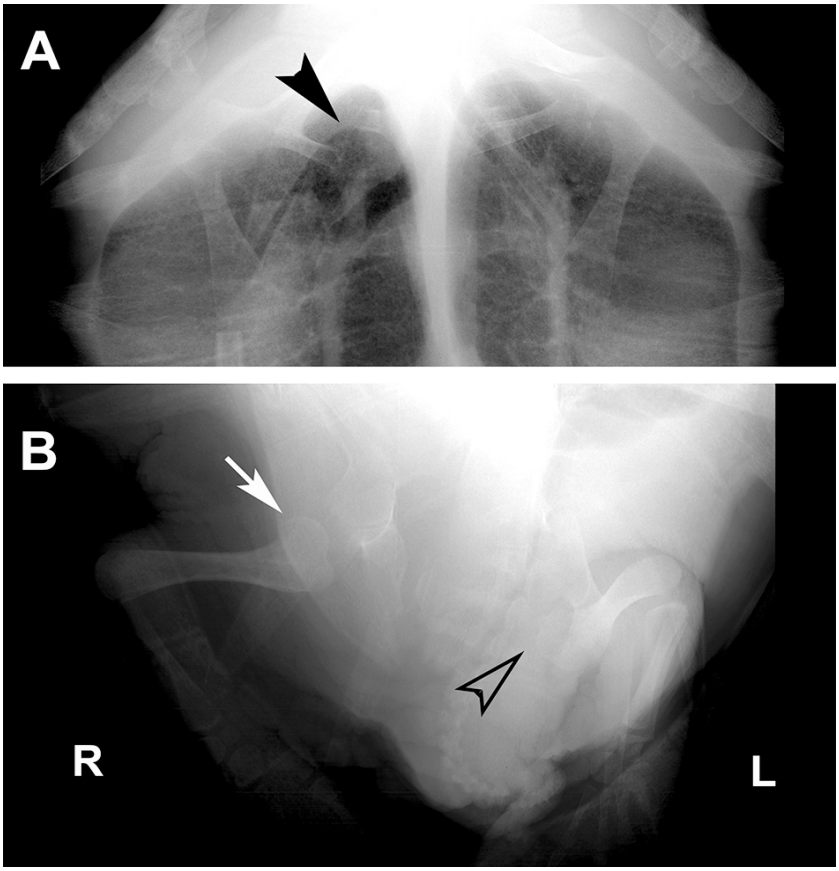

Fig. 2.1 Radiographs in dorso-ventral view.

A: Pectoral Girdle. It is visible a fractured right acromion process (black arrowhead).

B: Pelvic Girdle. There is a lytic left femoral head (empty harrowhead) compare it to the contralateral (white arrow).

R=right side; L=left side.

Image courtesy of Prof. Leonardo Meomartino.

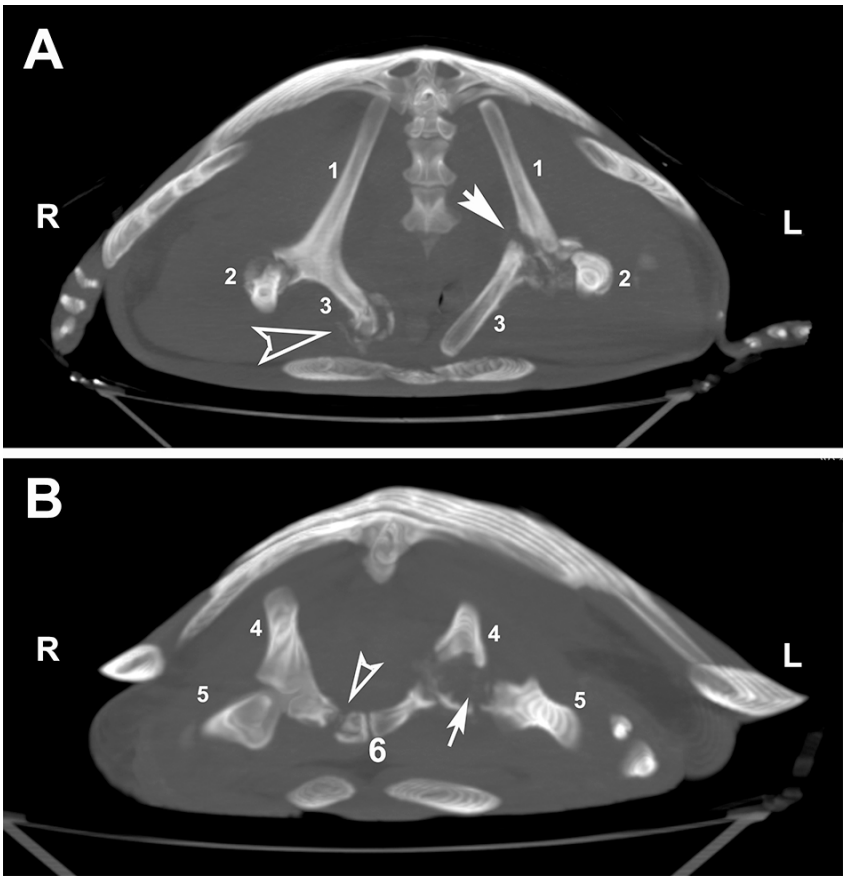

Fig. 2.2 Computed tomography Maximum Intensity Projections reconstruction.

A: Pectoral Girdle. It is visible bone lysis and pathologic fracture with a small callus of the right acromion process (empty arrowhead) and of the insertion on the scapula of the left acromion process (white arrow).

B: Pelvic Girdle. There are bone lysis of the left femoral head and acetabulum (white arrow) and a fractured right ischium (empty arrowhead).

R=Right side; L=Left side; 1=Scapula; 2=Insertion of the choracoid to the scapula (still not fused); 3=Acromion process; 4=Ileum; 5=Femur; 6=Ischium.

Image courtesy of Prof. Leonardo Meomartino.

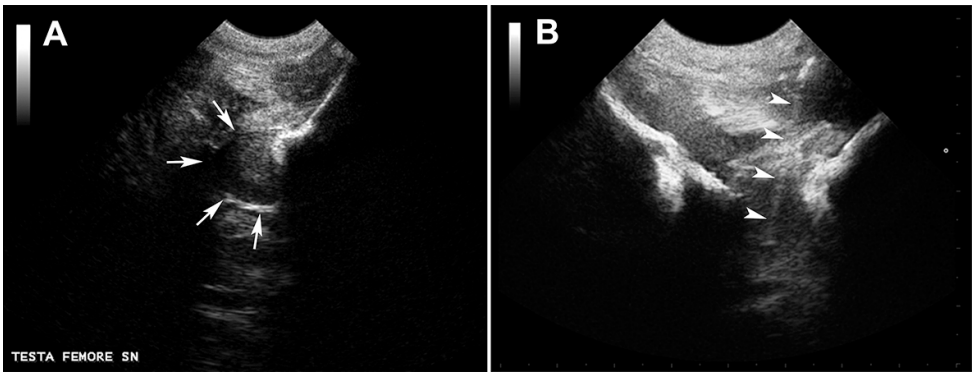

Fig. 2.3 Echo-assisted fine needle aspiration.
A: Transversal ultrasonographic scan of the left coxofemural joint in wich the lytic femoral head is hypoechoic (white arrowheads).
B: the same scan obtained during the fine needle aspiration from the left femoral head with the needle inserted (arrowheads).
Image courtesy of Prof. Leonardo Meomartino.

2.2.3 Bacterial Isolation

On the 19th February 2016, three samples from the affected left lower limb of the turtle were collected through needle aspiration and processed for bacterial isolation. The samples were split and enriched in Buffered Peptone Water and Alkaline Saline Peptone Water for 18-24h at 30°C, and susbequently plated onto different enrichment and selective agar plates. Cultures on Columbia Blood Agar (Oxoid) plates demonstrated large (≈3-4 mm of diameter), white, creamy, β-haemolytic and oxidase positive colonies; whereas on n. 3 MacConkey Agar (Oxoid) plates demonstrated lactose non-fermenting, oxidase positive colonies. No other microorganism was isolated from any culture. At the Gram staining, the isolates resulted in Gram-negative bacilli. The API 20E system (BioMerieux) was used to

identify the microorganism to the species level as *A. hydrophila*. The strain was submitted to antimicrobial susceptibility testing using the disk diffusion method, according to the CLSI documents [CLSI, 2012]. The antimicrobials tested were: Amikacin (30µg; Oxoid), Ceftazidime (30µg; Oxoid); Ciprofloxacin (5µg; Oxoid); Gentamicin (10µg; Oxoid); Doxycycline (30µg; Oxoid); Lincomycin (2µg; Oxoid). The inhibition zones were measured and scored as susceptible, intermediate and resistant, according to the CLSI documents [CLSI, 2014] and Lamy et al. [Lamy et al., 2012]. The *A. hydrophila* strain was susceptible to Amikacin and Doxycycline; intermediate susceptible to Gentamicin; resistant to Ceftazidime, Ciprofloxacin and Lincomycin.

2.2.4 Treatment

The turtle was treated with antimicrobials (Marbofloxacin 5 mg/kg SC q 48 h plus Ceftazidime 20 mg/kg IM q 72 h, for three weeks). Later in the course, given the persistence of symptoms, antimicrobials were adjusted, according with the results of the antimicrobial susceptibility testing. Thus, a treatment period of 16 weeks was initated with Amikacin (Amikavet 125 mg/ml at the dose of 5 and 2.5 mg/kg IM q 48h) plus Doxycycline (Ronaxan 250 mg/tablet at the dose of 25 mg/kg per os q 72 h). Overall the patient received about 6 months of antimicrobials.

Other support therapies consisted in:

- Analgesics (Tramadol 6 mg/kg per os q 72 h for 4 weeks and then under necessity);

- Fluid therapy 10 ml/kg (50% Ringer's solution; 25% saline solution and 25% glucose solution 5%, SC q 24 h 5 days/week) for the first 2 months. Then glucose was suspended and 7.5 ml/kg were administered 3 times/week for 4 months;
- Vitamin and mineral supplement (Stimulfoss 0.1ml/kg per week and Aquavits 1½ tablet per week).

The turtle's progress was monitored by bacterial examination, repeated on the 27th September 2016, and clinical evolution, as well as serial imaging studies, conducted on the 1st April and 18th May 2017. The bacterial examination led to negative results, indicating the resolution of the infection. The turtle's active movements improved whereas the radiology findings reduced in severity. Eventually the turtle was reintroducted in nature, on the 25th May 2017.

2.3 Discussion

The present study described a sub-adult loggerhead sea turtle with *A. hydrophila* inducing polyostotic osteomyelitis, and this is the first report of *Aeromonas* bone infection in a loggerhead sea turtle.

There are several cases of *Aeromonas* osteomyelitis documented in humans [Gómez-Garcés et al., 2011; Lee et al., 2003; Karam et al., 1983; Doganis et al., 2016; Gunasekaran et al., 2009; Mani et al., 1995], but fewer cases are reported in reptiles. *Salmonella* spp. is one of the most commonly cultured Gram-negative bacteria from reptiles with osteomyelitis [Ramsay et al., 2002]. Additional cases of osteomyelitis have been attributed to: *Serratia marcescens, Enterococcus* spp., *Pseudomonas*

spp., E. *coli, Proteus* spp., *Mycoplasma* spp., and fungi, which were reported in crocodiles, iguanas, snakes and lizards [Jacobson, 2007; Mitchell, 2006]. Only one case of *Aeromonas* spp. cultured from the lesion of a tibia affected by osteomyelitis was reported in an iguana [Mader and Bennet, 2006]. Osteomyelitis has been documented in Kemp's ridely sea turtles (*Lepidochelys kempii*), specifically in cases of cold-stunning [Solano et al., 2008]; penetrating injuries and pneumonia [Smith et al., 2000]; infections from *Mycobacterium chelonae* [Greer et al., 2003], *Vibrio alginolyticus* [Glazebrook and Campbell, 1990], *Nocardia* spp. [Harms et al., 2002], *Enterococcus fecalis* and coagulase positive Staphilococci [Smith et al., 2000].

Actually, *Aeromonas* spp. have been reported in sea turtles, mostly in association with other microorganims, from a variety of other lesions: salt gland adenitis [Orós et al., 2011]; pneumonia; nephritis; hepatitis and digestive lesions [Orós et al., 2005; Orós et al., 2004]; dermatitis syndromes [George, 1997] and from the US-OR-BP complex [Glazebrook and Campbell, 1990]. Captive reared sea turtles seem to be more susceptible to bacterial infections than their free-roaming counterparts [Milton and Lutz, 2002].

In humans the two major sources of infection are the environment-water-animals complex and the ingestion of contaminated foods [Igbinosa et al., 2012]. Osteomyelitis may arise through haematogenous spread [Lee et al., 2003], derived from mucosal defects or skin wounds [Karam et al., 1983; Holmes et al., 1996; Janda and Abbott, 1998]. Similarly, in sea turtles the most common routes of entrance are traumatic injuries and aspiration of water; bacteria can subsequently gain the bloodstream and disseminate

throughout the entire body [George, 1997]. Hypothetically, the loggerhead turtle here addressed, could have developped osteomyelitis from haematogenous spread of *A. hydrophila* either through abrasion of the oral cavity during grazing, or through depressed gastrointestinal immune response [Work et al., 2003]. Indeed, the bacteria already present as normal flora of the sea turtle, could have taken advantage of a condition of stress and subsequent immunosuppression [Higgins, 2002]. Ogden et al. [1981] suggested that bacteria normally found as bowel flora may occasionally enter the blood stream in sufficient quantity to cause osteomyelitis or septic arthritis, even in an otherwise normal individual. Alternatively, leeches could have been responsible for transmitting the infection, since leeches harbor *Aeromonas* species symbiotically, and few reports of leeches induced *Aeromonas* infections have already been reported in humans [Janda and Abbott, 2010; Mani et al., 1995; Janda, 1991].

Concerning the diagnostic imaging, radiography is acknowledged as a standard modality to assess skeletal abnormalities of turtles [Solano et al., 2008; Smith et al., 2000, Valente et al., 2007b]. Nevertheless, whilst radiography still represents the first exam used to assess skeletal disorders, computed tomography can be used when a better comprehension is needed, since the tomographic features of the computed tomography images fix the problems due to the superimpositions inevitably present in the planar radiographical images [Smith et al., 2000; Valente et al., 2007a; Valente et al., 2007b; Valente et al., 2007c]. Scintigraphy had been proposed as a complementary exam useful in demonstrating functional activities of lytic processes [Solano et al., 2008], however, the scarce

diffusion in veterinary facilities made this exam difficult to access. In the present case, radiography, computed tomography and ultrasonography were used to achieve the diagnosis. Despite radiography showed lesions on some bones, it demonstrated a lower sensitivity compared to the computed tomography. Ultrasonography was useful to collect the sample from one of the osteomyelitis sites since it permit interventional procedure under the real time modality [Arencibia et al., 2006].

With respect to treatment, the use of broad-spectrum antimicrobials and antifungal medications for cold-stunned turtles was suggested to be also effective on some osteomyelitic lesions [Solano et al., 2008]. On the other side, the implantation of antibiotic-impregnated polymethymethacrylate beads was discussed as the preferred treatment for osteomyelitis or septic arthritis, and also the application of hyperbaric oxygen therapy was reported effective on human bacterial osteomyelitis [Hernandez-Divers and McArthur, 2004; Wilkinson, 2004]. Most of *Aeromonas* spp. showed susceptibilities to aminoglycosides, tetracyclines, chloramphenicol, sulfamethoxazole-trimethoprim and quinolones, while resistances were detected to nalidixic acid, ciprofloxacin and norfloxacin [Igbinosa et al., 2012]. The present study detected moderate susceptibility to aminoglycosides and tetracyclines, and resistance to lincosamides, fluoroquinolones and third generation cephalosporins. Doganis et al. [2016] suggested a combination of an aminoglycoside and a cephalosporin as the appropriate therapy; whereas Igbinosa et al. [2012] described a synergistic effect of minocycline and cefotaxime in a murine model. The sea turtle with *Aeromonas* infection here presented, exhibited severe, diffuse, and multifocal osteomyelitis leading to severe handicap and was

successfully treated with a combination of Amikacin and Doxycycline for a prolonged period of time.

Interest in the pathogenic nature of *Aeromonas* spp. has been increasing over the last years due to their disease spectrum, antimicrobial-resistance patterns and ubiquitous presence. *Aeromonas* species are becoming appreciated for their implications in certain clinical situations, of both intestinal and extraintestinal disease [Thornley et al., 1997]. Given the zoonotic potential of this microorganism, vigilance should always be maintained, especially for population exposed, such as rescue center operators. Due to the paucity of reports of *Aeromonas* osteomyelitis in loggerhead sea turtles, the documentation of this case will increase knowledge and understanding in caring for these endangered animals. Prompt diagnosis and early antimicrobial treatment may improve the outcome of this serious infection, especially in this wildlife species, where the necessity of short recovery times is more strongly needed, in order to let them return to their natural habitats without further complications and delays.

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Chapter 3

Survey on bacteria and fungi isolated from unhatched loggerhead sea turtle eggs in Italy, and their influence on the embryonic development.

3.1 Introduction

Loggerhead sea turtles (*Caretta caretta*) have the most extensive nesting range of any reptile, covering temperate and tropical latitudes of both hemispheres [Pike, 2014]. In the Mediterranean, loggerhead nests are located mostly in the eastern basin (e.g. Turkey, Greece, Cyprus), though a few nests per year have also been reported along the coasts of southern Italy [Pritchard, 1997; Margaritoulis et al., 2003; Mingozzi et al. 2007; Maffucci et al., 2016].

The reproductive success in oviparous reptiles depends mainly on biotic and abiotic characteristics of the incubation environment, which have to be adequate to reach high hatching rates [Bárcenas-Ibarra et al., 2015; Bézy et al., 2015]. In sea turtles, the nesting beach represents the incubator, therefore successful incubation requires suitable conditions in the beach sand (e.g. temperature, humidity, salinity, gases) [Ackerman, 1997]. Usually, sea turtle eggs have high hatching rates, around 80% [Miller, 1997], but embryonic development represents a crucial stage in the life history of sea turtles, during which they are exposed to many different factors influencing their hatching success [Wyneken et al., 1988; Sarmiento-Ramírez et al., 2010; Broderick and Hancock, 1997].

3.1.1 Anthropogenic Threats

Important nesting sites in the eastern Mediterranean basin have been negatively affected by coastal development, mostly associated to touristic activities. Mechanical cleaning of beaches and beach installations are the

main responsible for direct damage to the eggs, but also for indirect alteration in sand characteristics (physical and geomorphological) [Arianoutsou, 1988; Margaritoulis and Panagopoulou, 2010; Kaska et al., 2010; Türkozan and Kaska, 2010; Spadola et al., 2016]

3.1.2 Predation

Eggs might be predated by several animals, which could act as primary or secondary predators, depending on whether they are able to open the nest by themselves (e.g. canids, mustelids) or they feed upon already exposed nests (e.g. rodents, birds) [Margaritoulis, 1988; Kaska, 2000]. A different type of predation has been described for plants, which in Greece have caused dehydration of several clutches through their invading roots [Margaritoulis and Panagopoulou, 2010; Margaritoulis et al., 2011].

3.1.3 Invertebrate Infestations

The role of invertebrate larvae has not been elucidated yet. Some authors have described a negative impact of Diptera larvae on sea turtle nests, attacking viable eggs and hatchlings, and resulting in a reduced hatching success [Lopes, 1982; Fowler, 1979; Vásquez, 1994]. On the contrary, other studies have reported that larvae feed only on dead embryos and hatchlings, actually helping in the removal of decaying material and preventing the risk of further infections [Andrade et al., 1992; McGowan et al., 2001a; Broderick and Hancock, 1997]. In the Mediterranean, Coleoptera, Diptera and Hymenoptera have been described to infest

loggerhead sea turtle nests [Baran and Türkozan, 1996; McGowan et al., 2001a; Broderick and Hancock, 1997]. Diptera, especially members of the Sarcophagidae family, were the most frequently detected, in particular *Sarcotachina aegyptiaca*, which probably outcompete other species' larvae. Eggs laid further from the high water mark and more deeply in the sand appear to be less prone to infestation [McGowan et al., 2001b].

3.1.4 Temperatures

The incubation duration, as well as sex determination, depends on temperatures of nest sand [Godley et al., 2001; Mrosovsky et al. 2002]. Therefore, changes in nest temperatures could directly influence hatchling phenotypes, but also embryonic mortality [Tapilatu and Tiwari, 2007; Pike, 2014]. The optimal temperature of egg incubation typically ranges from 24°C to 33°C [Spadola et al., 2016], and eggs incubated at temperatures lower than 23°C or higher than 33°C, have been seldom reported to hatch [Miller, 1997]. Actually, in the Mediterranean, viable hatchlings have been recorded from nests with mean temperatures as low as 26.5°C [Casale et al., 2012] and as high as 33.2°C [Godley et al., 2001]. In a future scenario of global warming, the hatching success has been predicted to increase in the Mediterranean by 2020, but a global decline will follow by the 2050, throughout the 2080 [Pike, 2014]. Moreover, high temperatures have been suggested to promote bacterial and fungal infections, as well as congenital malformations (though it has not been demonstrated) [Bárcenas-Ibarra et al., 2015].

3.1.5 Malformations

Congenital malformations in sea turtles are relatively rare, but they could negatively influence the hatching success, as malformed embryos have been reported to be unable to break out of their eggs [Bárcenas-Ibarra et al., 2015]. On the contrary, the effect of scale abnormalities is still unknown. Despite being unlikely to cause mortality, anomalous scales are more frequently detected in unhatched embryos compared to hatchlings and to adults, suggesting a negative influence on the turtle fitness. Scale abnormalities have been associated to environmental conditions (e.g. high temperatures, low humidity, pollutants) and loss of genetic diversity [Phillott and Parmenter, 2014].

3.1.6 Heavy Metals

Heavy metal concentration might have a detrimental effect in the hatching success. In leatherback sea turtles, Selenium and Mercury concentration in the liver of hatchlings were associated to hatching and emergence success. In particular, Selenium deficiency has been correlated to an inadequate skeletal muscle development, as reported in other animals, influencing the ability of turtles to break out from eggs and emerge from the nest. Additionally, Selenium might be able to protect the embryo from Mercury toxicity during early developmental stages [Perrault et al., 2011].

3.1.7 Health Status of Mothers

Several parameters of sea turtle health (e.g. alkaline phosphatase, blood urea nitrogen, Calcium and Phosphorus concentrations, cholesterol, creatinine) have been significantly correlated with hatching and emergence success in leatherback sea turtles. Indeed, a sea turtle in suboptimal health status might lay eggs with decreased nutritional reserves, affecting the survival of the offspring [Perrault et al., 2012].

3.1.8 Microbial Contamination

Bacteria and fungi have been reported to play a significant role during embryonic development. [Awong-Taylor et al., 2008]. A vast majority of these microorganisms have been considered to occur naturally in the nest environment (e.g. *Achromobacter*, *Acinetobacter*, *Bacillus*, *Citrobacter*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Aspergillus*, *Chrysosporium*, *Cladosporium*, *Mucor* and *Penicillium)*, acting as saprophytes decomposing failed eggs. Nevertheless, they could turn into opportunistic pathogens for viable eggs [Keene, 2012; Bézy et al., 2015; Güçlü et al., 2010; Craven et al., 2007; Mo et al., 1990; Patino-Martinez et al., 2012]. Indeed, the nest environment provides a favourable setting for microbial proliferation (e.g. nutrients, temperature, humidity), and the optimal conditions for egg incubation represent ideal circumstances for microbial growth and colonization [Keene, 2012; Sarmiento-Ramírez et al., 2010; Sarmiento-Ramírez et al., 2014a]. On the other hand, environmental factors have been suggested to influence bacterial pathogens (e.g.

humidity, proximity to vegetation) as well as embryo's susceptibility to them [Craven et al., 2007; Honarvar et al., 2011; Sarmiento-Ramírez et al., 2014b; Keene, 2012].

Wyneken et al. [1988] were probably the first to address bacterial pathogens in loggerhead sea turtle eggs. They could not identify a specific causal organism, but high bacterial diversity was significantly correlated with low hatching success. Additionally all isolated bacterial species represented well-known reptilian pathogens [Wyneken et al., 1988]. Since then, studies on the presence of bacteria on sea turtle eggs have been increasing, and Sarmiento-Ramírez et al. [2014b] provided also a first insight into the bacterial community associated with sea turtles eggs, identifying Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes as the most representative phyla.

Concerning fungi, they seem to play a larger role than bacteria in influencing embryonic development [Bézy et al., 2015]. Failed eggs, which are the first to be colonized by saprophytic fungi, could promote the spreading of fungal hyphae to viable eggs, prejudicing the entire clutch [Phillott and Parmenter, 2001a; Keene, 2012; Phillott et al., 2001; Güçlü et al., 2010; Sarmiento-Ramírez et al., 2014a]. One genus in particular, namely *Fusarium*, has been associated with mass mortalities during embryonic development in several sea turtle species of different locations [Phillott and Parmenter, 2001a; Phillott et al., 2001; Sarmiento-Ramírez et al., 2010; Sarmiento-Ramírez et al., 2014a]. Despite its proven pathogenicity, confirmed by fulfilment of Koch's postulates, the presence of *Fusarium* also in unaltered eggs, points at other co-factors in the development of disease (e.g. microclimatic conditions, sand composition,

natural immunosuppression) [Sarmiento-Ramírez et al., 2010]. Indeed, the incidence of *Fusarium* disease in nests subjected to inundation or characterized by clay and silt was significantly higher than in dry sand nests [Sarmiento-Ramírez et al., 2014a].

Bacteria and fungi contaminating sea turtle eggs might be of either environmental or maternal origin. As a matter of fact, microorganisms detected in unhatched eggs have also been isolated from nest sand and from nesting females [Bézy et al., 2015; Wyneken et al., 1988; Zieger et al., 2009; Acuña et al., 1999; Mo et al., 1990; Güçlü et al., 2010; Keene, 2012]. The eggs might be contaminated during their permanence in the reproductive tract of the female, before the eggshell is formed, or during oviposition, as they pass through the cloaca [Al-Bahry et al., 2009; Al-Bahry et al., 2012; Craven et al., 2007]. Wyneken et al. [1988] detected a significant association between reduced hatching success and the presence of a bacterial species both in a nesting female and her clutch. Actually, the female cloaca could be in turn contaminated during copulation with males or right during oviposition, as described for many fungal species that were recovered from the cloaca of inter-nesting sea turtles [Phillott, 2002; Keene, 2012; Patino-Martinez et al., 2012]. Following oviposition, eggs might be contaminated by microorganisms already present in the surrounding sand, or by others newly introduced by the nesting female [Craven et al., 2007; Zieger et al., 2009; Patino-Martinez et al., 2012]. During embryonic development, many other sources can introduce new microorganisms to the nest environment (e.g. tides, rains, wind, predators) [Keene, 2012].

Microorganisms are usually able to colonize every component of the egg, from the shell to the yolk. Nevertheless, Al-Bahry et al. [2009] found lower concentration of bacteria in the albumen. This finding is likely to be ascribed to antimicrobial properties of the albumen proteins [Zieger et al., 2009; Keene, 2012]. Actually, Chakrabarti et al. [1988] isolated a small protein from egg white of a loggerhead sea turtle, similar to those found in other organisms which lack lysozyme, indicating potential antimicrobial properties [Chattopadhyay et al., 2006]. Analogous properties have been suggested for the fluid deposited by nesting females along with the eggs [Phillott, 2002; Keene, 2012; Soslau et al., 2011].

Given the impact of egg failure on the production of an adequate level of hatchlings to sustain the population through time [Miller, 1997], it is important to assess all the potential threats to this delicate stage of sea turtle life history. Therefore, this study was aimed at performing the first microbiological survey on unhatched loggerhead sea turtle eggs in Italy, in order to define the composition of microbial community in these eggs and to assess its potential pathogenic role on embryonic development.

3.2 Materials & Methods

3.2.1 Sampling

During the months of August, September and October of three consecutive years (2015, 2016, 2017) a total of 19 loggerhead sea turtle nests in nine different localtions, along the coasts of Campania and Lazio (south-

western Italy), were examined (Figure 3.1). Each nest was excavated after 72 hours from the last emerged hatchling, as suggested by the Italian National Institute for Environmental Protection and Research [ISPRA, 2013]. Nests were named from N1/15 to N3/17 for the purposes of the study and a variable number of unhatched eggs were collected from each nest, depending on the number of unhatched eggs in the clutch (Table 3.1). Two samples were collected from each egg, when the egg conditions at excavation allowed it: a surface swab and a fluid swab. The surface swab was collected at the moment of the final excavation of the nest, using a sterile cotton-tipped swab, subsequently inoculated into transport medium Amies (Oxoid). Eggs were handled with sterile gloves in order to avoid contamination of the surface. Collected eggs were then transported at 4°C to the MTRC (Stazione Zoologica Anton Dohrn of Naples, Italy), in order to collect a fluid swab and perform the embryonic stage assessment. The surface of the egg was sterilized with hydrogen peroxide, and povidone-iodine was applied to a portion of shell that was then aseptically removed with sterile forceps or lancet. A sterile cotton-tipped swab was used to obtain a fluid sample from the interior of the egg, and then inoculated into PBS (Oxoid). A total of 86 surface swabs and 152 fluid swabs were collected. All samples were subsequently transported at 4°C to the Experimental Centre of Poultry and Rabbits (Department of Veterinary Medicine and Animal Productions of the University Federico II of Naples, Italy).

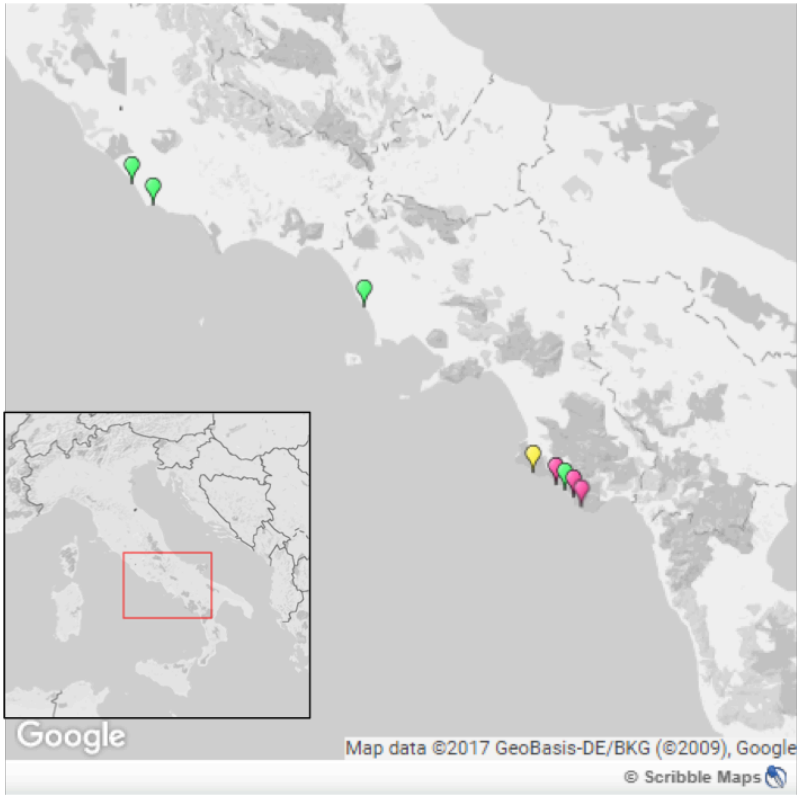

Fig. 3.1 Loggerhead sea turtle nests examined during Aug-Oct 2015, 2016, and 2017. The green marks indicate one examined nest; the yellow mark indicates two examined nests; the red marks indicate four examined nests.

Tab. 3.1 Number of surface and fluid swab samples collected from each nest site.

Nest Identification	**Surface swab samples (n)**	**Fluid swab samples (n)**
N1/15	0	14
N2/15	0	3
N3/15	6	5
N4/15	15	12
N5/15	0	9
N6/15	19	19
N1/16	8	9
N2/16	10	12
N3/16	0	8
N4/16	5	5
N5/16	2	2
N6/16	0	10
N7/16	2	2
N8/16	9	9
N9/16	0	9
N10/16	0	7
N1/17	2	2
N2/17	2	2
N3/17	6	13

3.2.2 Embryonic Stage Assessment

Each embryo detected at the opening of the eggs, during the collection of the fluid swab sample, was submitted to embryonic stage assessment. Embryonic morphometric parameters were evaluated through visual observation, and compared to the description provided by Miller [1985], in order to assess the stage of embryonic development. Embryonic stages

were classified into four categories for the purposes of this study: I) below stage 10; II) from stage 11 to stage 20; III) from stage 21 to stage 26; IV) above stage 27.

Sometimes, due to the effect of decomposition, it was not possible to classify the embryonic stage; in this case, embryonic stage was classified as Not Determined.

3.2.3 Microbial Isolation

Surface swab samples were transferred into Buffered Peptone Water (Oxoid). Concerning fluid samples, from each PBS suspension 50µl were transferred into Buffered Peptone Water and 50µl were transferred into Alkaline Saline Peptone Water.

Samples inoculated into Buffered Peptone Water were incubated at 37°C for 24h and then inoculated into Rappaport-Vassiliadis Broth (Oxoid), as well as plated onto *Pseudomonas* Cetrimide Agar (Oxoid); n. 3 MacConkey Agar (Oxoid); Baird-Parker Agar (Oxoid) and Sabouraud Dextrose Agar (Oxoid) with the addition of lactic acid.

Rappaport-Vassiliadis Broth were incubated at 42°C for 24h and then plated onto Brilliant Green Agar (Oxoid).

Samples inoculated into Alkaline Saline Peptone Water were incubated at 37° for 18-24h and then placed into *Aeromonas* medium base (Oxoid) and Thiosulfate-Citrate-Bile salts-Sucrose Cholera medium (Oxoid).

The *Pseudomonas* Cetrimide Agar, n. 3 MacConkey Agar, Baird-Parker Agar, Thiosulfate-Citrate-Bile salts-Sucrose Cholera medium and Brilliant Green Agar plates were incubated at 37°C for 24–48h; *Aeromonas* medium

base plates were incubated at 30°C for 24h; whereas Sabouraud Dextrose Agar plates were incubated at 30°C for 48 h and checked daily for a further week before discarding.

All isolated strains were primarily identified, selecting 2-3 colonies from each plate, on the basis of their colonial morphology, Gram characteristics, growth requirements, pigment production, tube coagulase test, and standard conventional biochemical and phenotypic tests. The isolates were confirmed using API systems (bioMérieux). *Staphylococcus* spp. isolates, which resulted positive to the tube coagulase test, were submitted to the rapid serum agglutination test with the mono-specific antisera for *S. aureus* (Biorad). *E. coli* isolates were serogrouped with poly-specific antisera (Sifin).

Fungal isolates were primarily identified on the basis of the size, surface, appearance, texture and colour of the colonies, and confirmed using API systems (bioMérieux).

3.2.4 Antimicrobial Susceptibility Testing

Bacterial isolates were submitted to antimicrobial susceptibility testing using the disk diffusion method, according to the CLSI documents [CLSI, 2012]. The antimicrobials tested were Amikacin (30µg; Oxoid); Ampicillin (10µg; Oxoid); Ceftazidime (30µg; Oxoid); Chloramphenicol (30µg; Oxoid); Ciprofloxacin (5µg; Oxoid); Colistin-sulphate (10µg; Oxoid); Doxycycline (30µg; Oxoid); Gentamicin (10µg; Oxoid); Nalidixic Acid (30µg; Oxoid); Streptomycin (10µg; Oxoid); Tetracycline (30µg; Oxoid); Trimethoprim-sulfamethoxazole (1.25/23.75µg; Oxoid). The

inhibition zones were measured and scored as susceptible, intermediate and resistant, according to the CLSI documents [CLSI, 2014]. When an antimicrobial molecule for a specific agent was not present in the CLSI documents, a similar antimicrobial molecule of the same class was used: specifically, Colistin breakpoints were used to evaluate CT sensibility. In order to evaluate the presence of ESBL producing bacteria, all strains belonging to the family Enterobacteriaceae were also submitted to the Combination Disk diffusion test, using CPD (10µg; Oxoid) and CD (10/1µg; Oxoid), and to the ETEST® ESBL (ESBL CT/CTL 16/1; bioMérieux).

3.2.5 Statistical Analyses

Bacterial isolation results were analysed to test differences between proportions of each bacterial family from surface and fluid swab samples and to evaluate possible association between bacterial families and stage of embryonic development. Similarly, fungal isolation results were analysed to test differences between proportions of fungi from surface and fluid swab samples and to evaluate a possible association with the stage of embryonic development. Additionally, results were analysed to assess the relationship between the presence of bacterial families and the presence of fungi. Chi-square analysis was used, except when numbers were too small to appropriately do so; in these cases, the Fisher Exact test was used. Statistical analyses were performed with Past3 and statistical significance was set at $p<0.05$.

3.3 Results

3.3.1 Embryonic Stage Assessment

A total of 93 embryos were classified as follows: 52 were below stage 10 (I); 15 were comprised between stage 11 and stage 20 (II); 2 were comprised between stage 21 and stage 26 (III); 24 were above stage 27 (IV). 59 embryos were not determined, because they were in too bad conditions to allow a reliable stage determination.

3.3.2 Microbial Isolation

All surface swabs samples (100%) and 149 out of 152 fluid swab samples (98.03%) were positive to at least one microorganism, yet the majority of samples yielded mixed microbial cultures. Results of microbial cultures are summarized in Table 3.2. Pseudomonadaceae were the most commonly isolated, both in surface and fluid swabs, followed by Enterobacteriaceae, and Staphylococcaceae, whereas Aeromonadaceae were the less frequently isolated.

Two *Staphylococcus* strains isolated from surface swabs resulted positive at the tube coagulase test, and were subsequently identified as *S. aureus* through rapid serum agglutination test.

Two *E. coli* strains isolated from fluid swabs were classified within enterohaemorrhagic *E. coli* of group I through rapid serum agglutination with poly-specific antisera.

The bacterial families Pseudomonadaceae and Staphylococcaceae were detected with significant different prevalence in surface and fluid swabs (respectively χ^2=9.5813 and χ^2=35.451; df=1 p<0.005), both characterized by higher prevalence in surface swabs than in fluid swabs (Table 3.2).

Tab. 3.2 *Prevalence of bacterial and fungal isolates from 86 surface swabs and 152 fluid swabs of loggerhead sea turtle unhatched eggs.*

Bacterial Family	**Surface Swabs (%)**	**Fluid Swabs (%)**
Staphylococcaceae	67.44	28.95
Pseudomonadaceae	97.67	84.87
Aeromonadaceae	0	1.97
Vibrionaceae	23.26	32.24
Enterobacteriaceae	72.09	68.42
Bacterial species		
CPS.[a]	2.33	0%
CNS	65.12	28.95
Pseudomonas aeruginosa	58.14	49.34
Pseudomonas spp.[b]	56.98	53.29
Aeromonas hydrophila	0	1.97
Vibrio alginolyticus	17.44	28.29
Vibrio fluvialis	8.14	5.92
Achromobacter denitrificans	4.65	1.97
Shewanella putrefaciens group	39.53	15.79
Citrobacter spp.	30.23	24.34
Enterobacter spp.	29.07	26.97
Escherichia coli	0	1.32
Klebsiella oxytoca	2.33	3.29
Klebsiella pneumoniae	10.47	6.58
Morganella morganii	10.47	8.55

Providencia spp.	0	0.66
Serratia spp.	1.16	7.89
Other coliform bacteria	18.60	15.13
Fungal isolates	**55.81**	**48.03**
Aspergillus spp.[c]	4.65	0.66
Candida spp.	27.91	19.08
Cryptococcus spp.	3.49	1.32
Fusarium spp.[c]	15.12	9.21
Penicillium spp.[c]	2.33	1.32
Trichosporon spp.	1.16	0
Other non identified fungi	30.23	29.61

[a] identified as *S. aureus*.

[b] with the exception of *P. aeruginosa*.

[c] identification only on the basis of colony morphology.

3.3.3 Antimicrobial Susceptibility Testing

Few bacterial strains exhibited resistance to antimicrobials. None of the bacterial strains submitted to antimicrobial susceptibility testing showed simultaneous resistance to more than two antimicrobials. The most frequently detected resistances were to Ampicillin (84.2% of tested strains), and Doxycycline (43.8%). Lower percentage of antibiotic resistance was detected towards Tetracycline (27%), Ceftazidime (8.62%) and Amikacin (1.54%). No resistance was detected towards Chloramfenicol, Ciprofloxacin, Colistin-sulphate, Gentamicin, Nalidixic Acid, Streptomycin and Trimethoprim-sulfamethoxazole (Figure 3.2). Strains scored as intermediate resistant were not included in the calculation of the percentage of antimicrobials resistance, previously reported.

The isolates that showed resistance to the greatest number of antimicrobials were the two enterohaemorrhagic *E. coli* (respectively resistant to 25% and 12.5% of tested antimicrobials). Lower proportion of antimicrobial resistance was detected for *Enterobacter* spp. (from 0% to 20%, mean 8.7%), *Citrobacter* spp. (0-20%, mean 7.5%), *Klebsiella* spp. (0-10%, mean 6.7%), and *P. aeruginosa* (0-40%, mean 5.7%). No strains of Enterobacteriaceae were detected to produce ESBL.

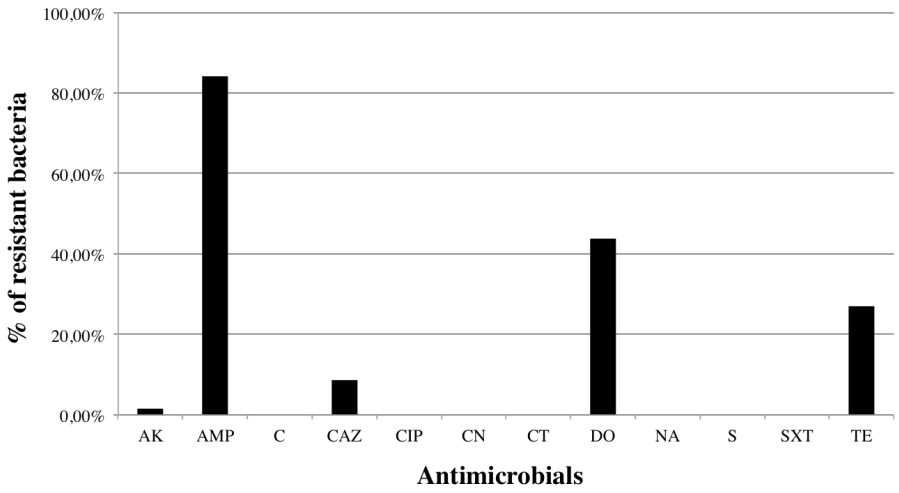

Fig. 3.2 Antimicrobial resistance of bacterial isolates from 86 surface swab samples and 152 fluid swab samples of loggerhead sea turtle unhatched eggs.

3.3.4 Influences Among Bacteria, Fungi and Embryonic Development

Significant association was detected between stage of embryonic development and microbial isolates. Specifically, in surface swabs, the prevalence of Enterobacteriaceae was found significantly (χ^2=13.441;

df=3; $p<0.005$) higher in earlier stages of development (I=73.08% and II=85.71%) than in later stages (III=0% and IV=26.67%). Opposite association, in fluid swabs, was detected for Vibrionaceae family and fungal isolates (respectively $\chi^2=16.708$ and $\chi^2=18.614$; df=3; $p<0.001$). Vibrionaceae family showed a trend of increasing prevalence along with the embryonic development (I=11.54%; II=40%; III=50%; IV=54.17%); similarly, fungal isolates showed a lower prevalence in earlier than in later stages of development (I=7.69%; II=20%; III=0%; IV=50%).

Concerning the relationship between bacterial families and fungal isolates, opposing associations were detected for Staphylococcaceae, Vibrionaceae and Enterobacteriaceae. Specifically, Staphylococcaceae were negatively associated with the presence of fungi on surface swab samples, showing a significant ($\chi^2=4.1045$; df=1; $p<0.05$) lower prevalence in samples with fungal colonization (58.33%) than in samples without fungal colonization (78.95%). On the contrary, Vibrionaceae and Enterobacteriaceae were positively associated with the presence of fungi both in surface and fluid swab samples. Vibrionaceae showed a significant ($\chi^2=16.226$; df=1; $p<0.001$) higher prevalence in surface swab samples with fungal colonization (39.58%), than in samples free of fungal colonization (2.63%), whereas no significative difference ($\chi^2=3.6063$; df=1; $p=0.058$) was detected in fluid swab samples, though a similar pattern was observed (39.73% in samples with fungal colonization, and 25.32% in samples free of fungal colonization). Enterobacteriaceae were detected with significant (in surface swab samples $\chi^2=12.817$, in fluid swab samples $\chi^2=20.783$; df=1; $p<0.001$) higher prevalence both in surface and fluid swab samples

with fungal colonization (respectively 87.50% and 86.30%) than in samples free of fungal colonization (respectively 52.63% and 51.90%).

3.4 Discussion

In this survey, bacterial and fungal contamination of loggerhead sea turtle eggs was investigated to assess their saprophytic or pathogenic role in sea turtle embryonic development.

Almost all the examined eggs yielded bacterial and/or fungal species. This high prevalence of contaminated eggs is in contrast with the lower one reported in green turtle eggs [Al-Bahry et al., 2009], but it is likely due to the sampling method rather than to the species, because it consisted in the collection of eggs during oviposition, thus drastically reducing the effect of environmental contamination. There are not many studies on sea turtle eggs similar to the present one; however, the majority of the bacterial and fungal isolates cultured in the present survey have already been described. Generally, Pseudomonadaceae and Enterobacteriaceae have been the most frequently isolated bacterial families [Zieger et al., 2009; Al-Bahry et al., 2009]. Within Pseudomonadaceae, the most commonly identified species was *Pseudomonas aeruginosa*, in line with a similar study conducted on olive ridley eggs [Keene, 2012], as well as within the Enterobacteriaceae, *Citrobacter* and *Enterobacter* represented the main identified genera, in accordance with other authors [Al-Bahry et al., 2009; Zieger et al., 2009; Keene, 2012]. Interesting was the absence of *Salmonella* spp. in this survey, because Al-Bahry et al. [2009] documented this bacterium in 19.2% of examined eggs, and Keene, [2012] reported its presence in both

failed eggs and sea turtle cloacal fluid. Indeed, the role of *Salmonella* spp. in sea turtles has always been controversial: sea turtles had not been shown to be a reservoir of this bacterial species [Johnson-Delaney, 2006], but *Salmonella* cases have been sporadically documented in sea turtles [George, 1997; Ives et al., 2017; Raidal et al., 1998]. Concerning other bacterial families, Vibrionaceae have been seldom reported in failed sea turtle eggs [Wyneken et al., 1988; Mo et al., 1990], though a severe case of human infection due to consumption of sea turtle eggs was ascribed to *Vibrio mimicus* [Acuña et al., 1999]. Similarly, the detection of enterohaemorrhagic *E. coli* and their high proportion of antibiotic resistance, give rise to public health concerns, as they are reported to be highly infectious to human beings, causing episodes of haemorrhagic colitis and haemolytic uremic syndrome, and they have increasingly been isolated from a growing spectrum of animal species [Caprioli et al., 2005].

Concerning antibiotic resistance, there is little information on bacteria from sea turtle eggs. Nevertheless, the results presented in this survey are consistent with those described by Al-Bahry et al. [2009] and Zieger et al. [2009], who both reported higher percentage of resistance towards Ampicillin, and lower percentage towards Amikacin and Gentamicin. Moreover, a very similar pattern of antibiotic resistance was reported in the previous study (Chapter 1) on bacteria isolated from oral and cloacal swabs of loggerhead sea turtles.

With respect to fungal isolates, the fungal genera reported here are partially confirmed in the literature, with *Aspergillus*, *Penicillium* and *Fusarium* as the most frequently described fungal genera [Güçlü et al., 2010; Mo et al., 1990; Patino-Martinez et al., 2012; Phillott, 2002;

Sarmiento-Ramírez et al., 2010], whereas *Cryptococcus*, *Trichosporon* and *Candida* have not been reported in sea turtle eggs. *Candida* spp. have been recovered in oral and cloacal swabs of sea turtles, yet they have been suggested to be unusual commensal microorganisms, rather associated with immunosuppression or promiscuity in rehabilitation facilities [Brilhante et al., 2015]. On the contrary, *Cryptococcus* and *Trichosporon* have not been reported in sea turtles, but cases of infections have been diagnosed in captive cetaceans and in terrestrial reptiles [Higgins, 2000; Paré and Jacobson, 2007].

A vast majority of the microorganisms isolated in this survey, as well as in previous studies, have been also detected in the sand from the egg chamber and in cloacal samples of nesting sea turtles, suggesting that the eggs become contaminated through either of these two routes [Al-Bahry et al., 2009; Bézy et al., 2015; Craven et al., 2007; Güçlü et al., 2010; Keene, 2012; Mo et al., 1990; Phillott et al., 2001; Phillott and Parmenter, 2001a; Sarmiento-Ramírez et al., 2014a; Spadola et al., 2016; Wyneken et al., 1988; Zieger et al., 2009; Phillott, 2002]. This hypothesis seems to be confirmed by the higher prevalence of Pseudomonadaceae and Staphylococcaceae in surface swabs compared to fluid swabs, which further implies the possible saprophytic role of these bacterial families, as they could pose a lower risk of inducing embryonic death if they do not penetrate the eggshell. The pathogenic potential of bacteria and fungi of sea turtles has long been debated. As previously discussed, several bacterial strains could be responsible for many different lesions in sea turtles, acting as opportunistic pathogens. Likewise, fungi could be responsible for diseases in sea turtles, as confirmed by cases of

hyalohyphomycosis and bronchopneumonia, ascribed to the genera: *Aspergillus*, *Paecylomyces*, *Fusarium* and *Penicillium* [Keene, 2012; Cabañes et al., 1997; Glazebrook and Campbell, 1990; Glazebrook et al., 1993]. In sea turtle eggs, the infection is commonly believed to be opportunistic, and the exact mechanism through which bacteria could affect the embryo has been difficult to determine [Mo et al., 1990; Bézy et al., 2015; Patino-Martinez et al., 2012; Keene, 2012; Craven et al., 2007; Spadola et al., 2016]. It has been hypothesised that high rates of microbial decomposition of organic matter results in increased temperatures and reduced oxygen concentration, altering the optimal range for embryonic development [Bézy et al., 2015; Spadola et al., 2016]. On the contrary, several mechanisms have been suggested for fungal pathogenicity. Indeed, fungal infection has been described to provoke loss of rigidity and thickness in the eggshell, likely as a result of enzymatic activity and the acidic metabolites. Other possibilities include: penetration of the eggshell and utilization of embryonic tissues as source of nutrients; impairment of gaseous exchange; depletion of Calcium reserves; production of mycotoxins and other metabolites; exhaustion of energy reserves by overstimulation of immune system [Phillott, 2002; Güçlü et al., 2010; Phillott, 2004; Phillott and Parmenter, 2001b; Phillott and Parmenter, 2014; Sarmiento-Ramírez et al., 2014a; Paré et al., 2006; Patino-Martinez et al., 2012].

Patino-Martinez et al., [2012] reported the final third of embryonic development to be less sensitive to the impact of microorganisms, probably because these latter do not have enough time to proliferate and compete with hatchlings. Whilst this assertion is consistent with the higher

prevalence of Enterobacteriaceae recovered from surface swab samples of eggs containing earlier stage embryos (categories I and II) compared to eggs containing later stage embryos (categories III and IV), it is in contrast with the finding of higher Vibrionaceae and fungal isolates recovered from fluid swab samples. It is possible that Vibrionaceae and fungi take more time to induce embryonic death, or that they affect already failed eggs before moving to the viable ones, allowing the vital embryos more time to develop. Indeed, fungal invasion does not kill the newly laid egg [Phillott and Parmenter, 2001a; Keene, 2012; Phillott et al., 2001; Güçlü et al., 2010; Sarmiento-Ramírez et al., 2014a]. It shall be specified that the findings presented in this survey only point at a significant association between stages of embryonic development and prevalence of microbial isolates, but do not imply a causal relationship. Moreover, studies correlating the embryonic death to the presence and abundance of microorganisms are still lacking [Bézy et al., 2015]. There is also the possibility that embryos arrested development due to other reasons (probably environmental, such as tides or temperature changes), and subsequently got colonized by bacteria and fungi already present in the surrounding environment, or, that precisely the environmental changes that provoked the embryonic development arrest were the ones to introduce the microorganisms or facilitate the microbial colonization [Craven et al., 2007; Keene, 2012; Sarmiento-Ramírez et al., 2014b;]. Similarly, the association between bacteria and fungi does not imply a causal relationship. The positive correlation between the bacterial families (i.e. Enterobacteriaceae and Vibrionaceae) and the fungal isolates could be related to several possibilities, including: a synergistic effect of the

microorganisms on each other, through mechanisms still to be disclosed; an increased susceptibility of the embryo, be it by one of the microorganisms or by environmental conditions; an external third factor promoting the growth of both microorganisms. On the contrary, the negative association between bacterial families (i.e. Staphylococcaceae) could be explained by competition for the available resources, or by the production of antimicrobial compounds, as suggested for certain bacterial taxa (Pseudomonadaceae, Flavobacteriaceae, Actinobacteria) which have been found to have antifungal properties in plants and insects. Curiously, these fungal antagonists have been described in hatched eggs, suggesting a potential protective role in sea turtle eggs against fungal colonization [Sarmiento-Ramírez et al., 2014b].

Indeed, bacteria and fungi are associated to egg failure in sea turtles, but additional studies are needed to elucidate the mechanisms through which bacteria and fungi interact with each other and with the embryos, in order to assess their role in the developmental arrest. Moreover, the risk that unhatched eggs could pose to humans and other animals should not be overlooked, as many of the microorganisms here described could represent potential zoonotic agents [Acuña et al., 1999; Zieger et al., 2009; Aguirre et al., 2006]. Therefore, biosecurity is always recommended when excavating sea turtle nests and handling sea turtle eggs. Further studies are currently been conducted to correctly identify the fungal isolates that were cultured in the present survey, and to better explore the role of additional factors (e.g. area of nesting, vegetation, climatic events, bacterial diversity, number of clutch) on microbial communities and hatching success of loggerhead sea turtles in Italy.

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Chapter 4

Screening for Herpesvirus and Chlamydiaceae in Mediterranean loggerhead sea turtles

4.1 Introduction

Sea turtles are susceptible to a myriad of infectious agents (bacterial, fungal and viral), yet the manifestation of disease depends on several factors, mainly involving the host, the pathogen and their environment. Healthy turtles in the wild seldom develop infectious diseases, whereas turtles in captivity, injured, or stressed for any other reason (e.g. environmental challenging condition, malnutrition, concomitant diseases) might frequently exhibit disease processes [George, 1997; Herbst and Jacobson, 2002; Wyneken et al., 2006]. Actually, as diseases can be present in a sub-clinical state, an appearing healthy sea turtle might have a sub-clinical process going on internally. Therefore, any stress, including captivity, could promote the manifesting of a sub-clinical disease, as well as the reactivation of a latent infection or the take over of innocuous opportunistic agents. This is the reason why several sea turtle infectious diseases have been described first, and sometimes exclusively, in outbreaks among captive sea turtles, including, as a matter of fact, herpesvirus and chlamydia associated diseases [Herbst and Jacobson, 2002].

4.1.1 Herpesviruses

Herpesviruses have been detected from several reptile species affected by a wide variety of lesions (e.g. oropharyngeal lesions, gastrointestinal necrosis, pulmonary edema, papillomatous lesions) [Ritchie, 2006]. All reptilian herpesviruses have been classified in the sub-family

alphaherpesvirinae [Stacy et al., 2008]. The majority of herpesviruses has a restricted host range, to which they have been adapting through a long process of co-evolution [Alfaro-Núñez et al., 2014]. Usually, herpesviruses establish a primary systemic infection characterized by viraemia, replication and shedding, which may be associated or not with signs of disease [Ritchie, 2006; Page-Karjian et al., 2015; Ariel, 2011]. More interesting is the ability of herpesviruses to establish a latent infection, through which the virus can persist in the animal life-long, and therefore in the population. Latent infections can reactivate later in the course, as a result of stressful events [Ritchie, 2006; Ariel, 2011]. Herpesviridae are probably the most studied family of animal viruses in sea turtles [Stacy et al., 2008; Alfaro et al., 2006]. To date, at least five herpesviruses (i.e. ChHV1, ChHV5, ChHV6, LOCV, and LGRV) have been identified, and associated to as many different syndromes [Ritchie, 2006; Jones et al., 2016; Stacy et al., 2008], as discussed in the general introduction. Several co-factors have been investigated as responsible for inducing disease (as in fibropapillomatosis) or for exacerbating the severity (as in GPD) [Alfaro-Núñez et al., 2016; Alfaro-Núñez et al., 2014; Aguirre et al., 1994; Aguirre et al., 2002; Aguirre and Lutz, 2004; Dailey and Morris, 1995; Herbst, 1994; George, 1997; Flint, 2013; Ariel, 2011; Rebell et al., 1975; Higgins, 2002; Ritchie, 2006]. Juvenile turtles have been reported as the most frequently affected, or the most severely affected [Jones et al., 2016; Ritchie, 2006; Origgi, 2006; Flint, 2013; Higgins, 2002]. Transmission usually occurs horizontally, through direct contact with an infected individual or its secretions (e.g. saliva, mucus, blood, urine, faeces, infected skin cells) [Alfaro-Núñez et al., 2014; Page-Karjian et al., 2015;

Ritchie, 2006]. Nevertheless, different authors have also suggested sexual transmission (e.g. LGRV) and mechanical vectors (e.g. *Ozobranchus margoi*, *Thalassoma duperrey*) as additional routes of infection [Stacy et al., 2008; Flint et al., 2009; Flint, 2013]. Diagnoses of reptilian herpesviruses could be achieved from different samples (e.g. oropharyngeal, choanal, conjunctival or cloacal swabs; liver, spleen, lung, brain, or gastrointestinal tract tissues), and through different techniques (e.g. serum neutralization test, Enzyme-linked immunosorbent assay, electron microscopy visualization, histologic examination, molecular-based method) [Ritchie, 2006; Barrows et al., 2004; Wilkinson, 2004; Ossiboff et al., 2015; Origgi, 2006]. In particular, ChHV5, which is responsible for sea turtle fibropapillomatosis, and probably the most studied herpesvirus in these animals, has been detected from several sea turtle species, in all major oceans, reaching epizootic proportions [Aguirre et al., 1994; Aguirre et al., 2002; Aguirre and Lutz, 2004; Flint, 2013]. Indeed, this virus has been detected both in healthy and affected sea turtles, and within these latter, both from normal and tumorous tissues. Actually, the virus might be unequally distributed throughout the body, with higher concentration in the affected tissues than in the normal ones [Alfaro-Núñez et al., 2014; Duarte et al., 2012; Page-Karjian et al., 2015; Fichi et al., 2016]. The detection of viral DNA in asymptomatic animals has been recommended as a mean to identify turtles that will eventually develop the disease, if subjected to the appropriate co-factors [Quackenbush et al., 2001; Alfaro-Núñez et al., 2016].

4.1.2 Chlamydiaceae

The order Chlamydiales consists of 4 families, one of which, namely Chlamydiaceae, include known pathogens for humans and other animals [Jacobson and Samuelson, 2007; Paré et al., 2006; Bodetti et al., 2002]. All chlamydiae are obligate intracellular bacterial pathogens, characterized by a developmental cycle consisting of two forms: an extracellular survival form (i.e. elementary body) and an intracellular replicating form (i.e. reticulate body) [Stacy and Pessier, 2007; Jacobson and Samuelson, 2007]. A recent reclassification of the family Chlamydiaceae resulted in nine species belonging to two genera (i.e. *Chlamydia trachomatis; Chlamydia suis; Chlamydia muridarum; Chlamydophila psittaci; Chlamydophila pneumoniae; Chlamydophila felis; Chlamydophila pecorum; Chlamydophila abortus; Chlamydophila caviae*) [Everett et al., 1999a]. All of them are genetically quite diverse, with at most 95% identity of 16S rRNA between *C. psittaci* and *C. trachomatis* strains [Bodetti et al., 2002]. Among pathogens, *C. pneumoniae* used to be considered the most common in humans, and restricted to them [Bodetti et al., 2002]. However genetically different isolates have been later reported in other animals, including mammals and, more recently, amphibians and reptiles [Storey et al., 1993; Wardrop et al., 1999; Berger et al., 1999; Bodetti et al., 2002; Jacobson, 2007]. Chlamydiae are being increasingly regarded as infectious agents in reptiles, as they have been detected from all major groups [Paré et al., 2006; Jacobson, 2007]. Indeed, chlamydial agents, included either in the Chlamydiaceae or other families (e.g. Parachlamydiaceae, Simkaniaceae), have been described in snakes, chameleons, iguanas,

crocodiles, tortoises and turtles [Jacobson and Samuelson, 2007; Paré et al., 2006; Corsaro and Venditti, 2004; Hotzel et al., 2005]. Several different lesions have been associated to chlamydial infections in reptiles: ocular infections, upper or lower respiratory infections, gastrointestinal lesions, heart infections and generalized granulomatous lesions [Paré et al., 2006; McArthur, 2004a; McArthur, 2004b; Soldati et al., 2004; Jacobson et al., 2002; Huchzermeyer et al., 2008; Stacy and Pessier, 2007; Murray, 2006a; Murray, 2006b; Di Ianni et al., 2015; Homer et al., 1994]. In sea turtles, only one case of chlamydiosis has been reported: an outbreak in a turtle farm, which caused the death of hundreds of juvenile green turtles. Chlamydiosis is apparently a systemic infection, but affected turtles showed non-specific symptoms (i.e. lethargy, debilitation, inability to dive). At necropsy, necrotic lesions involved mainly hearts and livers, and chlamydial agents were detected in tissue samples (i.e. *Neochlamydia*, *C. abortus*, and *C. pneumoniae*) [Homer et al., 1994; George, 1997; Bodetti et al., 2002; Jacobson, 2007]. Since culturing reptile chlamydiae is difficult, chlamydiosis diagnosis should be achieved by other means. Electron microscopy and immunohistochemistry are the most frequently recommended for reliability and rapidity, but PCR, targeting specific sequences (e.g., *omp*A, *omp*B, and *gro*ESL gene, fragment of the 23S rRNA gene, 16S rRNA signature region 16S-23S intergenic spacer) could help in the characterization to the species level [Bodetti et al., 2002; Jacobson and Samuelson, 2007; Homer et al., 1994; Hotzel et al., 2005; Soldati et al., 2004; Jacobson, 2007]. Because of the detection of chlamydial agents in so many different reptiles, their role as potential

zoonotic carrier, as well as their role as natural reservoir for this order of microorganisms, should be reconsidered [Hotzel et al., 2005].

Given the scarce literature on both herpesviruses in Mediterranean loggerhead sea turtles, with the exception of few anecdotal findings [Fichi et al., 2016; Alfaro-Núñez et al., 2016], and chlamydial infections, and considering the potential importance of these agents in clinically healthy animals, this study was aimed at screening healthy Mediterranean loggerhead sea turtles (*Caretta caretta*) for Herpesvirus and Chlamydiaceae, through molecular diagnostic techniques.

4.2 Materials & Methods

4.2.1 Sampling

Samples were collected from a total of 20 loggerhead sea turtles, coming from the southern Italy, and declared healthy by the responsible veterinary, following rehabilitation at the Marine Turtle Research Centre (Stazione Zoologica Anton Dohrn of Naples, Italy). One oropharyngeal swab, one conjunctival swab and one nasal swab were collected from each animal, using sterile cotton-tipped swabs. Samples were put into sterile, dnase free, rnase free cryovials, and stored at -80°C until shipment in dry ice to the Centre for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine (University of Lisbon, Portugal). The samples were then diluted in 600 µl of Hank's Balanced Salt Solution, incubated in dry bath at 37°C for 10 min and then stored at -80°C, until further use.

Animal handling procedures were performed according to the authorization by the Ministry of Environment and Protection of Land and Sea (Protocol n.0042848/PNM 09/08/2013 and Protocol n.0024471/PNM 22/11/2016).

4.2.2 Total DNA Extraction

Samples were processed for total DNA extraction using a DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions, and eluted in a final volume of 60 µl. Total DNA quantification and purity was determined using a NanoDrop 2000C Spectrophotometer (Thermo Scientific). The extracted DNA was stored at -80°C until further use.

4.2.3 Herpesvirus Screening

A pilot study was carried out to establish the perfect run protocol and mix and concentrations to apply, utilizing DNA extracted from previous positive samples (Figure 4.1). A conventional nested Polymerase Chain Reaction (PCR) targeting a partial sequence of the DNA-dependent-DNA polymerase gene was performed using previously described method [VanDevanter et al., 1996] with minor modifications. A previous positive sample for ChHV5 was used as a positive control of the PCR reactions, and negative controls were also included. The first round of amplification was performed in a total volume of 25 µl, with 7.5 µl of template DNA, 12.5 µl of 5 Prime Master Mix (5 Prime), 1.25 µl of MilliQ water and 1.25 µl of each primer (i.e. KG1, ILK, DFA, provided by Stabvida, Portugal) at

10 µM. PCR conditions were: 5 min at 94°C, followed by 50 cycles of denaturation at 94°C for 30 sec, annealing at 46°C for 1 min, and strand extension at 72°C for 1 min. After cycling, the reaction mixtures were incubated at 72°C for 7 min and then were held at 4°C. The same PCR conditions were applied to the second round of amplifications, which were performed utilizing 2.5 µl of the first reaction in a total volume of 25 µl, with 12.5 µl of 5 Prime Master Mix (5 Prime), 7.25 µl of MilliQ water and 1.25 µl of each primer (i.e. TVG, IYG, provided by Stabvida, Portugal) at 10 µM. Amplification reactions were performed in a Doppio thermal cycler (VWR). The PCR products were analysed in a 1.5% agarose gel stained with 0.05µl/ml of GelRed (Biotium) in 1x Tris-Acetate-EDTA, and visualized by ChemiDoc™ XRS+ System (Biorad).

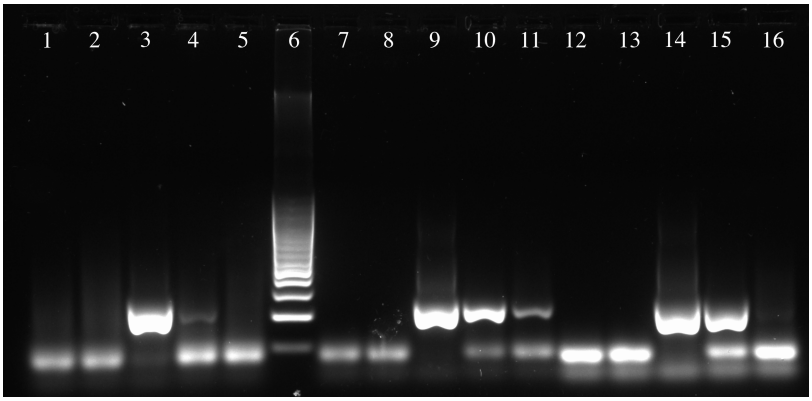

Fig. 4.1 Pilot study to establish the best protocol to apply.
Wells 1-5=Dream Taq Green Mix (Thermo Scientific), primers concentration=0.5 µM*; Wells 7-11=5 Prime Master Mix (5 Prime), primers concentration=0.5* µM*; Wells 12-16=5 Prime Master Mix (5 Prime), primers concentration=1* µM*. Wells 1-2, 7-8, and 12-13=Negative controls; Wells 3-5, 9-11, and 14-16=Positive controls (total nucleic acid concentration 82.9 ng/µl, 79.5 ng/µl, and 19.7 ng/µl, respectively); Well 6=NZYDNA Ladder V (NZYTech).*

4.2.4 Chlamydiaceae Screening

The detection of Chlamydiaceae DNA was performed by quantitative PCR (qPCR), targeting the 23S rRNA gene, according to a previously described method [Everett et al., 1999b] with minor modifications. A previous positive sample for *C. felis* was used as a positive control of the PCR reaction, and negative controls were also included. Amplification reactions were performed using 5 µl of template DNA in a total volume of 12.5 µl containing: 6.25 µl of SensiFAST^{TM} Probe Hi-ROX Kit (Bioline), 0.314 µl of MilliQ water, 0.312 µl of each primer (i.e. TQF, TQR, provided by Stabvida, Portugal) at 36 µM, and 0.312 µl of TaqMan^{R} probe (labelled FAM/TAMRA, Applied Biosystems) at 10 µM. Amplification conditions were: incubation at 94°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Amplification reactions were performed in a StepOnePlus thermal cycler (Applied Biosystems).

The amplified *C. felis* fragment was cloned into a plasmid vector with the Clone JET PCR Cloning Kit (Thermo Scientific) and serial tenfold dilutions of the recombinant plasmid DNA were amplified by qPCR in duplicate reactions and used to construct the standard curve.

Additional sequences from six positive samples (two oropharyingeal, two conjunctival and two nasal swabs, from two different turtles) were amplified by conventional PCR, targeting the 23S rRNA signature sequence of all Chlamydiales, using a previously described method [Everett et al., 1999b], with minor modifications. Amplification reactions were performed using 2.5 µl of the qPCR products in a total volume of 25 µl containing: 15 µl of 5 Prime Master Mix (5 Prime), 5.5 µl of MilliQ

water, 1 µl of each primer (i.e. U23S, 23SIGR, provided by Stabvida, Portugal) at 10 µM. Amplification conditions were: incubation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 51.5±7°C for 30 sec, and strand extension at 68°C for 30 sec. After cycling, the reaction mixtures were incubated at 68°C for 10 min and then were held at 4°C. Amplification reactions were performed in a Doppio thermal cycler (VWR). The obtained amplicons were analysed in a 1.5% agarose gel stained with 0.05µl/ml of GelRed (Biotium) in 1x Tris-Acetate-EDTA, and visualized by ChemiDoc™ XRS+ System (Biorad). Visible bands were purified using Qiaex II Gel Extraction Kit (Qiagen), quantified in a NanoDrop 2000C Spectrophotometer (Thermo Scientific), and cloned into plasmid vectors with the Clone JET PCR Cloning Kit (Thermo Scientific), according to the manufacturers' instructions. The amplicons were subsequently sent for sequencing by Sanger sequencing at Stabvida (Portugal) and the specificity of the nucleotide sequences was compared through Blast analysis at http://blast.ncbi.nlm.nih.gov/Blast.cgi with Chlamydiales sequences available in the GenBank.

4.2.5 Detection of Potential Zoonotic Chlamydiaceae

Three oropharyngeal swab samples were further analysed in order to detect Chlamydiaceae of potential zoonotic interest. A conventional nested PCR, targeting a partial sequence of the 16S rRNA of three Chlamydiaceae species (i.e. *C. trachomatis*; *C. psittaci*; *C. pneumoniae*), was performed as described by Messmer et al. [1997], with minor modifications. A previous positive sample for *C. trachomatis* was used as a positive control of the

PCR reactions, and negative controls were also included. The first round of amplification was performed in a total volume of 25 µl, with 5 µl of template DNA, 12.5 µl of Accustart II PCR Supermix (Quantabio), 1.25 µl of MilliQ water and 2.5 µl of each primer (provided by Stabvida, Portugal) at 20 µM. PCR conditions were: 2 min at 95°C, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 sec, and strand extension at 72°C for 1 min. After cycling, the reaction mixtures were incubated at 72°C for 5 min and then were held at 4°C. The same PCR conditions were applied to the second round of amplifications, which were performed utilizing 1 µl of the first reaction in a total volume of 25 µl, with 12.5 µl of Accustart II PCR Supermix (Quantabio), 6.5 µl of MilliQ water and 2.5 µl of each primer (provided by Stabvida, Portugal) at 20 µM. Amplification reactions were performed in a Doppio thermal cycler (VWR). The PCR products were analysed in a 1.5% agarose gel stained with 0.05µl/ml of GelRed (Biotium) in 1x Tris-Acetate-EDTA, and visualized by ChemiDoc™ XRS+ System (Biorad). Visible bands were purified using Qiaex II Gel Extraction Kit (Qiagen), and quantified in a NanoDrop 2000C Spectrophotometer (Thermo Scientific). The amplicons were subsequently sent for sequencing by Sanger sequencing at Stabvida (Portugal) and the specificity of the nucleotide sequences was compared through Blast analysis at http://blast.ncbi.nlm.nih.gov/Blast.cgi with Chlamydiales sequences available in the GenBank.

4.3 Results

4.3.1 Nucleic Acid Concentration

Extracted DNA samples yielded a mean nucleic acid concentration of 3.7 ng/µl (range from 0.9 to 16.1 ng/µl). In particular, the group composed by oropharyngeal swabs yielded a mean nucleic acid concentration of 5.4 ng/µl (range from 2.7 to 9.7 ng/µl; the group composed by conjunctival swabs yielded a mean nucleic acid concentration of 3.7 ng/µl (range from 1.3 to 16.1 ng/µl); the group of nasal swabs yielded a mean nucleic acid concentration of 2.2 ng/µl (range from 0.9 to 12.9 ng/µl).

4.3.2 Herpesvirus Screening

All 60 examined samples showed negative results to the screening for Herpesvirus (Figure 4.2 A-C).

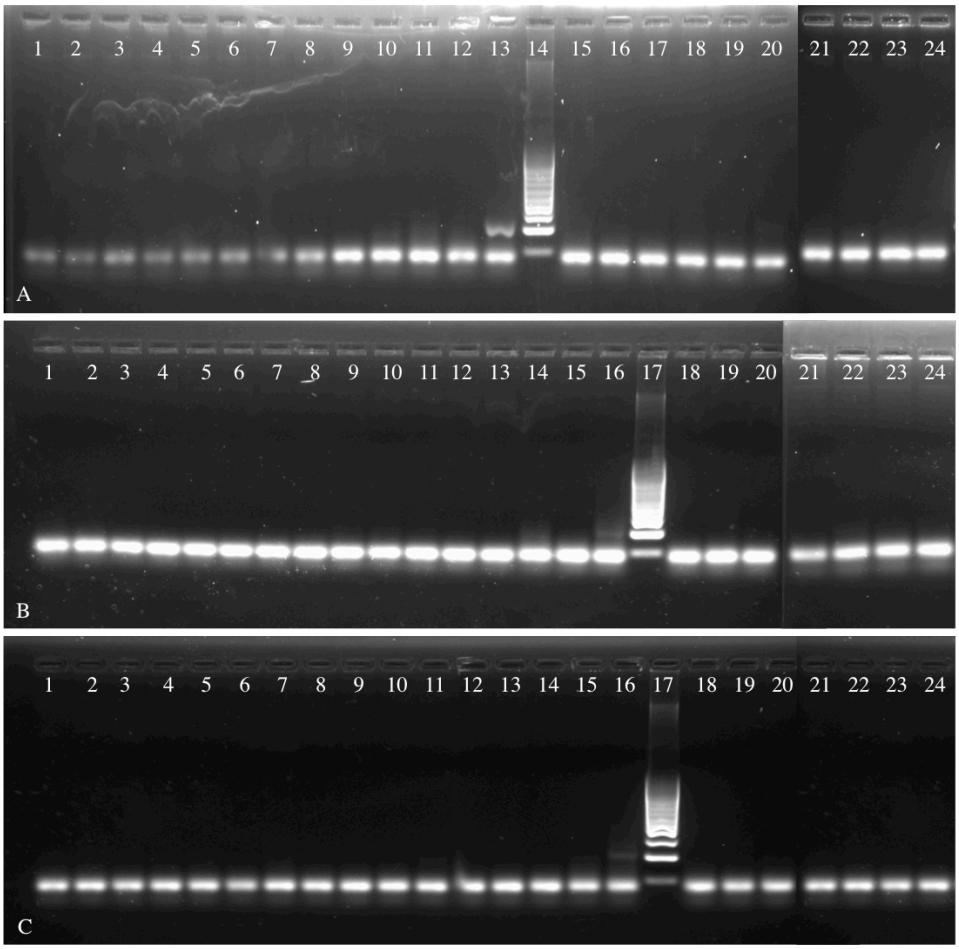

Fig. 4.2 Gel Electrophoresis of amplification products of the conventional nested PCR targeting the DNA-dependent-DNA polymerase gene.

A previous ChHV5 positive sample was used as a positive control. All samples showed negative results.

A: Oropharyngeal swabs. Wells 1-10 and 15-24=Samples; Wells 11-12=Negative controls; Well 13=Positive Control; Well 14=NZYDNA Ladder V (NZYTech).

B: Conjunctival swabs. Wells 1-13 and 18-24=Samples; Wells 14-15=Negative controls; Well 16=Positive control; Well 17=NZYDNA Ladder V (NZYTech).

C: Nasal swabs. Wells 1-13 and 18-24=Samples; Wells 14-15=Negative controls; Well 16=Positive control; Well 17=NZYDNA Ladder V (NZYTech).

4.3.3 Chlamydiaceae Screening

All 60 examined samples yielded positive results to the screening for Chlamydiaceae, with a mean threshold cycle (Ct) of 31.99±3.67 (ranging from 28,83 to 39,11). The standard curve showed high correlation (R^2=0.998) with a calculated efficiency of 105.283% (Figure 4.3).

Four out of the six selected samples resulted positive to further amplification for Chlamydiales (Figure 4.4), and the corresponding recombinant bacterial colonies, were sent for sequencing. Nevertheless, Blast analyses resulted in inconclusive identification.

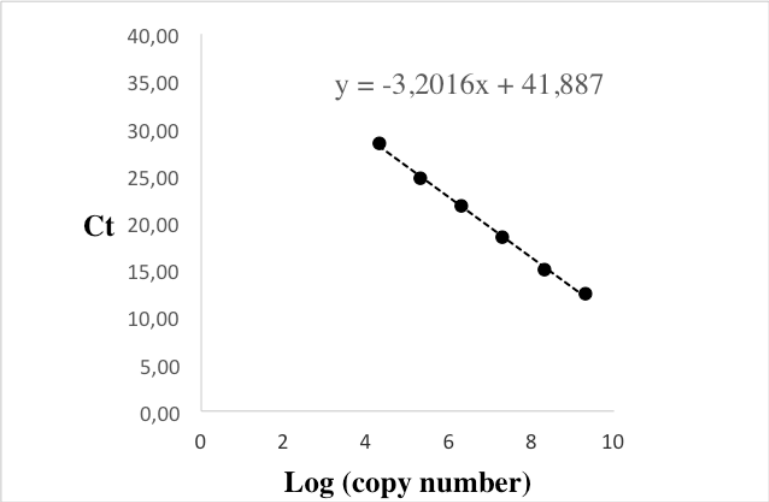

Fig. 4.3 Standard curve and equation for the determination of the efficiency of the qPCR for the molecular detection of Chlamydiaceae. Y-axis represents the mean Ct values obtained from the duplicates and X-axis represents the log_{10} of calculated copy numbers.

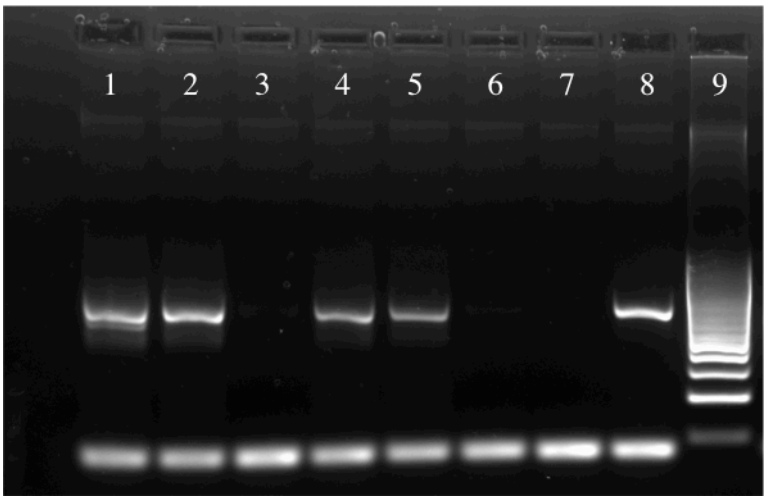

Fig. 4.4 Gel electrophoresis of amplification products of the conventional PCR targeting the 23S rRNA signature sequence of all Chlamydiales. A previous C. felis positive sample was used as a positive control. Wells 1-6=Samples; Well 7=Negative control; Well 8=Positive control; Well 9=NZYDNA Ladder V (NZYTech).

4.3.4 Detection of Potential Zoonotic Chlamydiaceae

None of the three oropharyngeal swab samples resulted positive to the amplification reaction for *C. trachomatis* (Figure 4.5 A). On the contrary, one oropharyngeal swab sample yielded an amplicons of the expected molecular weight both for *C. psittaci* and *C. pneumoniae*. Four purified amplicons, two for each positive reaction (Figure 4.5 B), were sent for sequencing, but Blast analyses resulted in inconclusive identification.

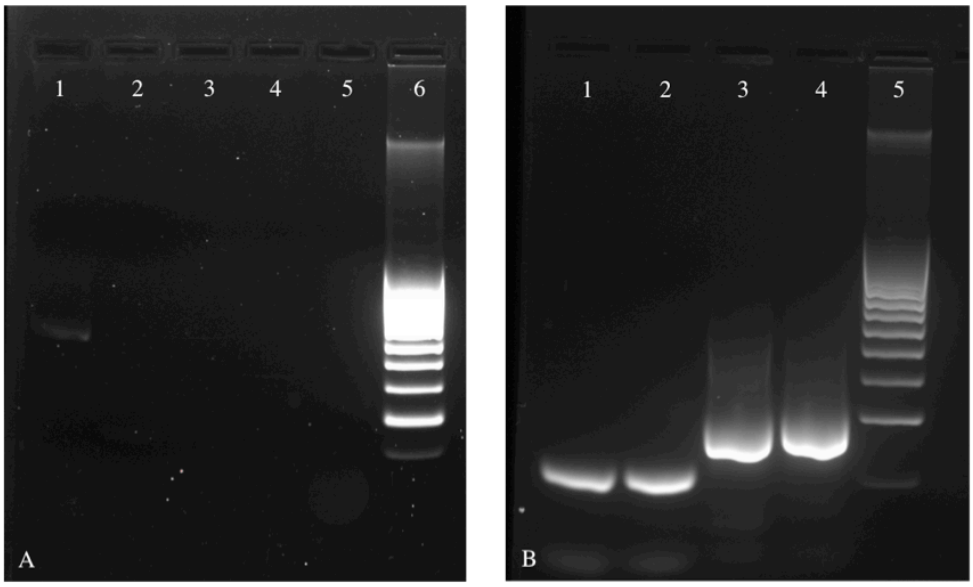

Fig. 4.5 Gel electrophoresis of amplification products of the conventional nested PCR targeting the partial sequence of the 16S rRNA signature sequence of three Chlamydiaceae species (i.e. C. trachomatis; C. psittaci; C. pneumoniae) with different molecular weight [412 base pairs (bp), 126 bp, and 221 bp, respectively].

A: Amplification products of C. trachomatis PCR. Well 1=Positive control (412 bp band); Wells 2-4=Samples; Well 5=Negative control; Well 6=NZYDNA Ladder V (NZYTech).

B: Purified amplicons of C. psittaci and C. pneumoniae PCR. Well 1-2=Sample in duplicate (126 bp band); Well 3-4=Sample in duplicate (221 bp band); Well 5=NZYDNA Ladder V (NZYTech).

4.4 Discussion

This study detected the presence of potential pathogenic microorganisms in oropharyngeal, conjunctival, and nasal swabs of apparently healthy sea turtles.

Concerning the screening for Herpesvirus, the absence of positive samples was remarkable, due to the almost ubiquitous nature of herpesviruses in

sea turtles, especially as regards ChHV5 [Alfaro-Núñez et al., 2016]. Nevertheless, in the literature there are no studies referring to the prevalence of fibropapillomatosis or ChHV5 in the Mediterranean Sea, but only few cases reporting the detection of the virus in clinically healthy turtles [Origgi et al., 2015; Alfaro-Núñez et al., 2016; Fichi et al., 2016]. Similarly, no historic records of fibropapillomatosis have been reported for the entire Arabian Gulf and Northern Indian Ocean, nor for the hawksbill population in Puerto Rico, suggesting a possible environmental difference or genetic resistance in these populations [Alfaro-Núñez et al., 2014]. Another possibility could be explained by the dissimilar distribution of herpesvirus in the body of their host, through the establishment of latent infections, and the consequent lower viral load in some tissues compared to others [Alfaro-Núñez et al., 2014; Quackenbush et al., 2001]. Indeed, Page-Karjian et al., [2015] detected greater amounts of ChHV5 DNA copy numbers in skin, blood and urine samples than in cloacal swab samples, whereas no ChHV5 DNA was detected in faecal samples and oral swabs. The last possibility would be to question the sensitivity of the technique, yet it has to be excluded as the method described by VanDevanter et al. [1996] have already been successfully used to detect Chelonid Herpesvirus in sea turtles [Lackovich et al., 1999; Quackenbush et al., 1998; Quackenbush et al., 2001; Aguirre and Lutz, 2004], as well as other herpesviruses in different animal species [VanDevanter et al., 1996; Marschang et al., 2006; Origgi et al., 2015; Anthony et al., 2013; Maness et al., 2011]. Further studies will be needed to assess the prevalence of herpesvirus among healthy loggerhead sea turtles in the Mediterranean Sea.

Concerning the screening for Chlamydiaceae, their presence in all samples from oropharyngeal cavity, nares and conjunctiva of loggerhead sea turtles opens up interesting scenarios. This is the first study to report the presence of Chlamydiaceae in sea turtles, save for the outbreak described by Homer et al. [1994]. Like for other wildlife species, the health impacts of these chlamydial infections remain unclear, especially on endangered species such as sea turtles [Burnard and Polkinghorne, 2016]. Despite the health status of the examined turtles should exclude any pathogenic role, Chlamydiaceae are well-known agents of a wide range of diseases in different animals species, humans included. Chlamydiales have been reported to cause enteritis, respiratory infection, polyarthritis, conjunctivitis, cardiovascular infections, urogenital tract disease and fertility problems [Bodetti et al., 2003; Bodetti et al., 2002; Blumer et al., 2011; Grayston et al., 1993; Longbottom and Coulter, 2003; Burnard and Polkinghorne, 2016]. Additionally, the progresses in molecular methods have led to the discovery of new uncultured Chlamydiales with pathogenic potential, indicating that knowledge of these infections is still limited [Bodetti et al., 2003; Taylor-Brown et al., 2015b]. Studies on chlamydial infections in wildlife are mainly represented by case reports and observational studies, but they have been detected in almost all wildlife vertebrates, including birds, mammals, fish, amphibians and reptiles [Kaleta and Taday, 2003; Salinas et al., 2009; Bossart et al., 2014; Stride et al., 2014; Blumer et al., 2007; Jacobson, 2007]. In wildlife, the most frequently detected species are members of Chlamydiaceae, likely because of the interest they attract as veterinary and human pathogens (i.e. *C. pecorum*; *C. abortus*; *C. psittaci*; *C. pneumoniae*) [Burnard and

Polkinghorne, 2016]. Indeed, the zoonotic potential of several chlamydial species is well established [Longbottom and Coulter, 2003; Dean et al., 2013; Burnard and Polkinghorne, 2016; Paré et al., 2006; Bodetti et al., 2002; Blumer et al., 2011]. In reptiles, the most frequently identified chlamydial species is *C. pneumoniae*, though other strains have been recently described [Bodetti et al., 2002; Taylor-Brown et al., 2015a]. Actually, the present investigation was not able to identify the detected Chlamydiaceae to the species level, but their extensive presence in loggerhead sea turtles suggests a role for these reptiles as important natural reservoir. Further investigations are currently being performed, in order to determine the exact taxonomic identity of these microorganisms and to better understand their pathogenic and zoonotic potential.

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Chapter 5

Enterobacteriaceae and gastrointestinal parasites in loggerhead sea turtles from Italian coasts

5.1 Introduction

Sea turtles may be affected by several infectious and parasitic diseases, be they bacterial, viral, fungal, endo- or ecto- parasitic. Nevertheless, the pathogenic nature of these organisms should be interpreted on a case-by-case basis, as many species may be present without causing significant pathology. Signs change with pathogen and condition, but generalized debilitation is the most common finding [Wyneken et al., 2006]. Among bacteria, Gram-negative strains are the most commonly isolated in sick reptiles as well as in healthy ones [George, 1997; Rosenthal and Mader, 2006]. Specifically, Proteobacteria was reported as the most abundant Phylum in cloacal samples of green turtles from the Gulf of Mexico [Price et al., 2017]. Similar results were obtained from Mediterranean loggerhead sea turtles, in whose feces samples a large presence of members of Gamma-Proteobacteria was described [Abdelrhman et al., 2016]. The family Enterobacteriaceae, in particular, is the most frequently cultured from cloacal samples (personal observation), as also outlined in a study on nesting green turtles from Costa Rica, in which this family accounted for more than 50% of the Gram-negative isolates [Santoro et al., 2006].

As regards parasites, helminths, protozoa, arthropods, and annelids are all included in the parasitic fauna of sea turtles. Some of them are very common, while others are rarely detected. Sea turtles usually act as definitive hosts, but sometimes they serve as intermediate (e.g. Cestoda) or paratenic hosts (e.g. *Anisakis* spp.) [Greiner, 2013; Santoro et al., 2010b]. Different biological and ecological factors (i.e. lifespan, feeding habits, site fidelity and migration patterns) influence the composition and richness

of endoparasitic communities. In particular, loggerhead sea turtles are mainly susceptible to digenetic trematodes and nematodes, mostly transmitted via invertebrates and fish as intermediate hosts [George, 1997; Gračan et al., 2012]; only a few protozoa have been reported [Greiner, 2013]. Sea turtle endoparasites may affect various organs, but the gastrointestinal helminths seem to be the most commonly recovered. Indeed, the majority of digenetic trematodes reside in the gastrointestinal tract [Wyneken et al., 2006; George, 1997; Piccolo and Manfredi, 2001]. In healthy hosts, endoparasites rarely cause problems. Nevertheless, stress associated with disparate causes (e.g. diseases, environmental imbalances, migration, nesting) could make sea turtles vulnerable to higher parasite intensities, and consequently susceptible to pathology [Wyneken et al., 2006; Santorto et al., 2007b]. Diagnosis of helminth infections is usually achieved through egg detection, both using faecal flotation and faecal sedimentation, or through collection of adult endoparasites. Whatever the technique might be, almost all of the parasitological surveys in the literature made use of samples obtained from carcasses, because of the endangered status of sea turtles [Greiner, 2013; Santoro et al., 2007a].

The gastrointestinal tract, in sea turtles as well as in other organisms, might be rightly considered as an ecosystem where bacteria, protozoa, fungi and endoparasites co-exist. Therefore, possible interactions among these different components should not be surprising [Berrilli et al., 2012; Lee et al., 2014]. The interplay between host, helminths and microbiota has attracted much attention owing to the potential for helminths to induce direct or indirect changes in the microbiota, and vice-versa [Kresinger et al., 2015]. Several studies have been conducted on distinct animal species,

including: bumblebees [Koch and Schmid-Hempel, 2011], fish [Bandilla et al., 2006; Pylkkö et al., 2006], tortoises [Dipineto et al., 2012], birds [Biswal et al., 2016], rodents [Houlden et al., 2015; Rausch et al., 2013; Walk et al., 2010], livestock [Rinaldi et al., 2007; Li et al., 2012; Dipineto et al., 2013; Wu et al., 2012], dogs and cats [Šlapeta et al., 2015], and even humans [Berrilli et al., 2012; Lee et al., 2014; Jones, 2016; Glenndining et al., 2014]. The outcomes of the parasite-bacteria interaction are contradictory: one common ground is an alteration of the gut microbiota, whether it be an increase or a decrease in bacterial diversity [Lee et al., 2014; Houlden et al., 2015; Glenndining et al., 2014; Holm et al., 2015] or in abundance of specific bacterial Phyla [Berrilli et al., 2012; Rausch et al., 2013; Walk et al., 2010; Reynolds et al., 2014]. Another common finding is the increased susceptibility of the parasite-infected host to pathogenic bacteria, or, vice-versa, the increased susceptibility to parasites in the presence of specific bacteria [Pylkkö et al., 2006; Li et al., 2012; Zaiss and Harris, 2016; Reynolds et al., 2015; Thakar et al., 2012; Galván-Moroyoqui et al., 2008]. Specifically, two different studies detected significanlty increased levels of Enterobacteriaceae in the intestinal compartments of parasite-infected mice compared to naïve mice, accompanied by a peak of pathology induced by the parasite infection [Rausch et al., 2013; Reynolds et al., 2014].

In order to better understand the ecological dynamics and the evolutionary basis of helminth-microbiota associations, it has been suggested to explore natural systems and naturally infected animals, where microbial and helminth communities are intact [Kresinger et al., 2015; Dipineto et al., 2013]. In sea turtles, no such study has been conducted. Nevertheless,

bacterial infections secondary to parasitism have often been reported in green turtles, and regarded as causes of illness and death [Raidal et al., 1998; Glazebrook and Campbell, 1990; Wolke et al., 1982]. As mentioned above, almost the totality of parasitological investigations conducted on sea turtles refers to samples collected from carcasses. This study was aimed at performing a parasitological survey on live loggerhead sea turtles, through a novel quali-quantitative technique which has already been succesfully utilized in several other animal species, concurrently addressing the possible correlation with Enterobacteriaceae bacterial family.

5.2 Materials & Methods

5.2.1 Sampling

A total of 30 loggerhead sea turtles, housed at the MTRC (Stazione Zoologica Anton Dohrn of Naples, Italy), was examined. All the recovered sea turtles were found in near shore environments along the coasts of Italy. Specifically, 20 turtles came from the middle-western coast (area W), corresponding to the Lazio and Campania regions, whereas ten turtles came from the southeastern coast (area E), corresponding to the Puglia region (Figure 5.1). In order to perform bacteriological and parasitological analyses, sea turtles were kept in individual tanks, and one cloacal swab sample and one faecal sample were collected for each animal. Cloacal swab samples were collected at the admission at the Centre, inoculated in PBS (Oxoid) and transported at 4°C to the Experimental Centre of Poultry

and Rabbits (Department of Veterinary Medicine and Animal Productions of the University of Naples Federico II). Seven cloacal swabs had to be excluded from this study, because of a suspect of contamination during the bacteriological analyses. Faecal samples consisted of the first faeces emitted by the turtles, which were collected in sterile containers and transported, at 4°C, to the Regional Centre for Monitoring Parasitosis (Department of Veterinary Medicine and Animal Productions of the University of Naples Federico II). All samples were preserved at 4°C, until further analyses, within 24h. Animal handling procedures were performed according to the authorization by the Ministry of Environment and Protection of Land and Sea (Protocol n.0042848/PNM 09/08/2013 and Protocol n.0024471/PNM 22/11/2016).

Fig. 5.1 Areas of recovery of 30 loggerhead sea turtles subject of study.
The blue line defines the middle-western coast (area W), corresponding to the Lazio and Campania regions (number of turtles=20); the red line defines the southeastern coast (area E), corresponding to the Puglia region (number of turtles=10).

5.2.2 Bacteriological analyses

Samples in PBS were analyzed in order to isolate and identify members of the Enterobacteriaceae family. Specifically, each sample was transferred into Buffered Peptone Water (Oxoid) and incubated at 37°C for 24h.

Subsequently, the samples were streaked onto n. 3 MacConkey Agar plates (Oxoid) and incubated at 37°C for 24h. All isolated strains were primarily identified on the basis of their colonial morphology, lactose metabolism, pigment production, and standard biochemical tests. The isolates were then confirmed using the API system 20 E (bioMérieux).

5.2.3 Parasitological analyses

Due to the paucity of faecal material and its dispersion in the individual tank, faecal samples were analyzed using the FLOTAC Pellet Technique [Cringoli et al., 2010]. This technique is performed for samples with an unknown weight of faecal material. In these circumstances, the weight of the faecal material to be analyzed can be inferred by weighing the sediment in the tube (pellet) after filtration (mesh size of 250 μm) and centrifugation of the faecal sample [Rinaldi et al., 2012]. Each sample was homogenized and filtered. Two 15 ml conical tubes were filled with the filtered suspension up to 6 ml and were centrifuged for 3 min. at 1,500 rpm. After centrifugation the supernatant was discarded and the two pellets (sediments) were weighed. Two different flotation solutions were used to resuspend the pellets: Sodium Chloride Solution (1200 s.g.) and Zinc Sulphate Solution (1350 s.g.). After homogenization, each of the two suspensions was poured into the two flotation chambers of the FLOTAC apparatus. The FLOTAC was closed and centrifuged for 5 min at 1,000 rpm; after centrifugation, the top parts of the flotation chambers were translated and each chamber was read under the microscope. Parasitic elements (eggs and oocysts) were counted, photographed and measured

using a light microscope at 10X and 40X magnifications (Leica DFC 490) and identified in accordance with Greiner [2013]. For each animal, eggs/oocysts per gram (EPG/OPG) of faeces were calculated using the following formula: EPG/OPG = (N x 1.2)/wp where N is the number of eggs/oocysts counted and wp is the weight of the pellet.

5.2.4 Statistical analyses

Parasitological results were analyzed in order to test differences between proportions of parasite species in the two areas (W and E) represented by the western and eastern coasts of Italy; Chi-square analyses were performed, except when numbers were too small, in which case the Fisher Exact test was used. Moreover, the possible association between parasites and Enterobacteriaceae was evaluated. Chi square analysis and Fisher Exact test were performed, as appropriate, to evaluate the relationship bewteen Enterobacteriaceae positivity and parasites positivity, whereas Spearman's r_s correlation was performed to evaluate the relationship between Enterobacteriaceae detection and parasitic burden (EPG/OPG). Statistical analyses were performed with Past3 and statistical significance was set at $p<0.05$.

5.3 Results

The bacteriological survey revealed that 78.3% (18/23) of the examined loggerhead sea turtles hosted members of the Enterobacteriaceae family. The species most frequently recovered and their prevalence is reported in

Table 5.1. The majority of turtles hosted either one species (5/18, 27.8%) or two (10/18, 55.6%); few animals hosted three (2/18, 11.1%) or more species (1/18, 5.6%).

Tab. 5.1 Prevalence of Enterobacteriaceae species isolated from 23 cloacal swabs of loggerhead sea turtles.

Bacterial species	**Number of positive animals**	**Prevalence (95% CI)**
Citrobacter spp.	14	60.9% (40.8-77.8%)
Enterobacter spp.	3	13.0% (4.5-32.1%)
Hafnia alvei	1	4.4% (0.8-21.0%)
Morganella morganii	10	43.5% (25.6-63.2%)
Proteus spp.	4	17.4% (7.0-37.1%)
Providencia rettgeri	2	8.7% (2.4-26.8%)

The parasitological survey revealed that 11 out of the 30 (36.7%) loggerhead sea turtles had parasites. Exclusively trematode eggs and protozoa oocysts were detected (Figure 5.2 A-F): their prevalence and mean parasitic burden (EPG/OPG) are reported in Table 5.2. In most of the turtles (9/11), just one parasite species was detected from each animal; only in two cases there was a co-infection of parasites (caused by two trematodes in one case, by a trematode and a protozoa in another).

Tab. 5.2 Prevalence and mean parasitic burden of parasites detected from 30 faecal samples of loggerhead sea turtles.

Parasite species	**Positive animals (N.)**	**Prevalence (95% CI)**	**Mean EPG/OPG (min-max)**
Angyodictium parallelum	2	6.7% (1.9-21.3%)	28 (8-48)
Cymatocarpus undulatus	1	3.3% (0.6-16.7%)	35 (35)
Eimeria carettae	3	10% (3.5-25.6%)	56 (48-70)
Enodiotrema megachondrus	3	10% (3.5-25.6%)	52.7 (14-80)
Pachypsolus irroratus	1	3.3% (0.6-16.7%)	180 (180)
Rhytidodes gelatinosus	3	10% (3.5-25.6%)	92 (24-140)

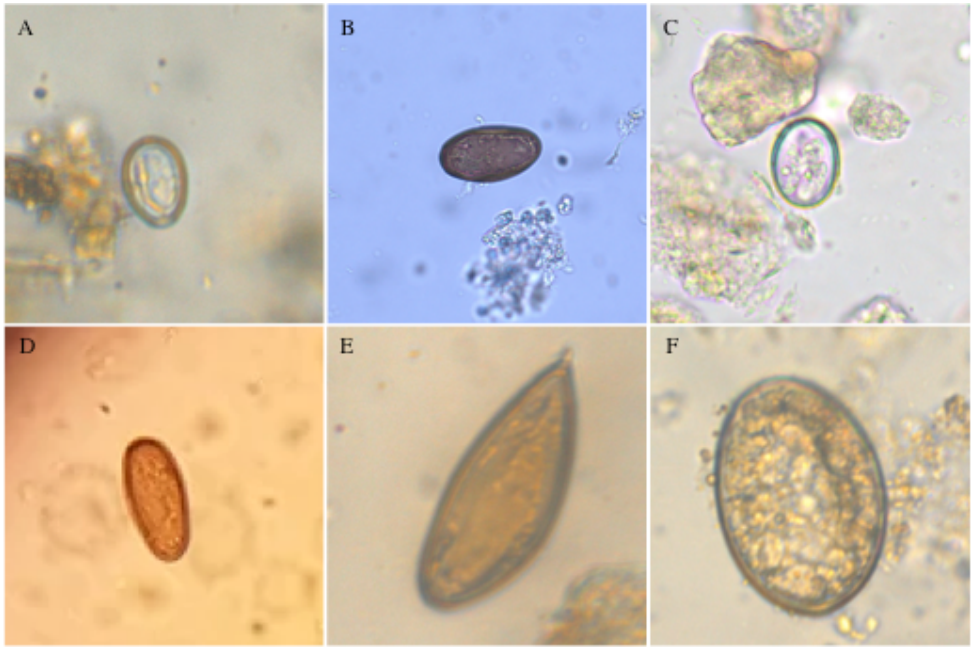

Fig. 5.2 Parasitic elements detected from 30 faecal samples of loggerhead sea turtles. A: Angyodictium parallelum egg, 25×15 µm; B: Cyatocarpus undulatus egg, 37×16 µm; C: Eimeria carettae oocysts, 25×22µm; D: Enodiotrema megachondrus egg, 42×21 µm; E: Pachypsolus irroratus egg, 51×18 µm; F: Rhytidodes gelatinosus egg, 75×53 µm.

Exclusively the prevalence of *Rhytidodes gelatinosus* was found significantly different (χ^2=6.6667; df=1 $p<0.05$) between the two areas, being higher in area E (10%) than in area W (0%). No other parasite species showed significantly different prevalence in the two areas.

Concerning the correlation between parasites and Enterobacteriaceae, from 30.4% (7/23) of turtles subject of study both parasites and Enterobacteriaceae were detected, whereas from 47.8% (11/23) of turtles exclusively Enterobacteriaceae were detected. From the remaining 21.8% (5/23) of turtles neither parasites nor Enterobacteriaceae were detected. No significant relationship was detected between parasites and Enterobacteriaceae.

5.4 Discussion

The present study showed a prevalence of 78.3% for Enterobacteriaceae and 36.7% for parasites in loggerhead sea turtles recovered along the coasts of Italy. With respect to Enterobacteriaceae isolation, previous studies conducted on loggerhead sea turtles in Italy show heterogenenous results, although few similarities in the distribution of species are present [Foti et al., 2009; Foti et al., 2008; personal observation]. The reasons for this difference could be researched in several factors, such as geographical distribution, feeding habits, and health status.

With respect to the parasitological analyses, the prevalence reported in this study (36.7%) is lower compared to other studies conducted on loggerheads sea turtles in the Mediterranean [Gračan et al., 2012; Piccolo and Manfredi, 2001]. In particular, the absence of nematodes was

unexpected, as they were detected with prevalence from 16.6% to 71.4% in loggerhead sea turtles from the Tyrrhenian and Adriatic Sea [Santoro et al., 2010a; Scaravelli et al., 2005; Manfredi et al., 1998]. On the contrary, nematodes were not detected in loggerhead sea turtles from the Balearic and Ionian Sea [Santoro et al., 2010a; Aznar et al., 1998]. This finding might be ascribed to the sensitivity of the technique, yet it is unlikely, as it has already been succesfully used to detect nematode eggs in other turtle species [Dipineto et al., 2012]. Another explanation could be researched in different feeding habits of the loggerhead sea turtles, as the turtles subject of this study were mostly subadults, whereas the study of Santoro et al. [2010a], conducted in the same area, refers to larger animals, probably consuming a wider variety and a greater amount of preys. There is only one report of cestodes in Mediterranean loggerhead sea turtles [Sey, 1977], suggesting their minor role as parasites of loggerhead sea turtles in this area. Anyway, the technique presented in this study is not suitable for the detection of cestodes, as loggerhead sea turtles serve as intermediate host for these parasites, and do not shed their eggs in the faeces.

A comparison of the parasitic burden is not possible, because of the different methods to assess it: this study used the number of eggs/oocysts per gram of faeces, whereas other studies used the number of worms in infected turtles. Neverthless, the parasitic burden showed heterogeneous results with both methods. This wide variation has been attributed to the influence of several factors, such as life cycle, availability of intermediate hosts, iteractions among different parasite species, host immune response and turtle population density [Greiner, 2013].

Concerning coccidia parasites, *Eimeria carettae* is the only species described in loggerhead sea turtles, but there has been no reference literature since its description [Greiner, 2013; Upton et al., 1990]. The present study could raise the interest in this parasite species and its potential pathogenic role in loggerhead sea turtles.

Regarding trematodes, all the species detected in this study usually reside in the stomach and upper intestine of different sea turtles species. Worth to mention is the detection of *Angiodyctium parallelum*, because there are no other reports in loggerhead sea turtles. Nonetheless, it has been described in hawksbill and green turtles. Probably, this finding is due to the ingestion of an intermediate host, which, as for most trematodes of sea turtles, has not been identified yet [Santoro et al., 2010a]. In loggerhead sea turtles, *Enodiotrema megachondrus* is reported as the most common and widely distributed trematode [Greiner, 2013]. In particular, its prevalence was found higher in the Balearic and Ionian Sea than in the Tyrrhenian and Adriatic Sea [Gračan et al., 2012; Santoro et al., 2010a]. The remaining species were more restricted in geographical distribution. Interestingly, *Rhytidodes gelatinosus* was detected with significant higher prevalence in loggerhead sea turtles from the eastern coast of Italy than in those from the western coast. This finding is consistent with other studies, reporting low prevalences of *R. gelatinosus* in turtles from the western Mediterranean and the Tyrrhenian coasts of Italy [Santoro et al., 2010a; Aznar et al., 1998], and high prevalences in turtles from the eastern Mediterranean and the Adriatic coasts of Italy [Gračan et al., 2012; Santoro et al., 2010a; Scaravelli et al., 2005; Manfredi et al., 1998; Sey, 1977]. The differences in the parasite communities of sea turtles are mainly due to ecological and

ontogenetic factors (e.g. trophic conditions, deep/shallow waters, pelagic/benthic diet, food intake rate) [Santoro et al., 2010a].

With respect to the correlation between parasites and Enterobacteriaceae, these negative results are in contrast with the literature, as a mutual influence between intestinal bacteria and parasites is often reported in animals [Rausch et al., 2013; Zaiss and Harris, 2016; Reynolds et al., 2015]. Nevertheless, two investigations conducted in humans found no significant alteration in the bacterial community composition during helminth infections, consistently with the results presented here [Cooper et al., 2013; Cantacessi et al., 2014]. Indeed, the studies conducted on laboratory animals analyzed the microbiota within the intestinal tissue, which is generally impractical with humans or live endangered species, like sea turtles. Moreover, the cloacal swabs may not appropriately represent the bacterial community of the stomach and upper intestine, where the parasites detected in this study usually reside, and where they can have a stronger influence on the local microbiota. The failure to detect a relationship between parasites and Enterobaceriaceae could also be explained by the low parasitic burden. It was suggested that highest infection intensities might have a more evident effect on bacterial communities. A limitation of this study is the relatively small sample size and its heterogeneity: a great number of confounding factors could have influenced one or both the agents here investigated. Despite that, this study presents a novel technique for the detection of parasites in sea turtles, able to highlight the same parasite distribution patterns mentioned in previous surveys on dead sea turtle [Gračan et al., 2012; Santoro et al., 2010a]. The FLOTAC technique is not suited to detect parasites that use sea turtles as

intermediate or paratenic hosts, but it is appropriate for the diagnosis of the majority of parasitosis of the intestal tract sea turtles. The harm that these parasites could cause to sea turtles is not completely understood: the intensity of damage could vary from case to case depending on, among other factors, the parasite species, the parasitic burden, and the host's immune system [Greiner, 2013]. The FLOTAC technique, being applicable on live animals, could relate the clinical manifestation of the parasitosis to a specific agent and its load, improving the knowledge on sea turtle parasitosis, and also allowing proper follow-up of animals in rehabilitation facilities, in line with the common aim to conserve these endangered species.

5.5 References

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Chapter 6

Conclusions

6.1 Conclusions

Sea turtle diseases have long been an understudied area, and many efforts have been recently done to address the issue. Nevertheless, they have not significantly increased the understanding of wild sea turtle health. Diseases have been mainly described from captive or dead sea turtles, and experimental studies are problematic, to say the least, because of the endangered status of sea turtle populations. On the other hand, surveillance programs mainly focused on establishing basic parameters of biology and ecology, overlooking the characterization of disease processes [Flint, 2013; Herbst and Jacobson, 2002; Alfaro et al., 2006].

This study consisted in a microbiological and parasitological survey on loggerhead sea turtles from the Mediterranean Sea, resulting in the following main outcomes: 1) it provided additional information on the bacterial and parasitic communities of wild sea turtles; 2) it highlighted the potential pathogenic role of these opportunistic agents, and the risk they could pose for sea turtle conservation; 3) it underlined the interdependence of these various pathogenic agents; 4) it strengthened the role of sea turtles as sentinel for their ecosystems.

This survey outlined a general microbiological and parasitological framework of live sea turtles, providing a hint of the normal communities commonly found in wild populations, and how the environment could influence them. This information represents valuable data to be used in the establishment of those baseline parameters that are considered fundamental to assess both the health of individual sea turtles and the potential impacts on sea turtle populations, in order to apply the appropriate conservation

efforts in time [Flint, 2013; Herbst and Jacobson, 2002]. The vast majority of the microorganisms detected in this study are opportunistic agents, becoming pathogenic when the animal health is already compromised for other reasons [Higgins, 2002; Flint, 2013; Wyneken et al., 2006]. Pathogens, even when affecting the individuals, might pose a risk to the whole population. In the context of sea turtle conservation, as already mentioned, every single animal is important for its reproductive value [Ullmann and Stachowitsch, 2015]. Additionally, pathogens could affect a sensitive life stage of a population, as in the case of embryonic development, reducing the chances of surviving to the adult stage from the beginning [Wyneken et al., 1988]. It would be important to better understand the role of infectious diseases as primary mortality factors in the population ecology of sea turtles, because, when combined with a population weakened by habitat loss, climate change and anthropogenic pollution, pathogens could result in severe outbreaks and ultimately lead to extinction of species [Herbst and Jacobson, 2002; Bogomolni et al., 2008]. The health status of an animal is dependent on a complex balance among the host, the pathogen, and the environment [Daszak et al., 2000; George, 1997]. It is now clear that many different pathogens (i.e. bacteria, fungi, viruses and parasites) could interact in determining health and disease in sea turtles, for example: spirorchiid trematodes have been listed along the co-factors in the etiology of fibropapillomatosis [Dailey and Morris, 1995]; marine leeches have been suggested as mechanical vectors of herpesvirus and bacterial infections [Stacy et al., 2008; Flint, 2013; Janda and Abbott, 2010]; secondary bacterial infections are promoted by spirorchiid egg migrations and herpesvirus skin lesions [Raidal et al., 1998; Wolke et al.,

1982; Stacy et al., 2010; Work et al., 2003; Aguirre et al., 1994]. Further studies will be necessary to disclose whether some infections act synergistically or they represent an overall increase in infectious disease, as a result of other, likely environmental, factors [Flint et al., 2009].

The role of sea turtles in the environment is becoming increasingly acknowledged. A thorough understanding of health and disease in sea turtle populations could provide a critical link between turtle health and ecosystem health [Jones et al., 2016]. In particular, loggerhead sea turtles have been proposed as good candidate species to monitor the Mediterranean on a sub-basin scale [Galgani et al., 2014]. In conclusion, this study strengthen the role of loggerhead sea turtles as sentinels of their ecosystem – providing data that could apply to other animals that share the same habitats, including humans, and raising health concerns as carriers of potential pathogens [Jones et al., 2016; Ives et al., 2017; Warwick et al., 2013] – in the wider context of "One Health", which recognizes the interconnection among human health, animal health, and the environment in which they coexist [Flint, 2013].

6.2 References

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