

Developing sampling protocols for biomonitoring contaminants in Mediterranean seabirds

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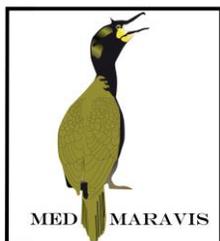
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Photo: F. Borghesi

PREFACE

Despite studies on seabirds and environmental contaminants in the Mediterranean Sea are relatively numerous, pollutants have mostly been studied considering birds as endpoints, whereas biomonitoring the marine environmental quality through seabirds is occasional. One of the main objectives of the EU Marine Strategy Framework Directive (MSFD, 2008/56/EC) is the development of standardized methods and the definition of indicators to be monitored in the long-term in order to measure whether a Good Environmental Status (GES) is reached (Law *et al.*, 2010; Galgani *et al.*, 2013). Therefore, the effects of contaminants on target species are intended in the MSFD as a tool to achieve repetitive indications of the environmental (marine) pollution levels, rather than a way to establish whether seabird populations are threatened. Dealing with the latter goal should entail investigating toxic effects on embryos or chick development, hatching success and chick behavior (Furness & Camphuysen, 1997), in association to studying population dynamics population.

The present document sees the light one year after the start of a period of close cooperation between Medmaravis and the Conservatoire du Littoral (CdL). In particular, the aim is presenting recommended and relatively simple sampling protocols to be applied in researches aimed at monitoring contaminants in the Mediterranean, using seabirds as bioindicators (chapter 4). The standardization of sampling methods is a crucial starting point in order to maximize the opportunity of comparing results obtained by different studies. This goal required a thorough study of the scientific literature regarding marine pollution and its relations with seabirds. This first step has led to results presented at the 2nd Symposium on the Conservation of Marine and Coastal Birds in the Mediterranean, held by UNEP RAC/SPA and Medmaravis in Hammamet (Tunisia) in February 2015 (Borghesi, 2016). About 300 papers and technical/scientific reports were collected, analyzed and entered in a database.

After this prospection phase, several sampling protocols have been tested in the Central Mediterranean during the breeding season of 2015 (9 sites, 3 seabird species). A subset of the samples collected in Italy and Tunisia have been analyzed by the Physical Sciences, Earth and Environment Department of the University of Siena, Italy (results and discussion in Annex A). Sampling procedures suggested in this work, therefore, have been directly inspired by literature and previous documented experiences and adapted when necessary to the characteristics of the Mediterranean (target species, breeding

phenology, selection of the breeding sites, etc.). They are expected to be suitable for monitoring programmes based on amount, concentration or molecular effects caused by some pollutant classes, such as heavy metals (HMs), legacy and emerging persistent organic pollutants (POPs and EPOPs), and plastics (PL).

While developing protocols, much attention was paid to feasibility by non-specialists, lack of adverse impact on birds and repeatability/consistence of sampling over years. Biomonitoring, being distinct from bioindication, does actually require long-term data sets, far beyond the 2-3 years normally covered by funds for most ecological researches. Simple and accessible sampling methods seem the only way to achieve a long-term data collection, especially when volunteers or non-professionals are used as a resource to decrease at least the costs of the field activity. Costs of sample preparation and analyses, however, remain relevant. Solutions to this limiting factor are beyond the purposes of the present work and seem only to be possible, on a relatively large geographical scale, when adequate resources are made available from the enforcement of international directives and conventions (e.g. MSFD).

Before giving the sampling protocols description, an introduction on the marine pollution and a brief review of the state of the art concerning research on contaminants in seabirds, especially in Mediterranean, is given in chapters 1 and 2, while most common methods used by researchers are summarized in chapter 3.

Samples that are collected by using the proposed sampling protocols can be used for immediate analyses - which is closer to the goal of the present report - or stored for future investigation. Vander Pol & Becker (2007) described the important role of specimen banking for temporal comparisons (especially of emerging contaminants), comparison of analytical techniques, and development of reference materials. Banking specimens organization is not trivial. Bank inventories and analytical data from banked specimens should to be readily available to interested researchers and functional to speed up research progress. The collection and processing conditions of banked samples should be consistent over time and eliminate any exogenous contamination. Relevant information and specimen bank inventories, if available, carefully chosen, collected and banked will allow researchers to determine past changes in the environment (Vander Pol & Becker, 2007).

1. THE MARINE POLLUTION

1.1 OVERVIEW

In common parlance, the word 'pollution' has a wide meaning. It is used to mean wastes, the occurrence of wastes in the environment, and the environmental damage caused by wastes.

This inaccurate definition is confusing, therefore the United Nations Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP) and the International Commission for the Exploration of the Sea (ICES) recommend to use 'inputs' to indicate 'wastes', 'contamination' for their occurrence, and 'pollution' to refer to the effects they have to the environment (Clark, 2001).

Inputs from human activities increase the concentration of substances (contaminants) in seawater, sediment, or organisms. Under a scientific point of view, pollution is a phenomenon that can be quantified.

Changes in concentration in the environment caused by the inputs from human activities and laboratory experiments showing toxicity of a contaminant to particular organisms may provide a warning signal. However, this does not prove necessarily that a harmful effect in the natural environment occurs (e.g. changes in the population parameters). When the damage is severe, a case of pollution is obvious. Conversely, when contamination levels are low, diffuse, or caused by unknown sources, demonstrating pollution may be difficult.

As a further complication, public perceptions of pollution changed over time, often based on inaccurate or wrong concepts of ecology. On the other hand, 'fashion' is an undeniable important factor, able to raise urgency of investigation (political pressure), drive funds for research (economic choices) and interest by scientists. Shortly after the industrial age started, concern on marine pollution started as well. Since then, issues such as hazards to human health, hindrance of marine activities, impairment of the seawater quality depending on its potential use, and reduction of amenities have been considering.

Nowadays, a wide branch of science is focused on the characterization of marine pollution, passing through the kind of materials discharged or resulting from human activities (when not deliberately

discharged), their effects on the marine environment and wildlife, implication for food resources, commercial activities, wildlife conservation, ecosystem structure and, consequently, human health of course.

Once characterized (sources, type, effects), pollution has to be removed or reduced. At present, researchers, institutions, NGOs, are suggesting what should be done, urging to do what can be done, disseminating what is being done. However, for most pollution, inputs still exist, and undesirable effects too, new ones having been discovered.

For these reasons, marine pollution is more and more an important issue for the EU legislation and this subject is the focus of the MSFD.

The MSFD asks to EU member States to take action to achieve or maintain a Good Environmental Status (GES) of their marine waters by 2020. According to MSFD, from mid-2012 to 2016 EU member States have to take six steps in order to develop a marine strategy for their waters (Galgani *et al.*, 2013):

- 1) assessment of the current environmental status
- 2) determination of the GES
- 3) establishment of a set of targets and associated indicators
- 4) establishment and implementation of a monitoring programme
- 5) development of a programme of measures designed to achieve or maintain the GES
- 6) start the implementation of the measures

The Annex I of the MSFD lists 11 qualitative descriptors to be used for GES evaluation. Of the 11 descriptors established by the Directive, Descriptor 8 and 10 are relevant for contaminants and plastics, respectively. Descriptor 8 has been defined as “Concentrations of contaminants are at levels not giving rise to pollution effects”. Descriptor 10, “Properties and quantities of marine litter do not cause harm to the coastal and marine environment” (see Law *et al.*, 2010; Galgani *et al.*, 2013).

MSFD gives a high importance to the scientific characterization of the pollution, a priority definition and precise deadlines. Despite this clear objectives and priorities, two problems should be considered. As said, the main threat to the sea, as it has been perceived, has changed during time. Initially, oil pollution

and then heavy metals were perceived as the major threats. Later, radioactive discharges, eutrophication, and certain chemicals became the most feared threats by people. In chronological order, the last fashionable subject is the marine litter. Finally, but not less important, project funds that are normally short-term can hardly allow studies to be supported over time. Hence, while new discoveries (ore the emergent cases) are usually placed under the spotlight, monitoring of the real impact of a pollution phenomenon or measuring changes over time of the environmental levels, are rarely conducted for more than a few years.

1.2 SOURCES, EFFECTS AND TYPES OF MARINE POLLUTION

Sources. Pollution reaches the sea through obvious and less obvious pathways, involving many possible sources. Pollutants deposit on sea surface through atmospheric inputs, falling with rain or consisting in particulate fallout. Organic wastes, pesticides and fertilizers, petroleum and oils, and any land-based materials can reach the sea through pipes discharging directly into the sea, or rivers transporting pollutants collected over the catchment area and flowing to the sea through their estuaries.

However, ships carrying toxic substances can pollute as an effect of accidents, or discharging (often illegally) substances such as oils, liquefied natural gases, pesticides, industrial chemicals and plastics. Offshore inputs exist in designated dumping zones. This may include dredging spoil, sewage sludge, fly ash from power stations, radioactive wastes.

Effects. The first target of toxicants or carcinogens are individuals. The biological response may vary according to the individual sensitivity. In extreme cases, the effect of intoxication is death. A typical contaminant with toxic properties gives a dose-dependent effect. The more toxic is the substance, the smaller is the lethal dose for an individual. Under a lower threshold of concentration, a substance may appear innocuous. Carcinogens are considered to behave stochastically. There is strong evidence that cancer usually arises from a single transformed cell. However, certain effects caused by radioactivity raise from the killing of many cells in the affected organs as a non-stochastic effect (Upton, 1987).

However, in most cases the exposure does not reach lethal doses. More often, organisms receive sub-lethal doses, which are more difficult to be measured because they are less evident or definitely subtle.

Sub-lethal effects may include major physiological stress, tumors, development abnormalities, that may result in early death, provoked by a wide range of causes, including trauma.

Metabolic processes are in part capable of avoiding toxic effects, by excreting, isolating or modifying substances in less harmful compounds. Such processes take place at cellular scale, and can be specific to the toxin. Often, the contact with contaminants implies the extra production of detoxifying agents, or causes the alteration of the shape of certain molecules. The concentrations of such biological elements, when measurable, can give an indication of the exposure to toxins and are named molecular biomarkers.

From an ecological point of view, what happens to an organism is not the main focus, but the fate of the survivors is more important, because a slow prolonged negative effect on a population is of more serious concern than a rapid short/punctual mortality of some (even many) individuals.

Pollution impact may be measured at the population or community levels. Although many species can be affected, attention should be focused on a few key species of high conservation interest. However, it may be convenient to address the effort also to species of commercial interest, species whose presence or absence can alter the community, or species that are suitable to be used as indicators, being particularly sensitive to pollutants or convenient to be monitored.

Types. This document will focus on some pollutants diffused at sea, such as heavy metals (especially mercury), hydrocarbons (halogenated and polycyclic aromatics), some emergent contaminants (e.g. flame retardants), and plastics. On the other hand, some pollution effects (albeit important) will not be considered, such as radioactivity, eutrophication, heating, acidification or alkalization. The main reason of this choice is that the present work is focused on seabirds, which are less studied as bioindicators of these types of effects.

This should not lead to forget what has been previously stated. All cited pollutants, beyond fashions, are still present in the environment, where they still introduced, in a larger or smaller amount. To some extent, all of them have been acting on the marine ecosystem at the same time. Focusing only on some of them is functional to the aim of our work: suggesting some standardized sampling procedures on Mediterranean seabirds, in order to achieve the capacity to better explore their suitability as bioindicators and share results at a regional (or even broader) scale.

1.2.1 Heavy metals and metalloids

The attention on metal pollution at sea is much smaller than in coastal environments (lagoons and brackish wetlands, estuaries, rivers, soils). Metals, as marine pollutants, are almost never on fashion, except mercury. Biogenic sources, such as volcanic activity and weathering of ore minerals, can account for 30–50% of the global baseline emissions of trace metals. The human activity (e.g industry, mining, agriculture) causes their excess in the environment which can make them toxic and harmful for wildlife. Among metals, the natural fluxes of those more toxic are small compared with emissions from industrial activities, implying that mankind has become the key agent in the global atmospheric cycle of trace metals and metalloids (Nriagu, 1989). Metals are conservative pollutants. What does it mean? Bacterial attack substantially does not affect the fate of so-called conservative contaminants in a timescale referable to the target endpoint. So, metals can be considered as permanently added to the marine environment. However, plants and animals have evolved in close contact with metals. On one hand, organisms developed biochemical processes depending on some essential elements for metabolic processes, while, on the other hand, they developed protection mechanisms against the toxic possible effects. In general, most metals are either un-reactive and therefore excreted as they are, or regulated to be proficiently used by the organism itself. Some metals are more toxic than others, and even essential metals can become toxic above a very limited threshold and the quantity that exceeds the natural capacity to be excreted tend to be accumulated in tissues.

Bioaccumulation is a possible effect of a conservative contaminant (most metals, but also persistent organic compounds) (Bryan *et al.*, 1979). It is the process that makes an organism reach, through time, the lethal dose of a substance without ever introducing a single lethal dose of it. In other words, bioaccumulation is a process provoking a higher concentration of a xenobiotic compound in an organism, in respect of the concentration in the environment (water, sediment, soil). When an organism is consumed by its predator, the latter can accumulate in turn part of the metal burden of the prey. When the prey population is diffusely affected by metal contamination (even at low levels), predators are unable to excrete the metal at the same rate of intake, and acquire an even greater body burden. Biomagnification is this special process of bioaccumulation along the trophic web, simply an effect of feeding on bioaccumulators. As a consequence, the trace element concentration in the tissues of an organism may be higher than the concentration in its food. This is often the case with mercury.

The biomagnification of methylmercury (the molecular form of mercury metabolized by animals) has been demonstrated (Lindqvist *et al.*, 1991; Watras and Bloom, 1992). Also cadmium can be accumulated by organisms through biomagnification (Reinfelder and Fisher, 1991), while lead, despite being extremely toxic and bioaccumulable, is not biomagnifiable (Hodson *et al.* 1978). Mollusks and fishes are perfect examples of mercury bioaccumulators. Seabirds, cetaceans, turtles, and humans are examples of top predators exposed to bioaccumulation and biomagnification.

Aluminum, arsenic, mercury, are widely dispersed by natural processes, at a larger extent than by anthropogenic inputs, while cadmium, lead, copper, and zinc enrichments in the environment are mostly caused by human activities. These are only general examples: each situation has to be assessed for metal contamination by defining a clear hypothesis to be scientifically verified. However, significant amounts of metals are released at sea by human activities. No biological function has been attributed to cadmium, lead and mercury and, therefore, they are of major concern because they are only highly toxic.

Cadmium is ubiquitous in the Earth crust but at very low concentration. In sea water it varies between 5 and 26 ng/l (OSPAR, 2000). Normally, it represents impurity in zinc materials. Cadmium is widely used in industry and the environmental presence has increased in the last decades. The sources of cadmium of anthropogenic origin are zinc-galvanization of plastic, paint industries, waste incinerators, contaminated phosphate fertilizers. Plastic industry, smelters, batteries may increase cadmium concentration in the environment. Recent studies assessed increasing bioaccumulation of cadmium in humans probably as an effect of atmospheric and water pollution, contamination of food, smoke of cigarettes (Ghidini *et al.* 2000). In the sea biota, cadmium is accumulated from the base of the food web (phytoplankton and plants) to top predators.

Lead is mainly transported by air and is particularly high in levels near urbanized areas and industrial belts. Natural concentration in sea water ranges between 30 and 100 ng/l from oceans to coasts (OSPAR, 1996). Rather rare as a product of natural rock weathering and volcanic activity, it can be found preferably associated to zinc, cadmium, silver and copper. Thousands of tons of tetraethyl-Pb have been dispersed worldwide as additive of gasoline before the ban in most countries (Green *et al.*, 2003), but despite of the harmful effects for biota, lead is still used for a number of industrial applications (batteries, paints, wires, jewelry, glass) and locally accumulated as metallic lead reserves by hunters,

shooters and anglers. The use of the leaded fuel has not been completely abandoned and avgas by aircrafts probably represents an important input of lead to the atmosphere (García-Tarrasón *et al.*, 2013, Miranda *et al.*, 2011).

Mercury in natural conditions is measured around 20 ng/l in sea the environment. It is highly volatile and its permanency is due to the chemical modification and association to sulphur, chloride, oxygen and hydroxyl ions. It can be bound in organic compounds as methyl-Hg by bacteria leaving in the sediment. This is the most biologically active form of mercury and the most likely to be bioaccumulated (Wright and Welbourn, 2002). Fishes of the Mediterranean have higher concentrations of methyl-Hg than Atlantic species (Cubadda *et al.* 1998). This is the effect of the high natural concentration of mercury in the Mediterranean basin: here 65% of the resources of mercury in the world is present (Cossa *et al.*, 1997). However, smelters, paper industries, and combustion of carbon are important polluting sources of this metal. As for copper, lead and arsenic, in the past mercury has been used as a main component of some pesticides, but this practice has now been abandoned.

1.2.2 Persistent organic pollutants (POPs)

POPs include families of organic compounds that are chemically stable. Their common feature is high toxicity and persistence in the environment. The most known (and dangerous) are polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), chlorinated pesticides (including DDT), some polycyclic aromatics hydrocarbons (PAHs), hexachlorobenzene (HCB).

However, the list of POPs evolves because discoveries continuously add 'new' chemicals among the candidates for such a group (see, for example, the two reviews on Dechlorane Plus, DP: Sverko *et al.*, 2011; Xian *et al.*, 2011).

Despite their organic molecular character, chemicals belonging to this group have to be considered as conservative pollutants, because they are very refractory to the oxidation and bacterial action. Halogens bounded to the molecule can be chlorine, fluorine, bromine, or iodine, but chlorinated compounds (OCs) are largely more abundant, so that halogenated hydrocarbons are often reductively identified as chlorinated hydrocarbons or organochlorines.

Like metals POPs can be bioaccumulated and 'permanently' added to the environment, but unlike metals their only source is the human activities, except for compounds (with molecular weight very low) produced by marine algae and part of PAHs.

The higher molecular weight of chlorinated hydrocarbons, and of some brominated emergent contaminants are probably the most worrying, because of their attitude to be bioaccumulated in fatty tissues. For this reason, marine animals are particularly subjected to be an endpoint of this class of pollutants, which includes a wide number of pesticides (the most known are DDT, Lindane, Exachlorobenzene, Toxaphene), polychlorinated biphenyls (PCBs) and flame retardants of new conception. Among pesticides, a number of compounds are grouped in the class of 'drins': aldrin, dieldrin, endrin, heptachlor, and many others. Drins are feared among pollutants as they are capable of generating degradation products that are equally toxic or even more. PCBs are not pesticides but industrial compounds which are used for various purposes, included dielectrics, hydraulic fluids, lubricants, paints, flame retardants. As many as 209 compounds belong to the PCB class (having 1-10 atoms of chlorine). Suspected carcinogenetic properties and hazard for biota are the reasons that led to the ban of their use in many countries. They also act as endocrine disruptors and may cause damages to the immune system. However, incinerators, dispersion of dredging muds for water depuration, combustion of exhausted oils are still polluting seas with such chemicals.

Dioxins and furans are very harmful halogenated hydrocarbons, which originate as unintentional byproducts of the combustion of organic material (domestic heating and incineration of wastes), but also come from the manufacture of herbicides and wood treatment. This group (the exact names are polychlorinated dibenzo-para-dioxins and polychlorinated di-benzo-furans), includes 210 chemical compounds with planar structure and 1-8 atoms of chlorine. They are very stable, odorless, insoluble in water, highly soluble in fatty liquids. In sediments they are rather immobile, but they tend to be adsorbed by mineral particles (and litter) and transported by currents and air worldwide (Fiedler, 1999). All developed countries have indiscriminately polluted the world with these carcinogenic compounds for decades and from decades they are making efforts to eliminate or reduce their dispersion. Food (especially fish) is the main source and, again, the endpoint in biota are fatty tissues of animals.

Brominated flame retardants (BFRs) have been added in May 2009 to the Annex A of the Stockholm Convention on persistent organic pollutants (POPs)¹. They are: a) Hexabromobiphenyl (HBB), b) Tetrabromodiphenyl ether, Pentabromodiphenyl ether, Hexabromodiphenyl ether, and heptabromodiphenyl ether. The polybrominated diphenyl ethers are usually abbreviated as PBDEs; the compounds cited here can be briefly referred as TetraBDE, pentaBDE, hexaBDE and heptaBDE, or, grouped as POP-PBDEs (Stockholm Convention, 2012). Like all POPs, these chemicals are toxic, persistent in the environment and can bioaccumulate in the organisms. They can be transported worldwide. When entered in the atmosphere and hydrosphere, they pollute soils, sediments and waters (Shaw *et al.*, 2010). An unimaginable amount of industrial and consumer products (especially electronics) contain flame retardants (Figure 1).

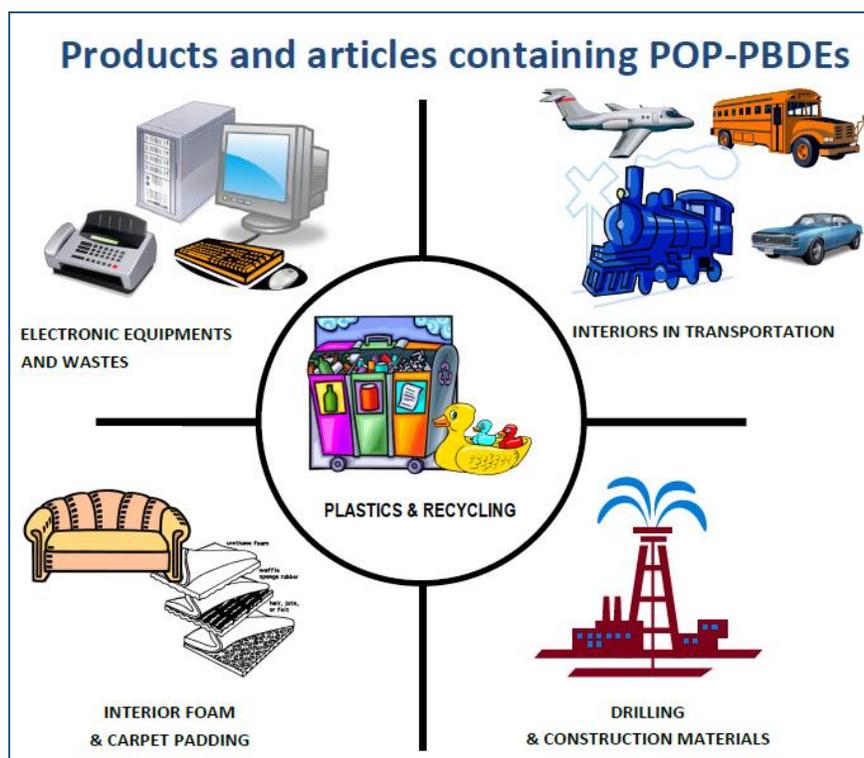


Figure 1 - Products and articles containing POP-PBDEs (from Stockholm Convention, 2012)

¹ The Stockholm Convention on POPs was adopted by UNEP on 22 May 2001 in Stockholm, Sweden, with the objective to protect human health and the environment from persistent organic pollutants. The Convention entered into force on 17 May 2004. For more information, see <http://chm.pops.int/Home/tabid/2121/>

PCDDs, PCDFs, PCBs, and BFRs are present in the environment as very complex mixtures. Changing the trophic levels, the relative concentrations change as well. For this reason, when the goal of a study is assessing the presence of such compounds in any matrix (soil, plants, animals) a great difficulty occurs. In a single sample contaminated by halogenated hydrocarbons, more than hundred different organochlorines can be found. It is, therefore, practically impossible to identify the responsible of an observed effect. As a possible solution, a single parameter, known as 'toxic equivalent factor' (TEF) has been introduced in order to achieve an overall risk assessment, based on TEQ, the 'toxic equivalent concentration' of molecules with a similar toxic mechanism (Pizzin and Bentley, 2006). In any case, POPs assessing poses a major problem to the analysts. Other than numerous, persistent, and toxic, these substances are sometimes present at very low concentrations. Despite this, they can contribute much to the risk level for biota. Rather than measuring concentrations, it can be convenient sometimes identifying biomarkers which indicate that an organism has been exposed to such chemicals, possibly measuring the magnitude of the damaging effect. Irrespective to the goal of any research, the selection of adequate (scientifically and economically) sampling and analytical protocols is necessary.

1.2.3 Emerging persistent organic pollutants (EPOPs) and pollutants of concern (EPOCs)

While some chemicals are being included in 'black lists', new alternative compounds are being produced and dispersed in the environment. This is the case, for example, of emerging brominated flame retardants: hexabromobenzene (HBB), pentabromoethyl benzene (PBEB), decabromodiphenyl ethane (DBDPE). Similarly, dechlorane 602 (Dec-602), dechlorane 603 (Dec-603), dechlorane 604 (Dec-604) and dechlorane plus (DP) were developed, after the 'feared' Mirex had been banned. There are already evidences that also these compounds tend to accumulate in the biota (Covaci *et al.*, 2011; Guerra *et al.*, 2012, Barón *et al.*, 2014).

Perfluorinated Compounds (PFCs) are produced since a long time and are known to have a big global warming potential (U.S. Department of State, 2007). Moreover, their increasing distribution in the environment may affect biota, and accumulation in organisms has been already demonstrated, although this aspect has not been much explored (Giesy and Kannan, 2001). Some PFCs, such as perfluorinated sulfonates and perfluoro carboxylic acids are widely used for consumer products and industrial processes. Temporal trends of PFCs concentrations in the seabirds of Norway showed a

significant increase from 1983 to 1993, followed by a leveling off in 2003 (Verreault *et al.*, 2007), but emissions in the atmosphere are still increasing (USEPA, 2015).

A new category is under study in these years. Emerging pollutants of concern (EPOCs) are mostly still not regulated by the normatives, but they are likely harmful to organisms and human health. This group includes: pharmaceutical active compounds (PhACs), drugs, industrial products for domestic cleaning such as detergents and for body care such as deodorants, lotions, cosmetics, toothpastes, suntan creams, body lotions, and nutraceuticals (food enriched of nutritive substances), altogether said pharmaceuticals and personal care products (PPCPs). In addition, hormones and new products have to be added to the Endocrine Disruptors category (EDs). Such micro-pollutants are released at low concentrations, but continuously, in the environment by municipal wastewater treatment plants.

1.2.4 Petroleum hydrocarbons

Oil pollution at sea is caused by two different types of event: oil spills and chronic oil pollution. The term "chronic oil pollution" is used to describe a persistent release of oil at low concentrations as a consequence of inefficient extraction, transportation, and consumption of oil. Differently, the presence of large amount or layers of crude or refined oil on water, as an effect of accidental and significant release of oil from a tanker, offshore drilling rig, or underwater pipeline, is said "oil spill". The explosion in 1991 of the "Haven" tanker which was anchored 7 miles off of Genoa (Italy) released more than 145,000 tonnes of petroleum leaking its remaining oil into the Mediterranean for years (Martinelli *et al.*, 1995). This event, probably the most important in the Mediterranean history, has caused effects on seabirds and impacted Italian and France coast. Among the 20 largest oil spills in the world history, there are also the "Independenta" and the "Irene Serenade" accidents in 1979 and 1980, near Navarino, Greece (100,000 tonnes), and in the Bosphorus (94,000 tonnes), respectively.

Chronic oiling affects marine wildlife through lethal and sub-lethal effects, including growth or reproductive rates, altered physiological functions, and molecular-level changes (hormone and DNA disruption) (Camphuysen *et al.*, 2007). Through the disruption of reproduction cycles and changes in other population dynamics, chronic oil pollution affects the stability of the marine ecosystem as a whole. Spilled oil has a considerable local or regional impact and can harm living things because its chemical constituents are poisonous. It can affect organisms by internal (ingestion) and external

exposure through skin and eye irritation. Oil can smother some small species of fish or invertebrates and coat feathers and fur. Seabirds are particularly vulnerable to oil because this substance damages the insulating properties of their plumage, which they require to survive in a marine environment. Even small amounts of oil in the plumage cause a bird to give up feeding and most casualties are due to starvation (Camphuysen et al., 2007).

Oil spills are often associated to beaching of dead marine animals, especially fishes and seabirds (Clark, 2001). In addition, oil pollution affects bathing beaches and therefore is often a televised subject. Also for these reason, among pollution events, oil spills raise the public attention. Despite recent declines in the amounts of oil released or spilled into the marine environment (Figure 2), chronic oil pollution is still a reason for concern (Camphuysen 2007), and within the European Union, major accidental oil spills (>20 000 tonnes) still occur at irregular intervals. Indeed, oil spills are only the most spectacular releasing events of hydrocarbons at sea. Many other routes have to be considered for this kind of pollution.

The estimated world input to the sea of petroleum hydrocarbons related to the human activity is 6,190 million t/y. Most inputs have been substantially reduced through the last decades, but evaporation from oil cargoes, previously ignored, appears to make a major contribution which returns to sea by rain out (Clark, 2001). Due to their physical properties, oil spilled on the sea forms a film on the sea surface. Thickness and spreading depend on the water temperature and oil characteristics. Even starting from a well-mixed fluid, the fate of each compound is different. Low molecular weight constituents evaporate into the air, while heavier compounds enter in the water column, dissolving or emulsifying in small droplets and are readily degraded by bacterial action. Tar balls, formed by the heaviest portions of the oil, resist longer and can travel through oceans.

Natural sources are more significant than anthropogenic inputs. A huge quantity of oil deposits is produced by plant remains that have become fossilized under marine conditions and living plants also produce hydrocarbons. The annual production of hydrocarbons by marine phytoplankton and by land plants is difficult to be estimated (Clark, 2001).

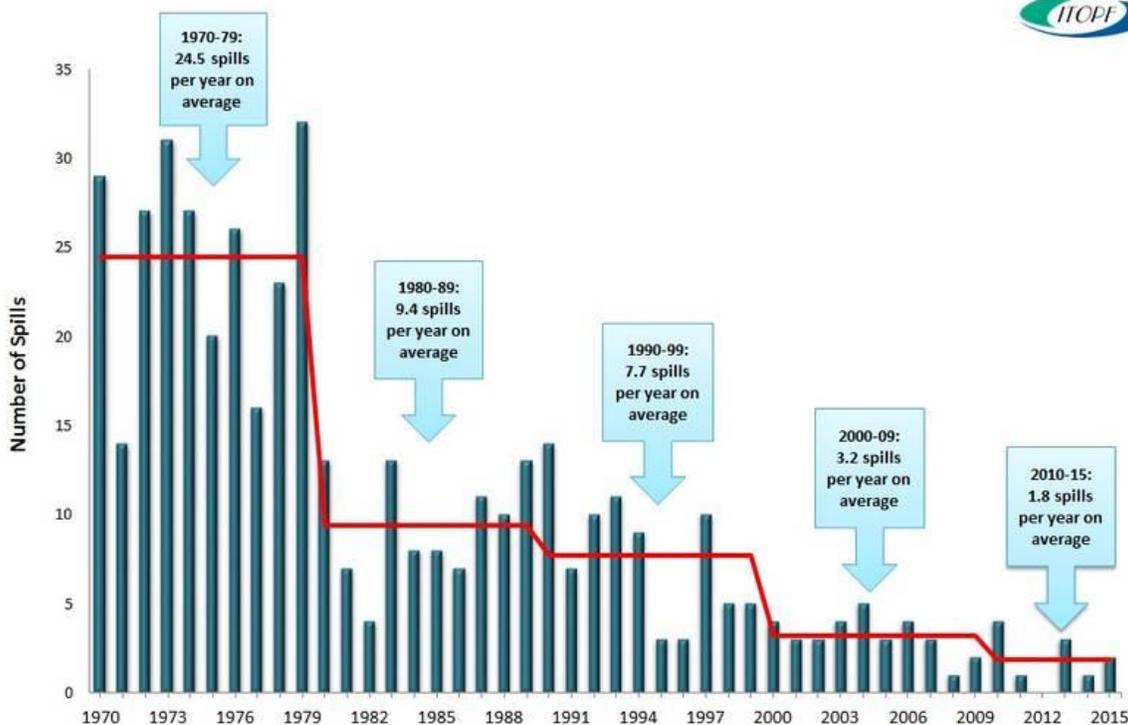


Figure 2 - Number of large spills (>700 tonnes) from 1970 to 2015 (www.itopf.com)

Oils are not conservative pollutants, as bacteria of a number of species can digest, at varying rates, all petroleum hydrocarbons. Also yeasts and fungi can also metabolize them. High molecular weight compounds degrade slower than lighter ones. Crude oil receives additives during its refinement, which may worsen the pollutant effects of the original mixture. In fact, crude oil is a complex mixture of hydrocarbons of many different molecular sizes. Cyclic chemical forms normally include aromatic rings having the capacity to bind each other in polycyclic aromatic hydrocarbons (PAH): 13 of them are known to be potent carcinogens (Bocca *et al.* 2003; EFSA, 2008) and are included in the POP family (see the previous paragraph).

From human side, PAHs are invariably produced by incomplete combustion of organic material. They may be released also by natural processes of geochemical transformation of molecules being part of petroleum and carbon. The former are named pyrogenetic PAHs, the latter petrogenetic PAHs. Relatively to PAHs, the anthropogenic source is orders of magnitude more important than natural ones (Baek *et al.* 1991; Mastral and Callèn, 2000). Accidental oil spills and port activities can add petrogenetic

PAH to the mixture (Fabbri and Vassura, 2003). In water, they are hardly susceptible to degradation and tend to be incorporated into sediments with long half-lives (MacRae and Hall, 1998). Especially heavier pyrogenetic PAHs are soluble in fat and accumulable (Menichini, 1994, Meador *et al.*, 1995). In the NW Mediterranean, the levels of PAHs were recently assessed near the Ligurian coast, and concentrations were similar to contaminated or slightly contaminated sediments in other parts of the Mediterranean (Cannarsa *et al.*, 2014; Bertolotto *et al.*, 2003). About toxicity, aromatic compounds are more harmful than aliphatics, and middle molecular weight constituents are more toxic than higher ones (Clark, 2001).

Toxicity of the water-soluble components additives from refinement can vary a lot but the dangerousness may regard most living organisms.

1.2.5 Plastic litter

All seas worldwide have been polluted by (practically) indestructible debris (taking 10 to 500 years to be degraded). Most of it, about 80%, is plastics (Gabrielides *et al.*, 1991; UNEP, 2005). Plastic pollution started many decades ago and production increased from 1.7 million tons in 1950 to 299 million tons in 2013, but only recently the marine pollution due to plastic litter has become fashionable (see Derraik, 2002 for a first comprehensive review of the plastic debris environmental problems). A recent study by Jambeck *et al.* (2015) estimated that 192 coastal countries generated 275 million metric tons of plastic waste in 2010 of which 4.8 to 12.7 million metric tons entered the oceans. As a consequence, plastic debris in the marine environment has been identified by the United Nations Environment Programme (UNEP) as a critical emerging global environmental issue (UNEP, 2011; 2014). However, many questions regarding the abundance, distribution and composition, main sources (changing over time), impacts (also changing) are far to be completely understood (Ryan *et al.*, 2009). In open ocean waters, denser plastic debris (e.g. polystyrene-styrene copolymers) gradually sinks to the denser, cold mid-water layers of the water column. This quota has been difficult to be estimated (Azzarello and Van Vleet, 1987), and likely it is still underestimated at present.

Plastics doesn't not only come from land. A lot of garbage comes from shipping and fishing at sea (about 20%), but this is potentially a more controllable source (Lentz, 1987). A large number of items is eventually stranded on the beaches or reaches the seabed getting in touch with the benthic

environment. Stranded litter has economic aftermath, acting as a deterrent to tourists (a big resource for Mediterranean countries).

The International Convention for the Prevention of Pollution from Ships (MARPOL) has come into force in 1978 to address the increasing problem of marine pollution. Annex 5 of MARPOL since 1988 bans the disposal of waste other than food to the sea by ships, but it is widely disregarded (Clark, 2001). At the Mediterranean scale, the Protocol for the protection of the Mediterranean Sea related to the Barcelona convention includes the elimination of persistent synthetic materials at sea. Primary and secondary packaging, plastic bags, cups and bottles, tampon applicators and polystyrene spherules are the most common types of man-made plastic litter. Fishing activity is responsible of lost net pieces, ropes, plastic straps, long lines. The distance of a beach from a population center seems to be a factor controlling the quantity of litter on a beach, but exceptions exist (Gabrielides *et al.*, 1991). Litter originated by ship-based sources increases moving off-shore, while total litter loads decreases, but in some circumstances aggregations may occur in gyres (Figure 3) (Pichel *et al.* 2007). Since 1999, Algalita is a non-profit organization focused on plastic pollution and its impacts on marine life and ecosystems. To get more information about this topic, it may be useful to visit the website www.algalita.org.

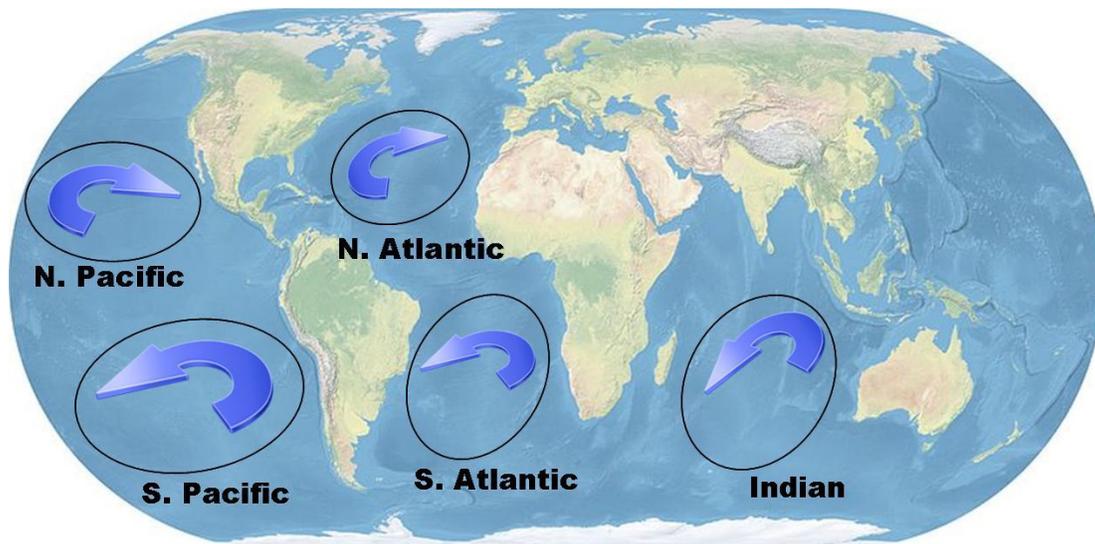


Figure 3 - The 5 subtropical gyres, gigantic whirlpools where waste concentrates as an effect of slow-swirling currents. Courtesy of Algalita (www.algalita.org).

Plastics are ingested by fishes, turtles, mammals and birds with surprisingly high frequency (Figure 4) and with various harmful effects (Figure 5). Another risk for animals is entanglement. Marine litter can cause alteration of benthic habitats (Katsanevakis *et al.*, 2007) and assist invasions of alien species (Barnes, 2002). In fact, plastics floating at sea may host a fauna of various encrusting organisms such as bacteria, diatoms, algae, barnacles, hydroids and tunicates (Winston *et al.*, 1982; Derraik, 2002). Gregory (2009) reviewed the environmental implications of plastic debris, just focusing on entanglement, ingestion, and hitch-hiking of alien species.

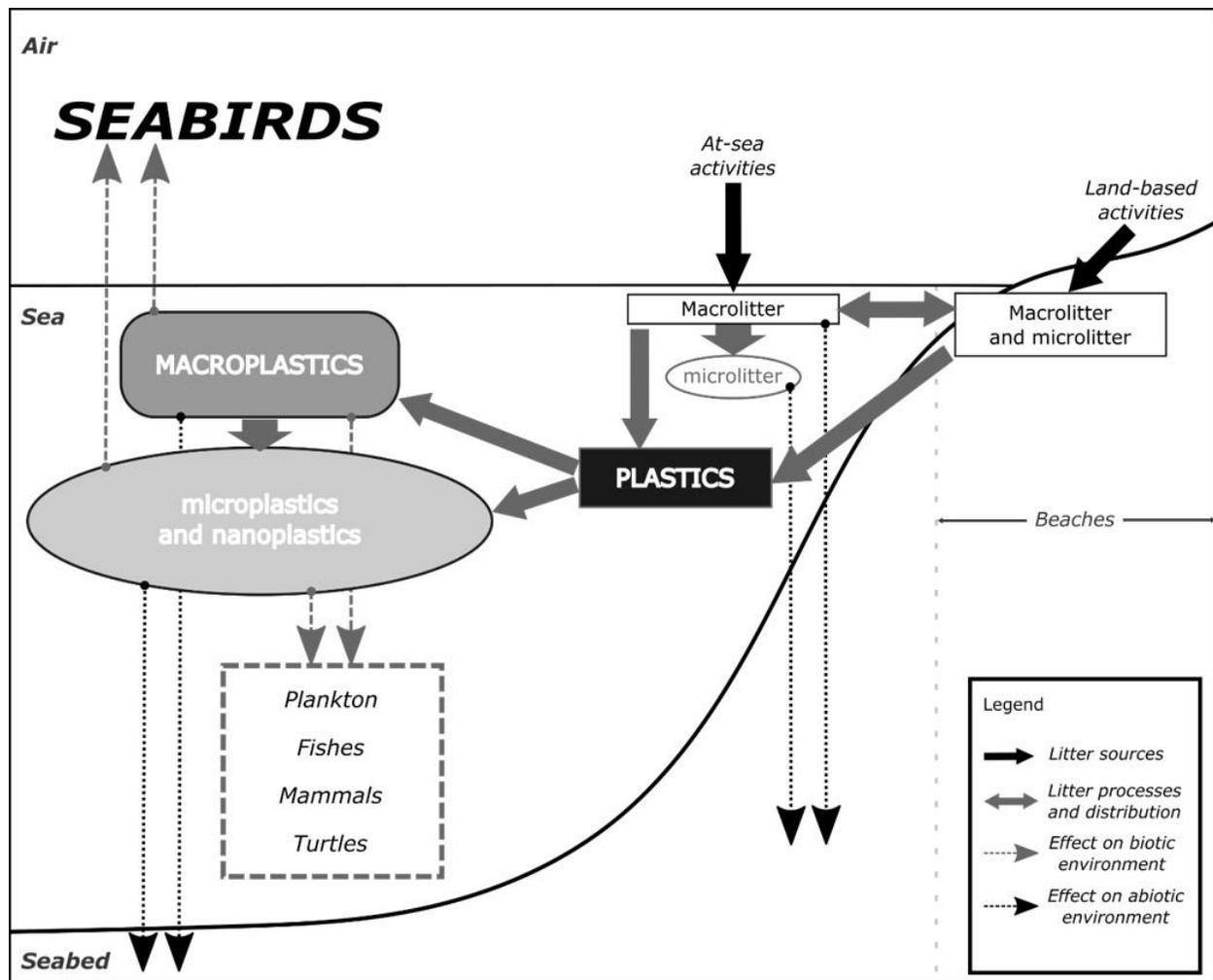


Figure 4 - A schematic cycle of litter at sea focused on plastics and seabirds. Re-designed by Beltrami R. and Borghesi F. from Galgani *et al.*, 2013

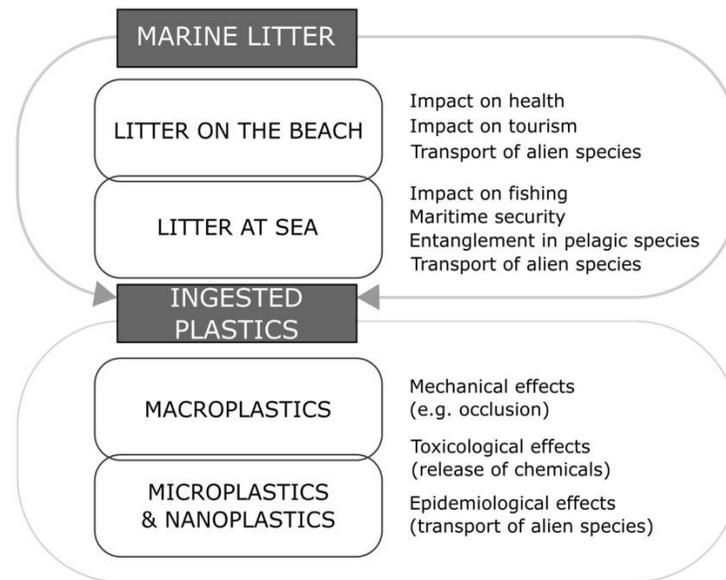


Figure 5 - Major impacts of marine litter in the environment and effects of ingested plastics in organisms. This is a part of the holistic assessment of the impacts of anthropogenic pressures on the components of the marine ecosystem which inspired the definition of MSFD indicators. Modified by Beltrami R and Borghesi F. from Galgani *et al.*, 2013.

Microplastics (<5 mm *sensu* UNEP, 2014) can be even worse for animal health due to the tendency of very tiny particles (included the so-called nanoparticles < 20 µm) to accumulate in the water column and sediment (Thompson *et al.*, 2004; Barnes *et al.*, 2009). Everywhere in the oceans, small pellets of 3-4 mm in diameter of polyethylene, polypropylene, and polystyrene can be found and accumulate on beaches (Figure 6). In addition, most macroplastic items (>20 mm) break down (very slowly) as an effect of photodegradation, oxidation and mechanical abrasion (Dixon and Dixon, 1981; Andrady, 2003), therefore also weathered fragments of macroplastic items must be included in the microplastics category (Figure 4) (Barnes *et al.*, 2009). Even the so-called 'biodegradable' plastics can improve pollution, because they contain starch which facilitates the material to break down, but also generates a proportion of plastics that gets sooner dispersed as micro-plastics (Klemchuk, 1990). Spatial and temporal heterogeneity of plastic debris raise difficulty in monitoring (Ryan *et al.* 2009). As a general indication coming from long-term bird monitoring projects in the North Sea, in bird stomachs a global decrease in the abundance of virgin pellets has occurred, replaced by more fragments of plastics from objects commonly used by people. Data collected from 1979 to 2012 suggest an increase in ingested plastics from the mid-1980s to peak values in the mid-1990s in both mass and number of items,

followed by a decrease in mass, but not in number. Eventually, the number and mass of plastics are apparently stable. (van Franeker et al, 2011, Galgani *et al.* 2013, van Franeker and Law, 2015). However, new insights in Svalbard colonies reveal a worse situation in this area (Trevail *et al.*, 2015).



Figure 6 - Microplastic accumulated on a Mediterranean beach (Photo: N.Baccetti)

Plastics are not just items with mechanical adverse effects (entanglements, intestinal occlusion when ingested, etc.). They contain organic contaminants, most of them toxic or disruptors of the endocrine system, including PCBs, PAHs and petroleum hydrocarbons, OCs, polybrominated diphenylethers (PDBEs), alkylphenols and bisphenol A, at concentrations from sub ng/g to mg/g. Alkylphenol exothylates (APEs), which have been widely used as detergents and as a component of plastics and rubbers, have similar effects of OCs, without containing halogens. Such chemicals can be transferred to the trophic web, being capable to penetrate into cells and interact with biologically important molecules (Teuten *et al.*, 2009). Photo-oxidation and photolytic reactions are the most important factors modifying plastic surface and, consequently, they alter the capacity of plastics to sorb/desorb contaminants.

According to Prof. Ad Ragas' presentation to the Massive Open Course on Marine Litter organized from October 2015 by UNEP, GPA, GPML, and The Open University of The Netherlands, the marine litter challenge can be summarized in 8 facts:

- 1) Land-based sources are the main cause of marine litter pollution
- 2) Plastics are the largely major component of marine litter
- 3) The annual industrial production of plastics is still increasing
- 4) Disposable plastic bags are still widely daily used by people
- 5) Plastics are long-lived at sea, up to half a century, becoming microplastics in their intermediate state
- 6) Plastic items can be transported by marine currents over distances, in some cases concentrating in gyres
- 7) Plastics are a threat to wildlife, human health, and economy
- 8) Microplastics are a potential carrier of toxic substances which can be transferred into the marine food web
- 9) Plastics at sea cause damage to fishing, shipping and tourism

In March 2011, the Fifth International Marine Debris Conference (5IMDC) brought the marine litter community together to develop and create a document known as the Honolulu Strategy, a framework for a comprehensive and global effort to reduce the ecological, human health, and economic impacts of marine litter (<http://www.unep.org/esm/Portals/50159/Honolulu%20Strategy%20Final.pdf>).

1.3. MARINE POLLUTION AT THE MEDITERRANEAN SEA SCALE

The Mediterranean Sea covers an area of 2,965,000 km². Tides are averagely low (except in the N Adriatic corner and in S Tunisia) and much of the basin is more than 200 m deep, with deep trenches deeper than 3000 m. The eastern and western Mediterranean are rather different in fauna and flora with the Sicily channel separating the two sub-regions (Figure 7). The most enclosed portions are the Aegean and the Adriatic seas (Clark, 2001).



Figure 7 - The Mediterranean Sea, Black Sea and their regions. By O H 237, via Wikimedia Commons.

The Atlantic water entering at Gibraltar compensates the strong evaporation, which is about three times greater than the input from precipitations. The Eastern Mediterranean has a higher salinity, while the western part is near to the Atlantic levels. The north-western coasts host the most industrialized areas and millions of tourists each year.

As elsewhere, Mediterranean big cities and industrial settlements are source of pollution at sea. However, more than other basins, the Mediterranean receives important pollution from tourism. This is far to be sustainable at large scale, urging a neat separation between conservation areas and tourism developments.

All parts of the Mediterranean are chronically polluted with tar balls (agglomerates composed by the heaviest components of petroleum), adversely affecting touristic localities. The main source of this kind of pollution are the shipping operations of deballasting and tank washing. Secondly, a contribute comes from refinery wastes. Since 1976, this problem has been reduced through international conventions and law enforcement.

Regarding conservative pollutants, in the past it was thought that in the Mediterranean anthropogenic inputs by rivers and direct discharges were a far more important source than atmospheric inputs. At present, atmospheric inputs of lead, zinc, chromium, nickel, and mercury, other than organochlorine hydrocarbons seem to be as large. However, the most important rivers (such as the Rhone and Po) are still considerable sources of pesticide and herbicide residues, heavy metals, and PCBs. Concerns are due

to the many industrialized areas along the European coasts, but emerging realities on the North African coasts are also improving some kind of pollution. As a consequence, there are several areas of accumulation of conservative pollutants that still represent a threat (Clark, 2001).

Concerning plastics, most marine litter in the Mediterranean seems to come from land-based activities rather than at-sea sources (Gabrielides *et al.*, 1991, PNUE/PAM/MEDPOL, 2009). Plastics reach the shorelines from effluents from land and, hence, from here disperse at sea. The nature and quantity of litter in the Mediterranean was determined through a IOC/FAO/UNEP study, undertaken within the framework of the MED POL activities at the end of 1980s in 13 beaches of five countries: Spain, Italy (Sicily), Turkey, Cyprus, and Israel (Gabrielides *et al.* 1991). Stefatos *et al.* (1999) focused on eastern Mediterranean. The composition of marine litter in the Mediterranean is quite different from that of the North Sea and includes a higher percentage of plastic (Galgani *et al.*, 2013). The abundance trend is also difficult to be defined, and it seems to be locally discordant, for example in the Gulf of Lion (France) and Greece (decreasing and increasing, respectively) (Barnes *et al.*, 2009). Microplastics are of particular concern due to their high prevalence (for northwest Mediterranean see Collignon *et al.*, 2012) and slow rate of degradation at sea (Arthur *et al.*, 2009).

The United Nations Environment Programme (UNEP) aims to protect Mediterranean from pollution effects. The Barcelona Convention is the agreement established to take this action, involving 22 Mediterranean countries, which differ in the degree of economic development, approach to the environmental protection and sometimes political stability.

A full review of the knowledge regarding pollution in the Mediterranean Sea is not the aim of this document, therefore this paragraph must be intended just as a hint. More attention is deserved to ecotoxicological studies on seabirds, with a special focus on Mediterranean seabirds. For more information regarding oil pollution and more generally pollution caused by shipping in the Mediterranean, see the Regional Marine Pollution Emergency Response Centre for the Mediterranean Sea (REMPEC) website (www.rempec.org).

Barcelona Convention and the Action Plan for the Protection of the Marine Environment and the Sustainable Development of the Coastal Areas of the Mediterranean (UNEP/MAP Phase II) can be consulted at www.unepmap.org.

2. CONTAMINANTS AND SEABIRDS

While the chemical industry is synthesizing, producing, and spreading new substances, many contaminants have been banned or restricted because of their harmful effects on the environment. Consequently, some types of effects determined by pollutants that are no longer discharged at sea are declining, while some others are still raising concern for their ecological impact. For example, new pesticide formulas are now producing even more subtle, sub-lethal effects, the significance of which for avian populations is far to be determined.

For seabirds, defined as birds that spend a significant proportion of their life in coastal or marine environments (Burger & Gochfeld, 2004), ingestion of food (including 'false' food, such as plastic items) and water are the main routes of exposure to pollutants. Seabirds are susceptible to bioaccumulate a wide range of chemicals, because they are top predators in the marine food webs. Such vulnerability, especially for lipophilic substances, makes seabirds potential indicators of change in the environment and can be used to monitor pollutants that bioaccumulate and/or amplified in concentration going up the trophic levels (Furness, 1993, Abdennadher et al. 2010, Abdennadher et al. 2011).

The colonial habits of seabirds have several practical benefits for monitoring contamination. They facilitate sampling and allow the collection of good quantities of data from a particular breeding site in a relatively short period of time (Mallory *et al.* 2006). In addition, since many species of seabirds return to the same nest and colony sites for years, contaminant loads of individuals can be studied over time (Burger, 1993). Furthermore, seabirds are in many cases sufficiently common for experimentation, are of interest to managers, conservationists, and public. Ultimately, seabirds, as bioindicators of certain contaminants, can provide an early warning to the human health (Burger & Gochfeld, 2004). In the Mediterranean area, most colonial seabird species are distributed across most coastlines and islands, thus allowing geographical comparisons between marine areas.

Normally, researchers focus on one or few chemicals in their studies. However, seabirds are endpoints for many elements and substances and there would be need of laboratory studies assessing mixtures of chemicals, but the complexity (and the costs) soon improve, limiting the possibility to explore interactions. Consequently, additive, antagonistic, or synergistic properties of different chemicals are

generally unknown (Burger & Gochfeld, 2004). For example, in literature concerning contaminants and seabirds, the effect of OCs or Hg are investigated relatively often, but very rarely in combination with HMs (Borghesi, 2016).

Moreover, stressors are not limited to contaminants, but multiple causes can interact with each other, provoking synergistic effects. Thompson and Hamer (2000) identified three broad categories of stress in seabirds: marine pollution, industrial fisheries (including effects of depletion of prey stocks and direct mortality), and climate change. They also pointed out that the impact of a particular source of stress on seabirds depends on the species in relation to foraging and breeding ecology. Changes in the industrial activity increased disturbance from shipping and fisheries activity, a warming climate and extreme meteorological events may modulate the relative importance of the various stressors, affecting ecological aspects such as type and movement of diseases and change in competition (including immigrated and/or alien species) (Mallory and Braune, 2012).

2.1 HEAVY METALS

Heavy metals in seabirds (in particular Cd, Pb, Hg) have been reported in many scientific articles, but detrimental effects only rarely could be proved. As metals occurs in the environment both from anthropogenic and natural sources, seabirds may accumulate high metal burdens reflecting background concentrations, or exposure to pollution, or a combination of both (see Thompson & Hamer, 2000 for a summary of metals in seabird tissues up to the late 1990s).

Once in the environment, inorganic mercury derived from ore weathering and volcanic activity may change in methylmercury (the organic form/state) which is assimilated by animals from consumed preys, whereas inorganic mercury is mostly excreted as it is. Methylmercury bioaccumulation propensity in the organisms is similar to those of other lipid-soluble organic compounds of environmental concern (Furness & Camphuysen, 1997). As a consequence, methylmercury is preferentially accumulated in tissues of seabird preys (Nisbet, 1994) acting as a neurotoxin and immunotoxin both in prey and its predator (Wolfe *et al.*, 1998). Critical observations about the high levels of Hg in Mediterranean fishes were presented at the FAO/WHO/IAEA/UNEP Meeting on the Biogeochemical Cycle of Mercury in the Mediterranean (Siena, Italy, 27–31 August 1984), and then published (Aston and Fowler, 1985). Pelagic seabirds show higher increases of mercury than most

coastal species, and increases have been greatest in seabirds feeding on mesopelagic preys (Furness and Camphuysen, 1997).

Because feathers allow non-destructive sampling and permit retrospective study, they are particularly convenient for monitoring of mercury pollution in marine food webs (Monteiro and Furness, 1995). Lewis and Furness (1991; 1993) showed that concentrations of mercury in feathers are related to the methylmercury levels in the blood at the time of feather growth. The researchers also found that since diet is the main via of entrance of Hg in seabirds and chicks are fed with prey from the breeding area, the methyl-Hg concentration in their feathers is directly related to the dietary intake. Mercury levels in feathers are also closely related to levels in liver (Thompson *et al.*, 1991). Levels in chick down correlate with those in the egg (Becker *et al.*, 1993; Stewart *et al.*, 1997) and are a good measure of the local food chain contamination with mercury in the area around the colony in which the birds fed during egg formation (Barrett *et al.*, 1985)². In adults, assessing mercury by using feathers can be more complex. For example, concentration in feathers may depend on sex, since females can excrete part of methylmercury burden into eggs (Lewis *et al.*, 1993). Adults undertake moult and during this process mercury can be partly transferred from soft tissues (in which it had been accumulated so far) to feathers in different amounts, according to the moult progression (Furness *et al.*, 1986). If the moulting pattern is known, feathers can be sampled at any time of the year to examine feeding habits and heavy metal intake in specific time periods and colonies (Ramos *et al.* 2009).

Museum feather samples have long been used to monitor mercury (Vander Pol & Becker, 2007). However, some questions regarding contamination from storage techniques have been raised (Hogstad *et al.* 2003), stressing the need of standardized procedures to eliminate extraneous contamination. Indeed, since metals are found on the surface of feathers as a consequence of external deposition (e.g. Hahn, 1993; Borghesi *et al.*, 2016), as well as incorporated into growing feathers from the blood (Burger, 1993), the use of feathers as bioindicators may be confounded by a combination of these two processes. A new approach to avoid misinterpretation due to the external contamination, based on geochemical information of the breeding site, has been suggested for colonial waterbirds (Borghesi *et al.*, 2016).

² However, a recent study highlighted that mercury levels depends on the egg in Yellow-legged gulls *Larus michahellis*, and concluded that females of this species could use also its own accumulated resources during synthesis of their eggs

In addition to mercury, cadmium and lead are of particular concern for marine ecosystems (Fowler, 1990). However, for most metals (including cadmium and lead), most concentration in feathers appears to originate from external contamination (Borghesi *et al.*, 2016), and only enter feathers in trace amounts (e.g. Walsh, 1990).

Several studies in the western Mediterranean (north-eastern Spain) assessed metals in Audouin's gulls comparing two breeding colonies (García-Tarrasón *et al.*, 2013; Sanpera *et al.*, 2007). The researchers used chick feathers. In both studies, the birds in the Ebro Delta showed the highest Hg burdens. In previous studies, high levels of Hg in Audouin's gull, were found in eggs (Sanpera *et al.*, 2000) and in feathers of dead adults of this species (Arcos *et al.* 2002). The latter study also investigated Yellow-legged gulls, Common tern and European Shag. A study on Audouin's gull was carried out also in the north-eastern Mediterranean (Goutner *et al.*, 2000). Taking samples from several Greek islands, Hg was assessed in chick feathers, and spatial and temporal variations were found.

Mercury biomagnification processes in several groups of Yellow-legged gull chicks at different trophic level (as assessed through isotopes) have been studied by Ramos *et al.* (2013). They included also lead and selenium in their study, and reported mercury and selenium concentrations to be lower than in more strictly piscivorous seabird species (Renzoni *et al.*, 1986; Arcos *et al.*, 2002; Sanpera *et al.*, 2007). For more studies on Hg, Pb, Se and Cd in feathers in the Mediterranean seabirds, see Abdennadher *et al.*, 2010; 2011 and references therein.

Metal exposure of shearwaters in the Mediterranean is little studied. Scopoli's shearwater (at the time considered as the Mediterranean population of Cory's shearwater) was investigated for mercury in liver by Renzoni *et al.* (1986) on the major colony of Linosa island (Italy). Mediterranean *Calonectris* have been studied for seasonal variations of Hg, Pb, and Se levels in comparison to Atlantic and Cape Verde species, by using both winter and summer feathers. More recently lead, cadmium, mercury and selenium were assessed in contour feathers of Yelkouan shearwater (Bourgeois *et al.*, 2011).

A complete screening of Hg in Razorbills wintering in the western Mediterranean Sea has been performed in a study based on dead birds caught by fishing nets, proposing feathers of this species as a valuable biomonitoring tool for Hg pollution in the Mediterranean (Espín *et al.*, 2012a). However, in

such case, Hg concentrations in feathers reflected the exposure period coincident with moult, having taken place on the breeding grounds i.e. outside the Mediterranean.

However, knowledge of contaminant loads in adult seabirds does not normally allow to identify the exact location of point-source pollution (Burger & Gochfeld, 2004) even though some species, that spend a long time in the Mediterranean, may be useful as bioindicators at the regional scale. In general, chicks and fledglings are more suitable to investigate local pollution, especially through feather analysis (Ramos *et al.*, 2013; Borghesi *et al.*, 2011, Goutner *et al.*, 2000).

2.2 PERSISTENT ORGANIC POLLUTANT (POPS)

The presence of POPs (organochlorines, OC) in birds was first evidenced in 1973 (Stickel *et al.*, 1973). POPs may affect bird condition, behavior, reproductive success and may lead to serious consequences for the development of the chicks (Morales *et al.*, 2012). As a new insight, PCBs and other POPs can contaminate seabirds as a consequence of plastic ingestion.

When birds have to use their reserves of fat, and this physiological function is repeated several times in a year, lipophilic compounds are newly mobilized and the contaminants circulating in the body increase with harmful effects. In seabirds and coastal ones, the higher residues are found in hychthyophagous species (Clark, 2001). Internal endpoint (e.g. fat, muscles, gonads) and body condition (feeding rate and quality of food) are the main factors driving the implications for health. For this reason, studies were focused on blood (e.g. Henriksen *et al.*, 1998; Bustnes *et al.*, 2001; Finkelstein *et al.*, 2006) and excreta (Sun *et al.*, 2006) from live birds, internal organs from dead birds (e.g. Espin *et al.*, 2012b), and eggs (e.g. Huber *et al.*, 2015). In Razorbills spending the winter in the NW Mediterranean, Espin *et al.* (2012b) found the highest OC levels in abdominal fat, followed by subcutaneous fat, liver and brain.

Sublethal effects are of great interest in birds, and some of them are well known, for example the interference on calcium metabolisms and therefore the alteration of egg structure provoked by DDT and PCBs (e.g., relating seabirds: Verboven *et al.*, 2008). Reduced hatchability and worse condition of chicks are also consequences of PCBs due to the toxic action on embryos (Bustnes *et al.*, 2000; 2008).

PCBs are endocrine disruptors affecting the reproductive system (up to sterilization) and altering the oestrogenic activity (Furness & Camphuysen, 1997).

As reviewed by Mallory and Braune (2012), long-term monitoring (especially based on egg sampling) has allowed to demonstrate that persistent organic pollutants show both spatial and temporal variation, partly attributable to differences in species' diets, and to differences in regional deposition patterns.

The ban on use of OCPs since 1980s caused a decrease in environmental and biological concentrations, and similar decreases were also observed for PCBs. Monitoring of contaminants in Canadian Arctic seabirds showed that legacy POPs are generally declining while Hg and several new, emerging compounds have been increasing over the past three decades (Butt *et al.*, 2010; de Wit *et al.*, 2010; Rigét *et al.*, 2010). Analysis of banked specimens have been carried out and shown that BFRs (new POPs since 2009, Stockholm Convention, 2012) increased in Common Murre eggs until the mid-to-late 1980s and then decreased again since 2001 (Sellström *et al.* 2003).

Assessing halogenated hydrocarbons on seabirds usually requires to sacrifice a certain number of individuals in order to analyze concentrations in internal tissues. Less impacting methods have to be found and improved. An alternative practice sometimes used is to analyze tissues from dead birds found stranded on beaches. This approach can raise the problem of the representativeness of the sample set, because it will mainly be composed by unhealthy birds. In particular, birds in a state of starvation reduce the mass of internal organs and mobilize pollutants (Bogan and Newton, 1977).

Eggs could be suitable for analysis of this kind of contaminants. Concentrations in eggs tend to reflect pollutant uptake by the female during the period before laying (Furness & Camphuysen, 1997). In the NW Mediterranean, studies and monitoring programs on POPs in gull whole eggs started in late 1970s (Vannuchi *et al.*, 1978) and a number of studies have followed (e.g. Focardi *et al.*, 1980; Lambertini and Leonzio, 1986; Focardi *et al.*, 1988, Pastor *et al.*, 1995a, 1995b; Gonzalez, 1991; Morales *et al.*, 2012). For an overview of the PCB levels detected in studies on Audouin's gull until mid-1990s, refer to Pastor *et al.* (1995b).

Studies in the eastern Mediterranean are much less numerous but more recent (Goutner *et al.*, 2001; Albanis *et al.*, 2003; Antoniadou *et al.*, 2007; Kocagöz *et al.*, 2014). Recent evidence was found that POP

concentrations in abiotic and biotic samples collected in Eastern Mediterranean are lower than levels measured in other parts of the basin, especially for PCBs (Albanis *et al.*, 2003; Kocagöz *et al.*, 2014). However, Albanis *et al.* (2003) found the PCB congeners 118, 138, and 180 (penta-, exa-, epta-chlorobiphenyl) the most elevated in gulls, according to previous studies carried out in the NW Mediterranean and even worldwide (see Goutner *et al.*, 2001 for further references). Morales *et al.* (2012) found that OC pesticides and PCBs were still the most abundant chemicals detected in Yellow-legged and Audouin's gull eggs in the NW Mediterranean, and Albanis *et al.* (2003) suggested that Audouin's gull and Cormorant eggs are suitable for this kind of biomonitoring in the eastern seabird colonies. Among DDT family, DDE mean levels in Tyrrhenian and Aegean seas are comparable in two studies (Leonzio *et al.*, 1989 and Goutner *et al.*, 2001, respectively). Cormorant eggs were also investigated for PCBs and OC pesticides by Kostantinou *et al.* (2000) in coastal and internal wetlands of Greece and, interestingly, the highest levels were found inland, though in both sites levels in eggs did not raise fear for biological implications.

The uropygial gland is located at the base of the tail feathers and produces an oil used by birds for functionality of the plumage. Such oil is capable to accumulate lipophilic pollutants, such as legacy and emergent POPs (van der Brink, 1997, Yamashita *et al.*, 2007). Since a long time preen oil has been attractive for monitoring organochlorines in birds (Johnston, 1976). A significant correlation between the total PCB levels in preen oil and adipose tissue in seabirds sampled in the North Pacific Ocean was recently found (Yamashita *et al.*, 2007).

Very recently, some waterbirds species were assessed for three main groups of POPs: organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs) and polybrominated diphenylethers (PBDEs). Even though not including any marine species, the study assessed contaminants in some coastal waterbirds listed in the Annex II of the Barcelona Convention such as Dalmatian Pelican and Mediterranean Gull. Yellow-legged Gull were also included in the study (Kocagöz *et al.*, 2014). In this research, the use of several types of samples as potential bioindicators for POPs were evaluated: preen gland oil, blood, eggs, and blood. Venous blood was verified to be a good potential biomonitor for the concentrations in liver and muscle especially for PCBs and the activities of antioxidant enzymes were correlated with the liver concentrations of several OCs.

Further, the latter study showed that venous blood can be a better indicator of PCB and other organic compound concentrations in liver and muscle of waterbirds. Some antioxidant enzymes in blood, proposed in the same study, were correlated with contaminant concentrations in internal organs and were, thus, recommended for biomonitoring by using birds as indicators.

Procellariiformes in the Mediterranean have been little studied for organic contamination (Renzoni *et al.*, 1986; Roscales *et al.*, 2010; Bourgeois *et al.*, 2011). Bourgeois *et al.* (2011), found in general low levels of about 100 contaminants in dead adult Yelkouan shearwaters, but PCBs and DDT were detected in two abandoned eggs. This was not unexpected because PCB and DDT concentrations were found to be higher in the blood of Scopoli's shearwaters than in other *Calonectris* species breeding out of the Mediterranean. Especially Crete and Hyères colonies showed the highest levels in a spatially comprehensive research (Roscales *et al.*, 2010).

Also feathers were proposed to be suitable for analysis of organic contaminants (Jaspers *et al.* 2007, Espín *et al.*, 2012b), but this opportunity is still poorly explored so far and methodological aspects have still to be improved (García-Fernandez *et al.*, 2013).

Measuring concentrations is the logical way to obtain quantitative data of a contaminant which is under investigation. However, chemicals that are causing adverse effects may be totally unknown, or the focus of a survey may be limited to only monitor how much a target species is stressed. Laboratory services are in general expensive and assessing the importance of the exposure to pollution before quantifying each possible pollutant could be the best approach. Especially when the goal is to evaluate the response of birds to the presence of contaminants is it possible to make sampling and environmental monitoring easier, simply by collecting blood smears and making observations through a microscope. In such a kind of investigation, visible abnormalities in the blood can be related to the exposure to some types of contaminants (especially PAHs) or used to infer the organisms' sensitivity to contaminants, as well as the environmental quality of an area (Sutherland *et al.*, 2004; Baesse *et al.*, 2015). Abnormalities may consist in an unexpected shape of some cell parts (presence of cell fragments), or an altered frequency of certain blood compounds (e.g. porphyrins, see below). Micronuclei are small DNA-containing bodies outside the cell nucleus, formed by anomalous fragmentation of the cell membranes especially during cell division (Heddle *et al.*, 1991). Micronuclei in the erythrocytes (red blood cells) reveal that birds may

be affected by genotoxic pollutants capable of mutagenic effects (Benner *et al.*, 1994). Blood samples for this purpose are easy to be collected and non-invasive, though the response to genotoxic agents may be lower than for other types of cells, since mature erythrocytes do not divide (Carrasco *et al.*, 1990). Studies evaluating micronucleus abundance rarely included wild birds. Higher micronuclei rates in birds sampled from areas with lower environmental quality (anthropized) found by Baesse *et al.* (2015) encourage to increase using this method for birds, but available experience is still scarce. Notably, three recent studies have been performed in Europe (Stončius and Sinkevicius, 2003; Quirós *et al.*, 2008; Skarphedinsdottira *et al.*, 2010), but some of them adopted a destructive sampling procedure. The first study was performed on Black headed gulls in Lithuania and measured the frequency of micronucleated erythrocytes in embryos. The latter study focused on Herring gulls from Sweden and Iceland after a mass mortality episode in the Baltic Sea. Blood was sampled directly from heart before sacrificing birds. This study confirmed that micronucleated erythrocytes is a useful biomarker in gulls but also suggested that other animal classes may be more suitable for this method. Quiros *et al.* (2008) sampled chicks of three heron species in the Ebro Delta, Spain, to evaluate micronuclei frequency in blood. However, they performed a complex sample preparation, in order to run nuclei and micronuclei in a flow cytometre, after a physical separation of these particles from membrane and cytoplasm.

Among compounds that are present in blood, porphyrins can be useful as biomarkers of the biochemical effects of the exposure to contaminants in vertebrates (De Matteis and Lim, 1994). Porphyrins are a group of organic aromatic compounds having very intense absorption bands in the visible region and, consequently, they may be deeply colored. Heme, the pigment in red blood cells, is a porphyrin, but the porphyrin group includes many other compounds, for example proto-porphyrin, an intermediate metabolite of heme biosynthesis, and uro- and copro-porphyrin, some of its oxidative byproducts. When the organism comes in contact with some classes of toxic compounds, an alteration of the heme synthesis occurs and, consequently, levels of excreted porphyrins change. The profiles of different metabolites (copro, uro and protoporphyrin) excreted or accumulated by the tissues will be different as a result of exposure to different toxic compounds such as POPs and trace elements. Other than in blood, they can be found in faeces of seabirds such as penguins, cormorants, and gulls (Casini *et al.*, 2001; Celis *et al.*, 2012; Bains *et al.*, 2015). Porphyrins can bind metals, and therefore can be used as

biomarkers both for organic toxic chemicals and metals (such as Pb, Hg, and As) (see the review regarding the various possible uses of porphyrins in birds, by Casini *et al.*, 2003). Assessing porphyrins combines affordability and information, because the results will allow to identify the best target for further research.

2.3 EMERGENT CONTAMINANTS

Emergent pollutants often require to involve new specialized laboratories and collaborators in order to include them in monitoring surveys (Mallory and Braune, 2012). Despite this, in recent years researchers are undertaking studies on this topic, also focused on Mediterranean region (for example, refer to Sánchez-Avila *et al.*, 2010 and Loos *et al.*, 2008 for estimation of PFCs in NW Mediterranean and Adriatic, respectively).

Emerging contaminants such as PFCs or short chain chlorinated paraffins (SCCPs) and polychlorinated naphthalenes (PCNs) do not occur naturally. They are a human-made chemical used in for many purposes. PFCs have been especially investigated in eggs. Studies included Common shag from Norway (Herzke *et al.*, 2009), Cormorant from Germany (Rüdel *et al.*, 2011), Little egrets from Asia (Wang *et al.*, 2008; Yoo *et al.*, 2008) and several bird species from North America (Custer *et al.*, 2009, 2010; Rattner *et al.*, 2004; Gebbink *et al.*, 2009, 2011). In all these studies, no matter the species or the geographical area, PFCs were detected in eggs.

Butt *et al.* (2007) demonstrated an overall increase in PFCs using archived livers of Thick-billed Murres and Northern Fulmars collected from Prince Leopold Island, Canada, between 1975 and 2004. Schiavone *et al.* (2009) argued that the accumulation of PFCs in marine animals is related to seawater pollution, feeding ecology and distance to land.

Barón *et al.* (2014) carried out a study in Southern Spain on bioaccumulation and biomagnification of emerging and classical flame retardants, basing on eggs of several bird species, among them Black-headed Gull and two species listed in Annex II of Barcelona Convention: Slender-billed Gull and Gull-billed Tern. Interestingly, they found PBDEs in all species, but the least contaminated were Charadriiformes, Anseriformes, and Strigiformes, the most were storks, herons and diurnal raptors.

Conversely, several chemical species of dechlorane (DEC) species were detected in all studied bird species, especially in gulls, while three emerging BFRs were not detected in any sample.

Dechlorane Plus (DP), most often reported in literature among the DEC category, was assessed before in Yellow-legged and Audouin's gull eggs in SW Mediterranean islands, close to the North African coast (Spain). Again, DP was detected in all samples, at a higher concentration in Yellow-legged gull eggs than in Audouin's gulls (Muñoz-Arnanz *et al.*, 2012).

In the Mediterranean, a spatial study has been carried out in eight sites all around Spain to evaluate the occurrence of PFCs in Yellow-legged gull eggs, namely perfluorooctane sulfonate (PFOS), perfluorohexane sulfonate (PFHxS), perfluorobutane sulfonate (PFBS), perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA). PFOS was the only compound detected in Yellow-legged gull eggs from all colonies, without affecting egg biometric parameters, while PFOA and PFNA were not found in samples from the investigated sites (Vicente *et al.*, 2012). In Audouin gull's eggs, PFOS was the most often detected among 5 PFCs, and its concentration decreased according to the laying sequence (Vicente *et al.*, 2014).

PFCs and SCCPs, other than legacy POPs, have been recently investigated in Yellow-legged and Audouin's gulls comparing several extraction methods from eggs (Morales *et al.*, 2012).

2.4 POLYCYCLIC AROMATIC HYDROCARBONS (PAHS)

Mutagenic, carcinogenic and toxic properties of PAHs make this class of pollutants of scientific interest (WHO, 1998). Despite this, and despite seabirds are frequently proposed as suitable monitors for persistent pollutants, in the case of PAHs studies are scarce and the role of bird as indicators of PAH contamination is still little explored. In addition, such a small number of efforts has provided controversial patterns concerning accumulation and biomagnification of PAH in seabirds (Roscales *et al.*, 2011).

Pérez *et al.* (2008) proposed Yellow-legged gull as a good indicator of PAH comparing blood levels from colonies polluted by the Prestige oil spill with non-affected areas. Later, blood parameters indicating hepatic damage in birds (AST enzyme) were used to infer adverse effects of the shipping accident on that species, correlating higher AST plasma levels with reduced red bill spot area (Pérez *et al.*, 2010).

Eggs were, then, used to assess spatial and temporal patterns of PAHs in Kentish plover in NW Spain as a consequence of the same oil spill (Vidal *et al.*, 2011). Eggs are considered to be good biomonitors of PAH contamination in birds (Malcolm and Shore, 2003), but pollution levels of these substances in eggs of seabirds are rarely investigated, and mostly out of Mediterranean (Lebedev *et al.*, 1998; Shore *et al.*, 1999; Stronkhorst *et al.*, 1993, and see Vidal *et al.*, 2011).

Roscales *et al.* (2011) compared 15 PAHs levels in several Procellariiform species, including Scopoli's and Balearic shearwaters for the Mediterranean. Livers for analyses were sampled in irreparably wounded adult birds accidentally captured by longliners. In their study, PAH burdens differed significantly among species rather than geographical areas, probably depending on feeding habits.

2.5 PLASTICS

Monitoring the impact on the biota provoked by plastic debris can be oriented in many directions. The two main issues concerning seabirds are entanglement and ingestion (Ryan *et al.* 2009).

Of the 120 marine animal species listed on the IUCN red list, 54% have been found entangled in plastic items or had ingested plastic debris. In total, 180 species of animals have been documented to ingest plastic debris, including birds, fish, turtles and marine mammals (Laist 1997). As many as 56 species of marine and coastal birds have been reported for entanglement worldwide (Katsanevakis *et al.*, 2007). Entanglement is mostly caused by fishing gear lost at sea, a relative minor problem in the Mediterranean if compared to some oceanic zones (Gabrielides *et al.* 1991). Derelict nets continue to exert "ghost fishing", catching animals even if they sink or are lost on the seabed (Laist, 1987; Matsuoka, 2005).

Ingestion of plastic items may cause harmful mechanical effects, such as gastrointestinal blockages (Baird & Hooker 2000), ulceration (Fry *et al.* 1987), internal perforation (Mascarenhas *et al.* 2004). These are often causes of death. Also when damage is not so large, seabirds eating large plastic amounts will consequently reduce food intake, with detrimental effects on general fitness, especially limiting fat deposits, egg laying, movement capacity (Ryan, 1988; Spear, 1995). Plastics can be roughly divided in 'industrial' or 'user' (Provencher *et al.*, 2015). Pellets are the primary source for plastics industry, and they are accidentally released into the marine environment, during shipping (Gregory and Ryan, 1997).

Pellets are the raw material for production of all user plastic items. Microplastics (which include pellets) and nanoplastics are used in beauty scrubbers and exfoliates. Seabirds interact with all kinds of plastics (Provencher *et al.* 2009; van Franeker *et al.* 2011).

If ingested, beside causing mechanical problems in the digestive tract, plastics release chemicals included in the manufacturing process (plasticizers, e.g. phthalates, organotin compounds, alkylphenols, Bisphenol A) and also hydrophobic pollutants (e.g. alkylbenzenes, chlorinated hydrocarbons, PAHs, PCBs, DDT) adsorbed from the environment (Teuten *et al.*, 2007). In addition, platinum group metals seem to have affinity for plastics (Cobelo-Garcia *et al.* 2007). In general, once in the bird stomach, acidic conditions will enhance desorption of metals from plastics. Chemicals adsorbed by plastics and released into the digestive fluid can be transferred to the tissue of birds with adverse effects (Mato *et al.*, 2001; Teuten *et al.*, 2009; Colabuono *et al.* 2010). This is confirmed by a positive correlation observed between the mass of ingested plastic and the PCB concentration in the fat tissue of a shearwater species in South Atlantic Ocean (Ryan *et al.* 1988). Biomagnifiable contaminants released by ingested plastics and preys concur to worsen the accumulation along the food web. For a comprehensive scenario about types of contaminants detected in marine plastics, see Teuten *et al.* (2009).

Beside Ryan *et al.* (2009) work, other scientific reviews were published to discuss the biological and ecological effects of plastics in seabirds. A comprehensive review of plastic ingestion by marine birds has been provided by Day *et al.* (1985) and improved by Azzarello and Van Vleet (1987), by including different aspects and species involved in this phenomenon. However, several concepts regarding the modes of ingestion and consequent effects have been revised and improved in following years. Plastics have been included among the numerous stressors that affect seabirds in the review cited above by Thompson and Hamer (2000). Another recent review reported plastic ingestion and nest incorporation in Canada (Provencher *et al.*, 2015).

Studies on ingestion rates. Seabirds are active samplers of plastics, as proven by peck marks in 80% of plastic debris on the Dutch coast (Cadée 2002). Plastic particles, however, are not only ingested directly by surface feeding, but also via secondary predation in the food web. It is known that ingestion of plastic debris by seabirds occurs much more frequently than supposed for long time, although the first

evidence dates back to 1960 (Bennett, 1960). Indication is available that ingestion of plastic has been increasing since the 1960s to recent years (Robards *et al.* 1995; Ryan *et al.* 2009).

Among seabirds, especially shearwaters and petrels can accumulate plastics in their stomach (Ryan *et al.* 2009). Procellariiformes can feed by surface-seizing and pursuit-diving, two feeding strategies that, more than others, expose birds to plastic ingestion (Azzarello and Van Vleet, 1987). These habits may represent a threat for birds, but also offer the possibility to monitor changes in the amount and composition of plastic debris at sea by using this group of species as bioindicators (Ryan *et al.* 2009).

For the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR convention), stomach contents of Fulmars have been used to assess temporal trends and to collect information about plastic feeding habits by this species (Iceland: Kühn and Van Franeker 2012; English Channel, various sites in the North Sea, and Faroe Islands: van Franeker and SNS Fulmar Study Group 2013; Svalbard Islands: Treveil *et al.*, 2015) (Figure 8). For the Netherlands, data are available from 1979 onwards; other North Sea countries have participated to the monitoring since 2002 (van Franeker and Law, 2015). As a quantitative indication, 95% of 1295 dead beached birds collected in the North Sea from 2003 to 2007 had plastic in the stomach. Bird stomachs contained an average of 35 plastic items, weighing a total of 0.31 grams. These studies seem to return good quality data (van Franeker *et al.* 2011). Hence, fulmars can be a powerful tool to monitor changes in the abundance and composition of small plastic debris at a regional scale.

North Sea is not the only region having a regular, coordinated monitoring scheme of plastic ingestion rates in seabirds, which is supported by normative tools and a network of researchers (van Franeker *et al.* 2011). Also in Canada, systematic assessments started in the mid-2000s, by using standard protocols (Provencher *et al.*, 2015). Canadian researchers are making efforts to estimate how many samples are required on an annual basis to detect changes in plastic ingestion over time (van Franeker and Meijboom, 2002; Provencher *et al.*, 2015).



Figure 8 - Plastics from the stomach contents of an individual of northern fulmar from Svalbard. From left to right: industrial pellets; probably industrial; fragments; sheets; threads; foam. Scale bar is 1 cm (from Trevail *et al.*, 2015, courtesy of van J.Franeker, IMARES).

However, in research studies aimed at establishing the links between plastic ingestion and health, beached birds are normally un-useable, because their cause of death is unknown and the type part of population under study cannot be statistically defined (Pierce *et al.*, 2004; Barbieri, 2009). A large, random sample of healthy pre-fledging Short-tailed Shearwaters (*Puffinus tenuirostris*) breeding in Tasmania has recently been investigated in order to relate plastic ingestion to body condition (Cousin *et al.*, 2015). This study was possible because a number of illegally harvested shearwater chicks was available. Notably, another study on the same species but from a different colony, including different age groups and sampling conditions (the study was based on beached fledglings), gave comparable results in terms of quantity and type of ingested plastics (Carey, 2011). The incidence of plastic-debris ingestion was about 95% in both cases. In the two studies (the first on fresh healthy corpses, the second on beached carcasses) plastic weight ranged from 113 to 148 mg per bird, respectively, with light-coloured user plastic more frequent than other types. Taking only the number of plastic pieces found in stomachs, Cousin *et al.* (2015) found 6 pieces per fledgling, while Vlietstra and Parga (2002) found 6.9 pieces per adult of the same species (Short-tailed shearwater). Another study based on a homogeneous sample set focused on the transfer of plastic debris from Cory's shearwater parents to fledglings was carried out in the Canary Islands comparing sexes, years and date of fledging and assessing the effect of plastics on the body condition (Rodriguez *et al.*, 2012). In the Canary Islands

every year a number of fledglings are recovered after being disoriented by light pollution (Rodriguez and Rodriguez, 2009). More than 80% of birds had ingested plastics; white items were the majority (about 51%) and tended to be shorter than other colors. However, none of the measured variables of plastics explained either body condition or body size, and no sign of perforation or ulceration was found in the sampled birds (Rodriguez *et al.*, 2012).

Another important issue to take in consideration when spatial and temporal changes in plastic ingestion are examined is the retention time of plastic particles in bird stomachs. This topic is just at present being discussed. While in Fulmars it has been recently suggested a retention time of about 1 month for the 75% of ingested plastics (van Franeker and Law, 2015), a commentary (in press at the time of this document) invokes caution for other Procellariiformes, in which plastics can take several months to pass in the intestinal tract, in absence of regurgitation (Ryan, 2015).

However, even live birds are not random samplers. Seabirds select the types of plastic fragments for specific shapes and colors, mistaking them for potential preys and depending on species, age, season, level of starvation (Day *et al.*, 1985; Ryan, 1987; Moser and Lee, 1992; van Franeker, 2005). In addition, selection can be a secondary effect due to the preferences of preys in certain plastic items, as in the case of fishes (Carpenter *et al.*, 1972).

For the Canadian waters, a complete review attempted to answer the questions: 1) which species are ingesting plastics at a higher incidence; 2) which species appear to be at low risk of plastics ingestion; 3) for which species data are available on the mass of plastic ingested; 4) for which species temporal trends can be assessed (Provencher *et al.*, 2015). In such a review knowledge gaps are summarized too.

Studies on chemicals released from ingested plastics. Phthalate esters are normally used by manufacturers in order to provide flexibility and durability to plastic materials. As they are not strongly bounded to the polymeric chain, phthalates are promptly released in the environment and animal tissues (Friocourt *et al.*, 1980). On this basis, these compounds can be used either for evaluating the toxic effects on individuals, or the ecological effects on a population, or even used to biomonitor the environmental quality by measuring the exposure level in an indicator species, such as seabird species that regularly ingest plastics. For the latter purpose, a non-invasive approach is needed, and the study of Hardesty *et al.* (2015) moved in this direction assessing dimethyl phthalate, dibutyl phthalate and

diethylhexyl phthalate (DEHP) concentrations in the uropygial oil of 5 seabird species. However, the authors warned that these plasticizers are not present in all plastic products. The most useful phthalate suitable as a marker for plastic ingestion in the investigated species seemed to be the DEHP. A limit of this innovative method is the lack of knowledge about time it takes to phthalates for being incorporated into the preen gland of seabirds, and the subsequent residence time after exposure ceases (Hardesty *et al.*, 2015).

Studies on plastic incorporation in nests. Plastic incorporation in nests is indicative of larger pieces of plastics that have been picked up by birds during nest construction and includes only macroplastics. Depending on species and sites, some hundreds of nests are required annually to detect a 10% change (Provencher *et al.*, 2015). Bond *et al.* (2012) compared the prevalence and composition of marine debris in nests at two Northern gannet colonies in Canada, in order to assess the potential impact of fishing plastic debris on birds, especially entanglement risk. Gannets collect almost all nesting material at sea (Bourne, 1976), therefore the amount of anthropogenic debris in nests is thought to provide an indication of the level of pollution in the surrounding ocean (Montevecchi, 1991).

Similar to nest incorporation by gannets, assessing plastic pollution in cormorant and shag nests may offer a relatively easy and noninvasive way to monitor plastic pollution (Podolsky and Kress, 1989; Cadiou and Fortin, 2015). A preliminary study on the presence of marine debris in European shag nests as an indicator of marine pollution was carried out by Cadiou *et al.* (2011) and the opportunity to use European shags as indicators of marine pollution and entanglement risk was presented at the 10th Meeting of the Intercessional Correspondence Group on Marine Litter (ICGML) organized by OSPAR in 2012 (Cadiou and Pouline, 2012). Cadiou and colleagues identified numerous types of hand-made items in shag nests, most of them originated from marine sources. On the basis of the abundance of items in nests, an evaluation of the local environmental quality (limited to plastics pollution) was carried out (Cadiou *et al.*, 2011). Monitoring has been continued in subsequent years in Brittany, and extended to other Brittany, Normandy, and Corsica colonies (Cadiou, 2013; Cadiou, 2014; Cadiou and Fortin, 2014; Fleuriat R. and Faggio G., 2014; Leicher and Fortin, 2015).

The studies on gannets in Canada have allowed to obtain useful information thanks to the high proportion of nests containing marine debris (50–90% with in average 470 g of plastics; Votier *et al.*,

2011; Bond *et al.*, 2012) and the large sample size (an assessment of at least 100 nests in each region per year are recommended by Provencher *et al.*, 2015). Studies on Kittiwakes in Denmark allowed to observe a temporal increase of plastics in nests (Hartwig *et al.*, 2007). However, not all marine species are suitable for this kind of monitoring, because of the ephemeral nature of nests in some species, extra- and intra-specific differences in nesting and breeding behaviour (Lavers *et al.*, 2013), and even individual preference for particular debris shapes and colours, an aspect far to be understood and modeled.



Photo: F. Borghesi

3. COMMON SAMPLING PRACTICES

The criteria suggested by Vander Pol & Becker (2007) in order to determine the seabird species and tissue types that are most appropriate for monitoring contaminants and banking samples can be similarly used to guide the definition of a general sampling protocol aimed to biomonitoring surveys:

- Species and tissues should bioaccumulate contaminants in concentrations that can be measured in relatively small amounts, but the tissue sample should be large enough for multiple analyses
- Species and tissues should be easy and relatively inexpensive to sample, so that multiple-year collections at the same sites and times may be obtained without causing an impact on individuals.
- The tissue samples must be properly collected and stored, to eliminate extraneous contamination or alterations in the contaminants.
- Species and tissues must be representative of the area to be monitored, consistently with the goals of the project.

A sentinel seabird species should provide an early warning of the effects of pollutants or their impact on the marine communities, or potential effects on humans, or all of these issues. Biomonitoring programs capable to give information about risks for human health have more opportunity to be medium-term funded (Burger & Gochfeld, 2001).

Tissues usually collected for contaminant studies in seabirds include blood, liver, kidney, brain, muscle and feathers. Blood reflects recent exposure to many substances. Feathers reflect exposure through the period between two moulting events. Liver has become a standard tissue for toxicity tests and for measuring concentrations of toxicants (metals and organic compounds). Muscle concentrations imply risks for predators (including humans), and brain and kidney levels are often indication of direct impact on the organism (Burger & Gochfeld, 2004).

For most seabird species, however, non-invasive techniques (e.g. feathers, blood, excrements), are preferable or often the sole possible, when sampling is carried out on most seabird species.

In relation to the aims of this document, which is to propose some repeatable, simple, non-invasive sampling protocols to be applied for medium-term biomonitoring projects using seabirds as bioindicators of the marine pollution levels, information based on literature reviewing is reported here. In particular, methods used for blood, feathers (including the down of chicks), and excreta have been considered.

From a conservation point of view, shearwaters, petrels, and some gull species (like Audouin's gull) are sensitive species. To minimize disturbance at the breeding sites, yearly-based monitoring programs should aim at combining sampling with other routinary activities that are carried out on the same colonies, such as, for example, censuses and ringing campaigns (Goutner *et al.*, 2000).

3.1 STUDIES ON MERCURY

Mercury concentrations in feathers is related to Hg in blood at the time of their formation (Jaspers *et al.*, 2006). Mercury binds with feather keratin in the form of methylmercury (Thompson and Furness 1989). Especially in seabird chick plumage, Hg concentration reflects the contamination level in the marine zone explored by adults to feed (Monteiro and Furness 1997, Abdennadher *et al.*, 2010).

Sampling of fledging Audouin's gulls in Spain was carried out by plucking a bunch of mantle feathers close to the neck (Sanpera *et al.*, 2007; Garcia-Tarrason *et al.*, 2013). Breast feathers were plucked in many other studies (e.g. Monteiro *et al.* 1999; Burger and Gochfeld 2000; Bourgeois *et al.*, 2011). Scapular feathers seem to be a good alternative due to their relatively large size. These were sampled in Yellow-legged gull fledglings in Spain (Ramos *et al.*, 2013), as well as on Greater flamingos (Borghesi *et al.*, 2011; Borghesi *et al.*, 2016). This type of feathers is suitable when shaft has to be analyzed separately from vane, or is the sole part of interest, being the rachis of mantle and breast feathers short and thin. Feather shaft may be a better indicator of internal tissue levels than feather vanes, even if concentration in vanes is normally higher. No washing procedure has been developed so far that completely avoids external contamination from substrate or faeces (Cardiel, 2011; Borghesi *et al.*, 2016), nevertheless washing is more effective for shafts than for feather vanes. In addition, correlation coefficients between internal tissues and shafts were found to be higher than between body burden and vanes (Espín *et al.*, 2012a).

Primaries of Audouin's gulls (Arcos *et al.*, 2002; Sanpera *et al.*, 2007), Yellow-legged gulls and Common terns (Arcos *et al.*, 2002) were sampled because the study focused on dead birds, and primaries of dead Razorbills (Espín *et al.*, 2012a), were sampled specifically for assessing mercury. Sanpera *et al.* (2007) found higher Hg concentrations in primary feathers, that did not correlate with Hg levels in mantle feathers. One study used primary and secondary remiges of breeding shearwaters to assess variation in trace elements between wintering and breeding season, including Hg (Ramos *et al.*, 2009). In general, however, sampling of flight feathers can be detrimental for birds and should be avoided on live individuals.

One chick per brood should be sampled in order to avoid undesirable interdependent data. Samples can be kept in polyethylene bags until analysis, but if feathers are collected for also organic compound analyses, plastic bags are not suitable (see below). To reduce external contamination, feathers must be cleaned before analysis. A number of washing methods are available in the literature. A good reference for the most accepted washing methods is "Best practice sampling and contaminant monitoring protocol for raptors" (Espín *et al.*, 2014), available from www.eurapmon.net.

If mercury has to be assessed in blood, a sample of 0.2-0.5 ml obtained by means of a puncture of the brachial vein of the wing seems to be enough to perform analyses of concentrations (depending on the instrumental technique). Whole blood can be kept in heparin-free vials and deep frozen (-20°C) until analysis (Sanpera *et al.*, 2007).

Information on the physical conditions of sampled birds can usefully be associated to the analysis of Hg concentrations, as the Hg body burden may also depend on body conditions other than environmental exposure (Espín *et al.*, 2012a). According to van Franeker (2004), physical condition can be scored on a four-point scale and an overall condition index calculated as the sum of scores attributed to subcutaneous fat, abdominal fat, and pectoral muscles.

3.2 STUDIES ON ORGANIC PERSISTENT CONTAMINANTS

As for other pollutants, organochlorine concentrations may seasonally vary, so that care is needed when developing a sound sampling strategy (Fryer and Nicholson, 1993).

A large number of sample types can be used for assessing lipophilic organic pollutants, including feathers (see previous paragraph for sampling procedures normally used in studies on Hg in feathers). Jaspers *et al.* (2006) discussed the external contamination of organic compounds in feathers (inner and outer primaries, vanes and shafts) of a raptor species and proposed the use of feathers also for biomonitoring of organic pollutants (Jaspers *et al.*, 2007). Feathers for assessing organochlorines were tested in the Mediterranean by using wintering Razorbill casualties as bioindicators (Espín *et al.*, 2012b). However, the use of feathers for this purpose is far to be perfected and needs further experimental studies.

Uropygial oil can be sampled with minimal impact on bird health and used as a non-invasive way to assess some organic contaminants released by plastics in seabirds after ingestion. This promising research method has been developed in the Aleutian Islands and Australia (Padula *et al.*, 2014; Hardesty *et al.*, 2015). A field protocol has been developed by the Marine And Atmospheric Research group of the Commonwealth Scientific and Industrial Research Organisation (CSIRO) (see www.cmar.csiro.au). The risk of sample contamination after collection is, however, very high. Samples must be kept far from any plastic item and placed in aluminum foil. The protocol suggests two methods depending on the condition of birds (dead or alive).

Roscales *et al.* (2010) sampled blood in shearwaters (including Mediterranean species) to assess PCB and DDT concentrations. A quantity of 0.5 mL from the brachial vein was sampled and transferred into a vial with 1 mL of absolute ethanol and preserved at -24 °C until analysis.

The removal of a single egg from nests can be acceptable for gulls (especially species of low conservation value), because they lay clutches of several eggs and negligible effect at population level can be expected from taking one of them. However, some studies avoided any impact by sampling unhatched eggs (e.g. Goutner *et al.*, 2001). In this case, only uncracked eggs were collected. However, the possibility that results obtained from unhatched eggs may be not comparable with those coming from recently-laid fresh eggs must be taken into consideration. Other factors of variability can be related to clutch effects (e.g., egg size and clutch size, egg laying order, etc.) and can influence the results, hence the sampling design must be designed depending on the aims of the study (Pastor *et al.*, 1995b). If sampling is carried out for a spatial and temporal comparative study (such as a long-term

biomonitoring involving more than one breeding site) standardization it is crucial to draw spatial patterns and reliable trends. Pastor *et al.* (1995b) provided some suggestions in order to select the most indicative eggs in gull clutches.

To minimize the risk of contamination of fresh eggs, Albanis *et al.* (2003) placed them in cotton-spread cartons (those used to transport hen eggs) and treated as soon as possible after collection, extracting the contents and pouring it in chemically cleaned glass jars, and immediately frozen to -20°C (Kostantinou *et al.*, 2000).

Baesse *et al.* (2015) sampled blood for erythrocytes micronuclei analysis through tarsal-metatarsal vein perforation with insulin syringes and performed a blood smear on a glass slide. When dry, the blood on slides were fixed in methanol or ethanol and coloured to obtain different colours for nuclear and cytoplasmic components of the erythrocytes (Mitchell and Johns, 2008). Each erythrocyte can contain one or more micronuclei and their identification can be follow the proposed methods by Schmid (1975) and Wolf and Luepke (1997), basing on the morphology of the fragments.

As said for Hg investigations, physical condition of specimens should be recorded, hopefully with standardized methods (van Franeker, 2004). In case of eggs, these parameters should be recorded: length and width, both weight of the whole egg and that of the dried shell, and shell thickness along the equator in order to obtain an eggshell index (Ratcliffe, 1967; Vicente *et al.*, 2012). With these parameters and a species-specific constant (for Yellow-legged gull see Oro *et al.*, 1995; Oro, 2008; Vicente *et al.*, 2012), the egg volume can be calculated (Hoyt, 1979). Helander *et al.* (2002) provided a formula for calculation of the desiccation index, useful to take evaluate the loss of weight from laying to the sampling date.

3.3 STUDIES ON PLASTICS

Seabirds are variously exposed to plastics, as they can be entangled, occluded, or poisoned by plastics. Therefore, biomonitoring contaminants can regard assessing the quantity and type of plastics ingested or the chemical and biochemical effects of plastic ingestion. In this paragraph, also assessing plastic items used by birds to build their nests is included, an issue that not necessarily impacts on bird's health, but which provides information on the abundance of macroplastics in the environment (Figure 9).



Figure 9 – This nest of Northern Gannet is near completely made of debris, in particular plastics dispersed by fisheries (nets and ropes) (Photo: M. Cozzo).

3.3.1 Sampling ingested plastics.

The usual ways to assess plastic ingestion by seabirds are lavage, necropsy or endoscopy (Hardesty *et al.*, 2015). Lavage is at times harmful for birds (Gonfriddo *et al.*, 1995), and it's always unsure whether it provides a complete sampling of the gastrointestinal content (Hardesty *et al.*, 2015). On the other hand, necropsy can be performed only on dead birds or through destructive sampling. Beached birds provide an opportunity for sampling ingested plastics, although not for all purposes, and the use of a beached bird surveys as a monitoring instrument has been debated. As a tool for monitoring seabird mortality rates, or population trends, beached bird surveys are certainly inadequate (Furness and Camphuysen, 1997). However, in some situations, beached birds can be useful to get statistically significant results over a span of 10–15 years of collecting data (for oil pollution see Camphuysen and van Franeker, 1992; for ingested plastic: Ryan *et al.*, 2009). A sample size of 40 birds is needed to assess changes in ingested debris rates over time using Fulmars in the Dutch seas (van Franeker and Meijboom, 2002). Provencher *et al.* (2015) performed a power analysis and estimated the sample sizes needed annually for measuring changes of 10% and 25%. Numbers of dozens to hundreds are required depending on species and areas. Unfortunately, the same protocols applied for beached birds surveys on the northern coasts seems difficult to be applied in the Mediterranean where stranded birds are usually found in small numbers (Baccetti, 2006).

Normally, plastics in seabirds are sampled (recovered) from the proventriculus and ventriculus (Auman *et al.*, 1997; Rodriguez *et al.*, 2012; Cousin *et al.*, 2015) and then classified. In Cory's shearwaters, Rodriguez *et al.* (2012) discarded particles shorter than 1 mm, but according to standard methods for bird dissections in the OSPAR monitoring program, the stomach contents should be rinsed in a 1 mm-mesh sieve and sorted under a binocular microscope. Smaller meshes tend to clog and sieving becomes difficult (van Franeker, 2004; Bravo Rebolledo, 2011; van Franeker and Law, 2015).

Plastics items can be categorized as industrial plastic, user plastic or other, according to many studies, such as Ogi (1990), Vlietstra and Parga (2002), Carey (2011), and Cousin *et al.* (2015). This allows comparisons between studies. Regarding colours, Cousin *et al.* (2015) improved the number of colour categories previously adopted by Vlietstra and Parga (2002) and Carey (2011). In the [Table 1](#) the colour classification according to Cousin *et al.* (2015) is reported. In gut of Cory's shearwaters from the Canary Islands, white, green, brown, black and blue items were more frequent, while orange, red, purple, transparent and grey items were rarer. Interestingly, the study on this species showed that white items were on average smaller than those of other colours (Rodriguez *et al.*, 2012).

Table 1 - Colour classification of plastic items found in gannets according to Cousin et al. (2015)

Light colours	Medium colours	Dark colours
Clear	Brown	Dark-grey
White	Blue	Dark-blue
White-yellow	Orange	Dark-green
Yellow	Green	Dark-red
Yellow-brown	Olive-green	Dark-red-brown
Peach	Red	Black
Light-green	Turquoise	
Light-blue		
Grey		
Pink		

Also for other previously reported studies, van Franeker's method can be applied in order to classify levels of muscle and fat of sampled birds (van Franeker, 2004).

3.3.2 Sampling uropygial oil.

Collecting uropygial oil is not difficult, according to the protocol developed by CSIRO and used by Hardesty *et al.* (2015). However, a considerable complication is due to the necessity to adopt extensive precautions before and after sampling, for example by keeping extremely clean all tools and items used for sampling in order to remove phthalate contamination. Hardesty *et al.* (2015) sampled live birds by using surgical cotton wool swabs. The procedure used to treat swabs in order to remove background phthalates, and clean all glassware is described in detail in Hardesty *et al.* (2015). According to CSIRO protocol, when sampling live birds, the uropygial gland must be massaged with hand until oil is secreted. Then, the gland must be wiped over with a swab.

3.3.3 Assessing plastics in nests.

Standardization in studies assessing plastics in seabird nests is a challenge. Studies are many, but very few have reached an advanced stage. However, most of them referred to the methods adopted by Montevecchi *et al.* (1991). For example, Bond *et al.* (2012) visually recorded the type of debris in each nest, when necessary using 10x40 binoculars or 15-60x spotting scopes. Objects were categorized as strapping (thin, flat pieces of plastic used to bundle items), heavy cord, rope, twine, monofilament line, netting, tape, plastic bag/sheet, and other (including hard plastic, straws, ballpoint pens, shotgun shells, and unidentified plastic). However, that study aimed to investigate the impact of fisheries, and therefore the researchers grouped debris from all colonies and years into two broad categories: debris typically originating from fishery operations and other. Similarly, Montevecchi *et al.* (1991) visually observed nests, and checked about 5% of them with a stick to expose plastics from within the nest structure, invisible from the upper surface. Here the categories were: fishing gear (rope, line, netting) package strapping, bag or sheet, hard plastic items, shotgun shell casing, lobster trap tag, other. The number of items found in each European shag nest was grouped in 6 abundance classes (0 items; 1-5 items; 6-10; 11-20; more than 20 items) by Cadiou *et al.* (2011); the researchers warn about the possibility that some items are left unnoticed, in their case dark strapping and wires, or confused with plastics, as in presence of rusty iron stems. When observations are performed during breeding, items should be evaluated as rapidly as possible to reduce increasing risk of predation or overheating.

4 PROTOCOLS TO BE USED FOR MULTI-SITE BIOMONITORING OF CONTAMINANTS THROUGH SEABIRDS

In this chapter, some protocols to collect samples from breeding colonial seabirds are suggested for biomonitoring contaminants in the Mediterranean. With respect to all the lines of study presented so far and related sampling methods, only protocols for collecting feathers for mercury, blood smears for toxic and genotoxic effects on blood cells, uropygial oil for phthalates, excrements for porphyrins, eggs for persistent contaminants, and evaluating plastics in nests are proposed. However, this chapter should be intended as the most 'dynamic' part of this document, because scientific knowledge continually evolves and methods as well. New methodologies will be experimented in the near future and probably old ones will be refined. It has to be expected that the paragraphs in this chapter will be improved or revised in future, in order to follow scientific progress. On the other hand, it may be useful also to recall that biomonitoring is different from research, and the procedures need to be maintained relatively constant over the years because the intrinsic value of monitoring is the possibility to compare results among years, areas, and, possibly, species.

4.1 GENERAL RECOMMENDATIONS

Studies on seabirds and environmental contaminants in the Mediterranean Sea are increasing in number (Borghesi, 2016) and proceeding in many directions, as shown in the present work. Standardization and homogeneity of sampling methods are, in general, poorly considered subjects, with heavy consequences on the comparison of the results obtained in different studies. Also in a view of long-term biomonitoring studies on the health of the Mediterranean Sea by using seabirds as bioindicators, as requested by the Marine Strategy Framework Directive, achieving a sampling protocol, applicable by non-specialists and repeatable at affordable costs, is strongly desirable.

Having this in mind, the protocols suggested here are relatively simple to be applied by collaborators with little experience and volunteers. However, it is important to point out that all monitoring activities should always be managed and coordinated by trained and authorized staff. Well before undertaking the field work, licenses and permissions must be obtained from the appropriate authorities, and roles within the team well defined, in order to assign responsibilities and ensure the circulation of clear and correct information.

As a general good practice, all containers that will be used for samples (or group of samples) should be labelled before they are used in the field indicating date, site, and name of sampler. Before, or immediately after the sample is collected, its container must be labelled also with a unique code. The rationale of the codes should be the same for all sites and adopted by all samplers.

The risk of contamination of samples after they have been collected is critical. Tools and all reusable material has to be pre-cleaned according to the instructions given by the laboratory that will process the samples. Use adequate protective equipment in order to protect samplers but also to avoid their contamination. Smoking, drinking, eating, spraying insect repellents during sampling must be avoided.

Especially in seabird colonies, nests can be difficult to access. Safety requirements for boating, climbing and hiking should be fulfilled. In some risky conditions, despite protocols being simple, only experts should be asked to take samples.

Finally, birds welfare and safety should be a priority for coordinators and samplers. All kinds of unnecessary stress to birds should be avoided. Some precautions, such as cover bird head, avoid noise, exclude from sampling nests in unfavorable conditions, be quick in sampling, should be considered in relation to the specific case.

Sample size is also crucial. Collecting too many samples than needed may cause unnecessary stress to the colony, but also too few may thwart the efforts and the sampling campaign. The adequate sample size is related to species, objectives of monitoring, colony size, laboratory requirements, statistics that will be used and other factors.



Photo: F. Borghesi

4.2 PROTOCOL 1: COLLECTING FEATHERS FOR MERCURY CONCENTRATION

This sampling protocol aims to use feathers or down for trace element analysis, especially mercury. Studies on metals in feathers are numerous and the usefulness of feathers as bioindicators of many elements has been debated. However, it is broadly accepted that feathers reflect the exposure level to mercury. External contamination is a minor problem when mercury is the target.

Targets. Metals and trace elements, especially mercury.

Species. All Mediterranean seabird species.

Quantity. Normally, laboratories require about 100 mg of feathers per sample, but depending on the analytical technique the necessary sample weight ranges from 20 to 200 mg. An amount falling in the range 50-200 mg is normally reached by taking 4 primary coverts coverts from adult shearwaters, or 6 scapulars from adult gulls. If only shafts will be used for analysis, the number of feathers should be doubled. Down from chicks is also suitable for assessing mercury levels. In the present protocol filling two Eppendorf tubes of chick down is recommended. Fledglings that have lost their down and are near fully feathered have to be sampled as adults, but using some precautions (see below).

Sampling chicks and fledglings. When sampling down, gently pluck it especially on the back and flanks, where it is the longest and loosest. Avoid the ventral zone (dirtier). Press the down inside two Eppendorf tubes until full. The scissors tip can be a useful tool in this operation, since the down is, electrostatic and tends to flutter away. When sampling feathers in fledglings, cut them (and not pluck) at some distance from the skin, where the shaft starts to look unreached by blood; or, alternatively, choose feathers that are full grown (if available). Freezer plastic bags are suitable for metal analysis, so put the sampled feathers in one bag (one bag per individual bird). Finally, label the bag (externally) by using a permanent marker.

Avoiding contamination. Once in the laboratory, feathers will be cleaned by a thorough washing procedure, therefore no handling precautions are needed during sampling. The protocol suggested here may be suitable also when feathers are sampled for assessing organic contaminants, according to

Jaspers *et al.* (2007), but any plastic materials and tools should be avoided in this case and feathers should be placed in paper envelopes, instead of plastic bags.

Additional samples. Feathers integrate a longer period of exposure than blood, but can be affected by external deposition that is difficult to remove (in particular soil dust). Local geochemical information can be useful to interpret trace element concentrations found in feathers (un-necessary only for mercury). To obtain material for this purpose, collect about 200 ml (two handfuls) of soil from the shearwaters burrows or close the nest of gulls, using a plastic spatula or spoon, or hands, and put the samples in individual plastic bags. Avoid collecting samples that mainly consist of vegetal fragments, root lumps, pebbles, guano. Put all bags containing soil samples of the same site in larger freezer plastic bag (make sure they are robust enough, cheap bags are normally very thin).

After sampling. As soon as possible (within 24-48 hours) freeze samples at -20 °C. Brief periods outside the freezer are not a problem, for example transferring samples from one site to another or to the laboratory.

Sampling kit.

-
- | | |
|-------------------------------------|---|
| • Disposable gloves | • Stainless steel scissors |
| • Plastic bags (1 per sampled bird) | • 1,5 ml Eppendorf tubes (2 per sampled chicks) |
| • Permanent marker | • Pencil |
-



4.3 PROTOCOL 2: COLLECTING BLOOD FOR CELLULAR ABNORMALITIES

Owen (2011) completely reviewed the best practices for collecting, processing, and storing avian blood. Blood can be sampled for analysis of concentrations, or for visual examination under a microscope. In this protocol only the latter purpose is considered, explaining how to collect a blood smear for diagnostic tests aimed to search for abnormalities in the blood cells.

Targets. Not a specific contaminant, but the adverse effects on blood cells caused by several classes of contaminants (metals and/or organic pollutants).

Species. All Mediterranean seabird species.

Quantity. One drop of blood is enough to obtain a blood smear. Each sample must be done in double (two slides).

Sampling the bird. A drop of blood is collected by using an insulin syringe, after having pricked a brachial vein. The brachial/ulnar vein is located just beneath the ventral surface of the humeral-radial-ulnar joint (Figure 10). Extend the wing, possibly with the aid of a collaborator, and, to improve the visibility of the vein, clear the area of feathers around the ulnar-humeral joint using a cotton ball soaked in distilled water until the brachial vein is visible (avoid alcohol and hydrogen peroxide). Then, with a 23-25-gauge needle (internal diameter of about 0.3 mm) kept in orthogonal sense with respect to the vein, gently prick. Sometimes the blood flows poorly, because the needle tip was inserted too far into the vein or the needle bore size is inappropriate, causing blood to flow under the skin. Once the blood is flowing, remove the needle and use the syringe for collecting it. The next step (preparation of the blood smear) should be performed by another person, because blood immediately coagulates, but one has to be sure that bleeding stops. To facilitate the flow stop, press on the puncture site using cotton wool for half a minute. Allow the wing to fold naturally against the body, securing in that position to prevent flapping.

Preparing the smear. Put a drop of blood on the first slide and another drop in the second slide (Figure 11). The optimal slide has rounded corners, a frosted area where you can write a code, and no coverslip. Leave no more than a spot of blood of about 3 mm in diameter on the slide. As shown in the Figure 12, keep a third slide tilted and bring it near the drop of blood until it touches the slide and adheres to the

drop itself. Move the third slide so as to distribute the blood on the slide underneath. If the same side of the third slide is used for both replicates, it can be recycled (using the opposite side of it) for the next bird. Label the slides using a graphite pencil and avoid using a marker pen (its ink would be immediately removed by the following treatment). If the slides have no frosted area, write the code with a graphite pencil on a paper tape strip. Put carefully the slides in the slide box. Some slide boxes are easy to be accidentally opened during field operations. To keep the slides safe and avoid this inconvenient, use boxes equipped with a top-hinged lid with a clasp.

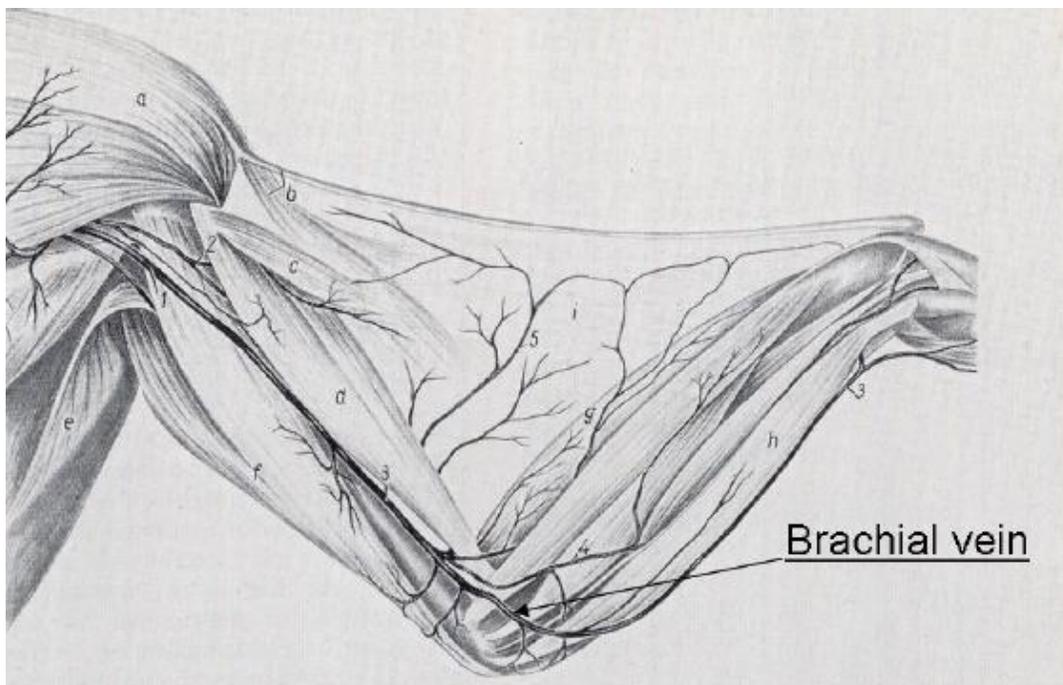


Figure 10 – Anatomy of a left wing indicating the brachial vein and the point of pricking (point of the arrow).



Figure 11 - Releasing of a blood drop on a glass slide for preparing a blood smear (photo: F. Borghesi)

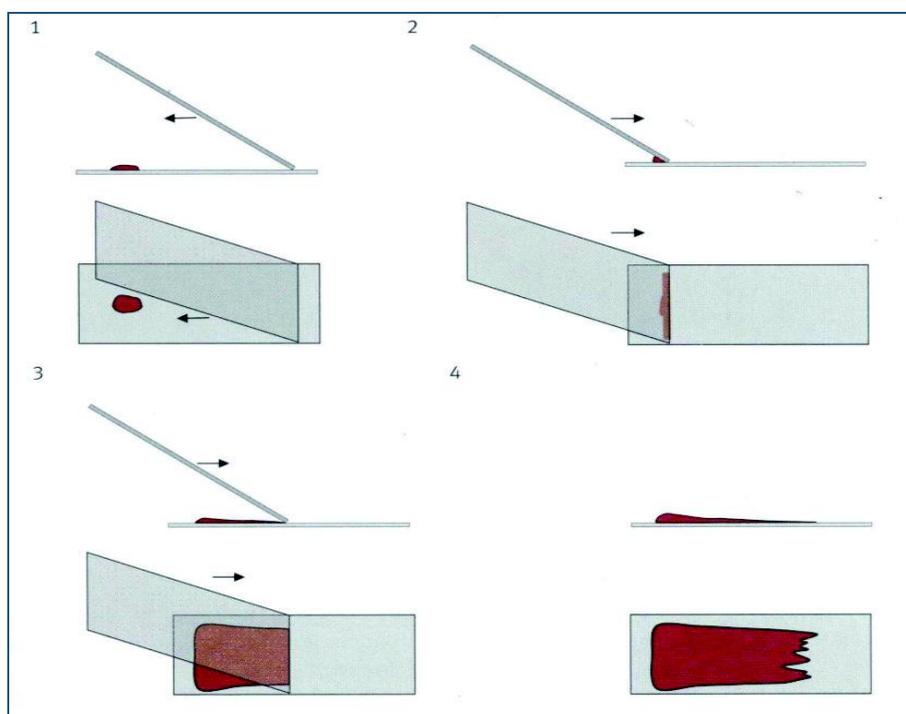


Figure 12 - Blood smear technique explained in 4 steps (from www.laboklin.de)

Avoiding contamination. As a blood smear will be analyzed visually, there is little risk to contaminate the samples. However, during field work, prevent soil dust from entering in the slide box or soiling the smear. It is important that the quantity of blood is not excessive, otherwise the analysis may result difficult or impossible.

After sampling. Slide fixing must be done after the slide is completely dry. Normally this can be done at the end of the day, or when back from field sampling. Immerse the slides in methanol for 10 minutes and let the slides dry to air. Buying pure methanol can be difficult and requires permissions, but also pure ethanol (not colored) can serve the purpose.

Sampling kit.

- | | |
|--------------------------------------|---|
| • Disposable gloves | • Insulin syringes (1 per sampled bird) |
| • Glass slides (2 per sampled bird) | • Slide box (25/50 slots depending on the case) |
| • Glass slides plastic staining rack | • Plastic box for immersion in Methanol/Ethanol |
| • Methanol or Ethanol | • Cotton wool |
| • Permanent marker and Pencil | • Paper tape |



4.4 PROTOCOL 3: COLLECTING UROPYGIAL OIL FOR PHTHALATE CONCENTRATIONS

The uropygial oil has been extensively used to assess lipophilic chemical concentrations (persistent organic pollutants) in bird corpses. In almost all works, the need of a considerable amount of oil required the preen gland dissection. On the contrary, this minimally intrusive protocol, set up by CSIRO Marine and Atmospheric Research, aims to collect a small quantity of oil from the uropygial gland of live birds (Figure 13), in order to detect phthalates. Phthalates are organic molecules that can be released by most plastics once ingested and then accumulated in fatty tissues.

Target contaminants. Phthalates released by plastics after ingestion.

Species. Theoretically all Mediterranean bird species, but this method has not widely tested.

Quantity. It is difficult to quantify the amount of oil to be collected. A small amount is required by this protocol. Refer to the sampling method described below.

Avoid contamination (before sampling): Due to the extensive presence of phthalates in many everyday items, this biomonitoring method requires extensive precautions to avoid contamination, especially before sampling. All glassware used in handling samples must be washed in laboratory before it is used for field operations. This implies that a link with the laboratory that will perform the analyses has to be established and the samplers equipped with containers and cotton wool swabs prepared by the laboratory, according to the methods of Hardesty *et al.* (2015).

Sampling. CSIRO protocol indicates two possible ways for live bird sampling. Here, the procedure adopted by Hardesty *et al.* (2015) is reported. Once the bird is kept in hand, gently massage the preen gland at the upper base of the tail. With bare hands, give a gentle squeeze after massaging the gland so that a small amount of oil can be obtained. The gland secretes a waxy substance, rather than a fluid oil as one could expect. Using a pair of metal tweezers that have not been in contact with plastic, remove the clean cotton wool from the glass jar. Gently massage the oil gland and wipe cotton wool over the gland 1-2 times to transfer the oil gland exudate to the cotton wool. Do this without touching latex gloves or other plastic items. Then, place the cotton wool back in a glass jar. Seal and label the jar.

Avoiding contamination (during sampling). Keep any plastic or latex objects away from the bird sampling area.

After sampling. Be careful with the glassware containing samples. To transfer samples from field to headquarters or laboratory, it may be useful to protect vials with packaging material, avoiding any plastic products even if vials are sealed. Use leather, corn or paper materials.



Figure 13 – The uropygial gland in a seabird (from CSIRO, 2013)

Sampling kit.

-
- Disposable gloves
 - Stainless steel tweezers
-
- Pre-cleaned cotton wool in Teflon-capped vials prepared according to Hardesty *et al.*, 2015
-
- Cotton wool
 - Permanent marker and Pencil
-



4.5 PROTOCOL 4: COLLECTING EXCREMENTS FOR PORPHYRIN CHARACTERIZATION

Bird excrements can be used for assessing both metal and organic pollutant concentrations. However, neither old faeces (guano), nor fresh faeces occasionally ejected during bird manipulation should be used for assessing environmental pollutants. Potential for contamination of faeces so collected is large and specific precautions are necessary. In fact, collecting faeces generally requires the individuals defecate (it may take minutes) on a clean object that will be protected from any contact with hands, soil, dirty tools. This kind of sampling is not described here. Instead, porphyrins are not present in the environment and even dry faeces can be used.

Targets. Porphyrins and related metabolites.

Species. All Mediterranean seabird species.

Sampling. Put dry or almost dry faeces (e.g. those present in the nest cup) in an Eppendorf or in a piece of aluminum foil, which will be closed as an envelope. Use a teaspoon or spatula (about 1 g is needed). Fresh faeces ejected during bird handling can be collected too, and put in a piece of aluminum foil, which will be closed as an envelope. Use paper tape and graphite pencil to label.

Avoiding contamination. Risk of contamination is little, however, avoid to put samples in contact with blood and do not mix excrement samples from different nests, or sites.

After sampling. As soon as possible (within 24-48 hours) freeze samples at -20 °C. Brief periods outside freezer are not a problem, for example while transferring samples from one site to another or to the laboratory.

Sampling kit.

- | | |
|-------------------------------|---------------------------------------|
| • Disposable gloves | • 1,5 ml Eppendorf tubes (1 per nest) |
| • Aluminum foil | • Stainless steel spatula |
| • Permanent marker and pencil | • Paper tape |



4.6 PROTOCOL 5: COLLECTING EGGS FOR TRACE ELEMENT AND POPS

It is important to have an unbiased sample set, and randomize sampling in order to apply statistics and have significant results. For this reason, most studies are carried out on whole, freshly laid eggs (by taking one egg per nest). The protocol suggested here regards unhatched eggs, and therefore it is thought not only for birds laying multi-egg clutches (e.g. gulls and cormorants), but also for shearwaters and petrels which lay single egg.

Targets: trace elements, persistent organic pollutants.

Species. All Mediterranean seabird species.

Sampling. Collect only deserted or addled eggs avoiding slightly cracked eggs, because they will easily collapse. If only the contents will be analyzed, a graphite pencil should be used to write information on both the eggshell and the container. Egg paper cartons are suitable containers for transportation but they are not resistant to crush (for example into the backpack) or wetness. To keep eggs safe, portable plastic cartons are a better solution. Record the nest contents: viable and addled eggs, nestlings (and their estimated age) in order to estimate the laying date.

After sampling. Do not freeze the eggs because they will crack, but keep them cool and process as soon as possible. Before opening, measure the length and width. Open the eggs along the equator and put the contents into a container, thoroughly washed with deionized water. Record the weight and homogenize the egg contents. The presence of an embryo and its estimated age should also be recorded and separated from the rest of the egg contents prior to homogenization. Finally, put the egg contents and the embryo at -20°C until analysis is done. If information on the eggshell are required, wash it thoroughly with tap water and then rinse it with deionized water (if chemical analyses are to be carried out). Then dry the eggshell at room temperature. Regularly check the weight and stop drying when the weight become constant (record initial and final weight). When dry, measure eggshell thickness at equator using a 0.1 mm caliper.

Avoid contamination. Contamination can occur if the container is dirty, inadequate or not sealed. If monitoring includes metals, use plastic containers, while a glass container (with Teflon cap) should be

used if organic contaminants are the target. The same strategy should be adopted for the tools used for opening, mixing or separating parts.

Sampling kit.

-
- Disposable gloves
 - Portable plastic egg cartons
 - Pencil
 - Manual hand mixer
 - Aluminum or plastic box (depending on the target contaminant) to freeze samples
 - Caliper with 0.1 mm graduation
-
- Portable plastic egg cartons
 - Glass beaker
 - Weighing scale with 0.1 g graduation
-
- Aluminum or plastic box (depending on the target contaminant) to freeze samples
-
- Caliper with 0.1 mm graduation
-



4.7 PROTOCOL 6: COLLECTING PLASTICS FOR ASSESSING MACROPLASTICS ABUNDANCE

Initially, the assessment of exposure to plastic pollution in seabirds has focused on dissecting the digestive tract in order to count and classify plastic items. This practice is still widely applied on stranded seabirds, especially in ongoing long-term projects in the North Sea and Canada (van Franeker *et al.*, 2011; Provencher *et al.*, 2015). In the present proposal, however, only sampling on live birds has been included, with paragraph 4.4 (protocol 3) 'Collecting uropygial oil' for chemicals released by plastics and, here, with the assessment of plastic items incorporated in nests, according to the method outlined for European shags in Brittany in 2010, and later extended to Corsican colonies by Cadiou (2013). It has to be considered that studies on plastics in seabirds have been increasing in number and this issue is likely the best candidate for new discoveries and modification of existing methods.

Targets. Frequency and abundance of macroplastics in the marine environment.

Species. Theoretically all Mediterranean species that built a nest with materials collected at sea notably gulls and cormorants. However, only shags have been tested for this protocol in Corsica (Fleuriau and Faggio, 2014; Cadiou and Fortin, 2015).

Visually assessing. Count the number of macroplastic items in the nest without further specific research into the cup of the nest. Only information on the quantity of the items has to be reported. Should an entangled bird be found on a nest, record the age, the kind of item, and the entangled limb. Observations should be limited to the incubation phase, because after hatching the nests are often damaged or even destroyed. This implies that the survey must be carried out quickly, avoiding to expose eggs to predation or overheating. Unfortunately, the type of monitoring can vary among colonies, depending on geomorphology and local conditions (distance of the colony from the observer, density of nests, etc.), hence, it is crucial for the observers to get a perfect understanding of the kind of results expected. For Shags, following Cadiou (2013), classify nests according to the following categories: having none, 1-5, 6-10, 11-20, or more than 20 visible plastic items.

Sampling (after breeding). After fledglings have left the nest, sampling of macroplastics can be carried out in order to examine the plastic items more in detail. The collected items from each sampled nest must be put in a single plastic bag and all bags put in a larger bag, labelled with site and date of sampling.

After sampling. The collected debris must be washed under tap water and classified according to OSPAR (2010) which provides a comprehensive description of plastic items from beach surveys. The aim of the classification is to statistically infer the relative importance of the origin (fishery, common users, industry, etc.).

Avoid contamination. No risk of contamination exists. However, there is risk to be injured by handling debris. Be careful and use protective gloves.

Sampling kit.

-
- | | |
|------------------------------------|-----------------------------------|
| • Binoculars 10 x 42 | • Permanent marker and pencil |
| • Cut resistant gloves | • Small rubbish bags (1 per nest) |
| • Big rubbish bags (some per site) | • Paper tape |
-



5 FINAL REMARKS

The present report is aimed at providing an overview for orientation into the vast and heterogeneous field of contamination monitoring in seabirds. It has to be stressed that not all aspects were addressed with the same level of detail, and already from the plain reporting of the different techniques and goals a particular focus on metals (especially mercury) and persistent organic pollutants, rather than for instance plastics or oil spills, is apparent. Planning the first steps of a future operative strategy or simply providing an answer to the 'what next?' question shouldn't reasonably change this initial heading. Our recommendation, therefore, is to keep on this track and, as a first target, promote the full use of the present exercise. This might steadily imply that the following actions are undertaken. These are listed according to decreasing priority and wise use of resources that have already been invested:

- Completing the analyses of all the samples that have already been collected, in order to achieve representative numbers and confirm the existence of differences between species/sites, that are the subject of annexes A and B of this document.
- Once the step above is reached, aim at a temporal target, i.e. adopt the same sampling protocol in the same study areas and species, in order to cover different years and establish if/how the analyses' outcomes vary in time. Five years might be a reasonable start for building a time series. This action could have a direct link to the 'Sentinel islands' project of PIM, and meet the targets requested – to European countries at least – by the EU Marine Strategy Framework Directive. For the latter aspect, the initiative should be presented to the ministries that are involved.
- Investigating the causes of local differences in contamination, e.g. assessing differences in seabirds diet and promoting local studies focused on the contamination of the food web and identification of pollution sources.
- Improving the geographical coverage e.g. sampling at other colonies/islands, and assessing the breeding performances at each of them. As a matter of fact, while there is little doubt that seabirds are good indicators of contamination in the environment, the effect of contaminants on bird populations has not been adequately investigated in the Mediterranean.

Adherence to a recommended protocol (and targets) should not, of course, mean being blind to other subjects, especially new ones. Whereas for oil spills a Mediterranean strategy is developing from other channels (Camphuysen et al., 2007; POSOW, 2013), plastic contamination is a theme that is well worth being developed. The Australian protocol for phthalate monitoring from the preen gland of live bird (see protocol no. 3, page 58.), as well as a profitable use of the corpses of selected species, obtained from ad hoc searches, are the best candidate for future enforcement in the Mediterranean area.

When biomonitoring contaminants, it's important to identify sources and polluted areas, in order to take proper actions. Though seabirds can travel across the Mediterranean (and even reach other seas) through their life, during the breeding season they have restricted foraging areas that are regularly used and from which they depend, as demonstrated by recent studies based on telemetry. Moreover, breeding seabirds are forced to stay within a given radius from their colony because travelling further is not practicable for energetic reasons. This behaviour can profitably be used by sampling on chicks, which can provide information on the geographical areas regularly frequented by their parents to feed, with little risk of reflecting pollution sources that are beyond them.

No less important than the points mentioned above, is considering the economic aspects, when long term campaigns are planned. Investigating the effects of bioaccumulable pollutants on top predators like Procellariidae is definitely cheaper than affording broader studies based on many sites, organisms, and environmental components. Indeed, seabirds, from their high position in the marine food web: 1) do the sampling for us across a marine sector, and concentrate it into their offspring; 2) amplify the pollution carried by their prey by bioaccumulating contaminants in their tissues, so revealing problems earlier than lower-ranking species; 3) are, in many colonies, already a subject of long-term studies (such as censuses) which may represent an opportunity to join competences and resources for multiple targets (limiting, at the same time, disturbance if the activities are properly coordinated).

In conclusion, the analysis of chemical pollutant concentrations or their biological effects on seabirds could provide an interesting tool to compare pollution levels between different marine areas. This work, however, is focused on the sampling phase. It has to be stressed once again that most laboratory analyses are quite expensive. Full costs should be properly evaluated at the moment when targets and methods of a biomonitoring project are defined.

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Photo: F. Borghesi

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Photo: F. Borghesi

ANNEX A – PRELIMINARY RESULTS FROM SAMPLES TAKEN IN 2015

TECHNICAL REPORT

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Preliminary analysis of mercury in feather's samples and analysis of frequency of abnormalities in peripheral erythrocytes in blood samples from three different species of seabirds sampled in different areas of the Mediterranean Sea.

1. INTRODUCTION

Seabirds are impacted by human-related threats including fisheries interactions, habitat destruction and pollution including oil spills. Some contaminants tend to bioaccumulate and biomagnify throughout marine food webs due to their properties. Marine organisms at high trophic positions are those at greater risk, because these compounds can have noxious effects. Sea birds have been proposed as useful bioindicators for contamination, mainly because many of them are top predators, breed at specific place and can integrate contaminants levels of their feeding areas (Burger and Gochfeld, 2004). It is possible to evaluate contaminant levels and biological responses from noninvasive biological material, such as blood, feather and excreta. The blood is the vehicle for transportation and distribution of pollutants following uptake: this makes blood suitable for screening contaminants in vertebrates. Some pollutants, such as mercury or polycyclic aromatic hydrocarbons, show certain bioaccumulation in blood, faithfully reflecting the environmental concentrations (Pérez et al., 2008; Mieiro et al., 2009). Frequently, the external concentrations of these and other pollutants are positively related to levels in blood. As blood is involved in the inter-tissue redistribution of contaminants, it can be particularly recommendable for monitoring pollution events in species with high mobility or in migratory stages (Pérez et al., 2008; Roscales et al., 2010). Analysis of frequency of abnormalities in peripheral erythrocytes in blood is a useful biomarker that allows to evaluate the impact of genotoxic compounds such as Hg, OCs, PAHs, dioxins and flame retardants in organisms.

Mercury concentrations in feathers reflect circulating concentrations in the body at the time of their formation, with a direct relation to mercury levels in the feeding areas that are used by moulting adult seabirds. In chicks, Hg concentration reflects the contamination level in the adults' feeding areas that surround the breeding sites (Monteiro and Furness 1997).

The aim of this preliminary analysis is to validate a common protocol based on a noninvasive sampling methodology and confirm the sensitivity of the analytical methods used for this monitoring effort. These were

the analysis of mercury in feathers, through spectrophotometric analysis, and the biological responses (biomarkers) by counting the frequency of abnormalities in peripheral erythrocytes of blood samples. The protocol was applied to Audouin's gull (*Ichthyaetus audouinii*) Yelkouan shearwaters (*Puffinus yelkouan*) and Scopoli's shearwaters (*Calonectris diomedea*) sampled in different areas of the Mediterranean Sea.

2. MATERIALS AND METHODS

2.1 SAMPLING

Sampling was carried out in the month of June 2015 in two countries placed in a central position within the Mediterranean Sea: Tunisia and Italy (see Annex B). In Tunisia, sampling of the three species was carried out in Zembra archipelago. In Italy, samples were taken in four areas: South Sardinia (all three species), North Sardinia (yelkouan and scopoli's shearwaters), Tuscan Archipelago (yelkouan shearwaters) and the gulf of Naples (audouin's gull). Three different areas were investigated in N Sardinia: the islands of Tavolara, Soffi and Barrettini; in Sardinia two areas were investigated: Nora lagoon and Cavoli island. Vivara islet near Procida was the sampling site on the gulf of Naples and Montecristo was the sampling site of yelkouan shearwater in Tuscany (presently not examined). Only adult birds were sampled for scopoli's shearwaters, and only fledglings for yelkouan shearwater and audouin's gull.

2.2 MERCURY IN FEATHERS

The samples of down and feathers of three species were subjected to compressed air jet and jet of deionized water in order to remove the coarse particulate which may be present. Following to completely remove the deionized water, the samples were frozen at -80 ° C and subjected to freeze-drying process. Aliquots of approximately 0.100 g of lyophilisate were subjected to acid digestion with HNO₃ and H₂O₂ in 4:1 ratio in a system in reaction (teflon bomb). The analytical determinations of mercury were carried out by means of atomic absorption spectrometry using the technique of cold vapors (Perkin Elmer mod. 400 FIMS). The statistical analysis of data (Mann Whitney U test and Kruskal-Wallis test) was performed using the software StatSoft, Inc. (2008), STATISTICA (data analysis software system), version 8.0.

2.3 BIOMARKER ANALYSIS: *Erythrocytic Nuclear Abnormalities (ENA) assay*

The smears were stained with 5% Giemsa solution in Sorensen buffer for 30 min and air-dried. The stained slides were analyzed using an Olympus BX41 microscope at final magnification of 1000x. The erythrocytic nuclear abnormalities were stored in 1000 mature erythrocytes for sample, according the criteria adopted by Schmid (1976), Carrasco et al. (1990), and Smith (1990), adapted by Pacheco and Santos (1997). Micronuclei were identified according to the following criteria: (1) round and ovoid-shaped no-refractory particles in the cytoplasm, (2) colour and structure similar to chromatin, (3) diameter of 1/3-1/20 of the main nucleus, (4)

particles completely separated from the main nucleus (Heddle et al., 1991). The morphology of the other nuclear abnormalities studied are showed in figure 1A.

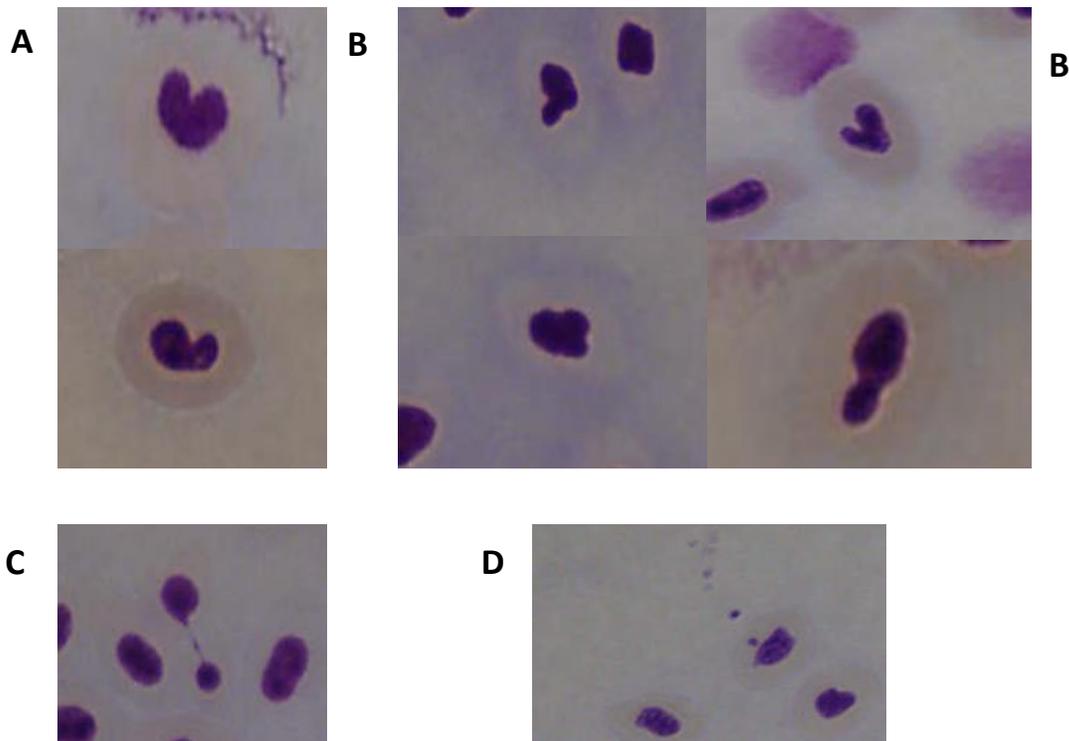


Figure 1A. Nuclear abnormalities in peripheral blood: A) erythrocyte with kidney, B) nuclear bud, C) fragmented erythrocyte, D) erythrocyte with micronucleus.

3. PRELIMINARY RESULTS

3.1 MERCURY ANALYSIS

COMPARISON BETWEEN SPECIES. Figure 2 shows the levels of mercury detected in the chick down of the yelkouan shearwater and in adult feathers of audouin's gull and scopoli's shearwater sampled in Tunisia. The levels of mercury detected in the down of the gull chicks were significantly higher than those in the yelkouan shearwater ($H = 7.75$; $p < 0.05$) (Figure 2). The levels determined in the adult's feathers of scopoli's shearwater sampled in Tunisia show intermediate than in the down of the yelkouan shearwater and audouin's gull (Figure 2A), while in Italy they are comparable with those of the gull chicks although with a higher variability (Figure 3A).

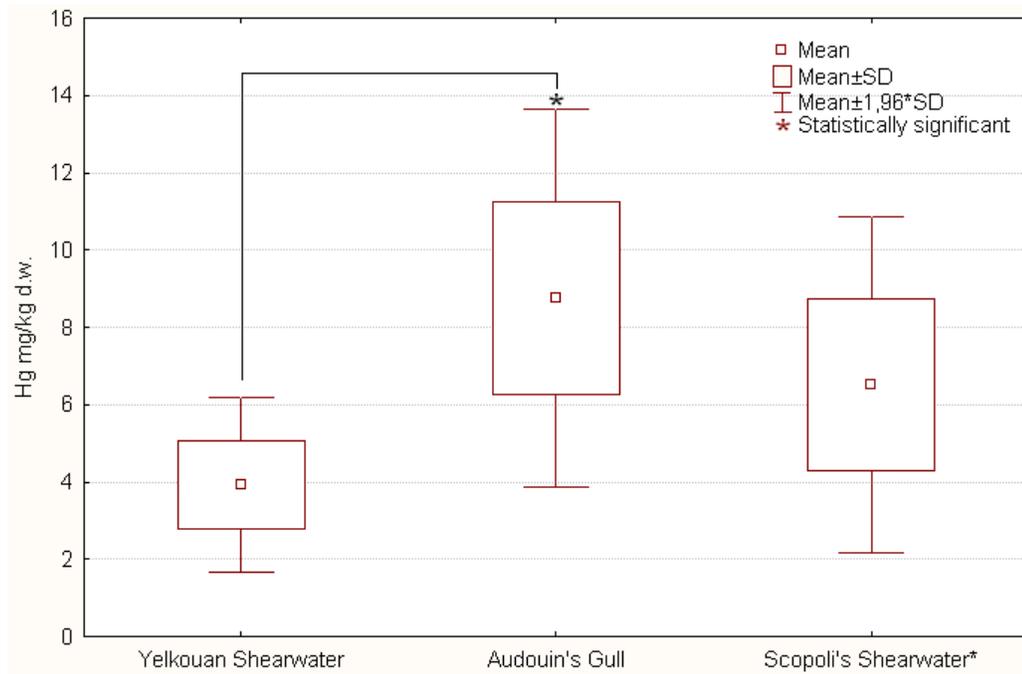


Figure 2A. Mercury levels (mg/kg dry matter) in down of yelkouan shearwater (n = 9), audouin's gull (n = 3) and in feathers scopoli's shearwater (n = 3) sampled in Tunisia. (* feathers of adults).

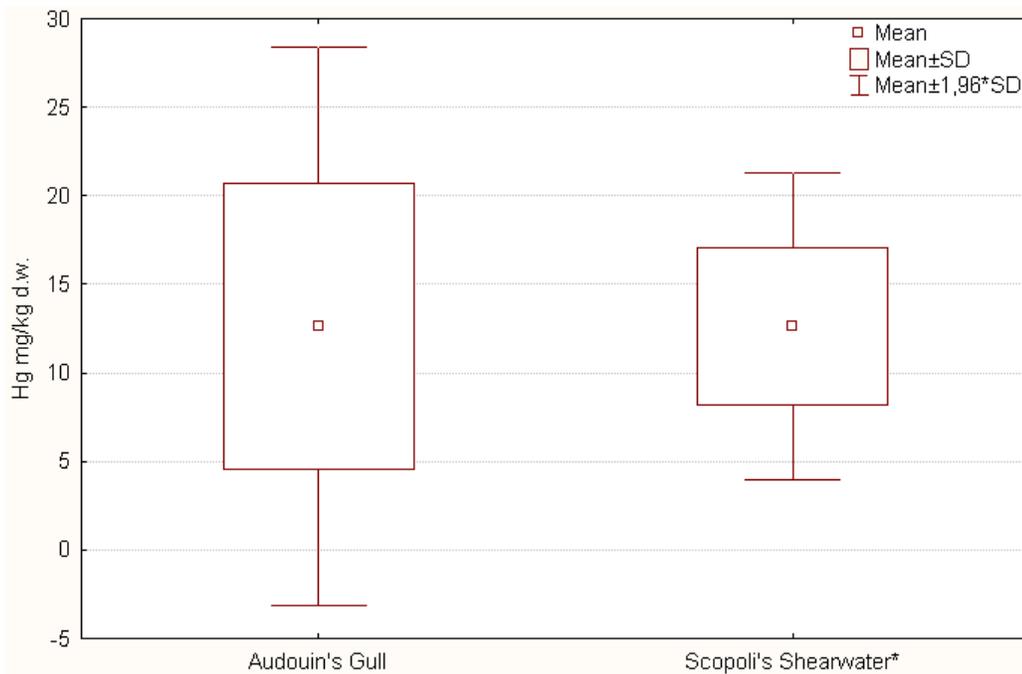


Figure 3A. Mercury levels (mg/kg dry matter) in down of audouin's gull (n = 3) and feathers of scopoli's shearwater (n = 3) sampled in Italy. (* feathers of adults).

COMPARISON BETWEEN SAMPLING MACRO AREAS (ITALY-TUNISIA). The levels of mercury detected in scopoli's shearwater sampled in Italy were significantly higher than those sampled in Tunisia ($U=0$; $p<0.05$) (Figure 4A). In audouin's gull the two areas did not differ significantly even if the levels of the individuals sampled in Italy showed higher average levels with concentrations reaching 21.09 mg/kg dry in the sample from Procida, (Figure 5A).

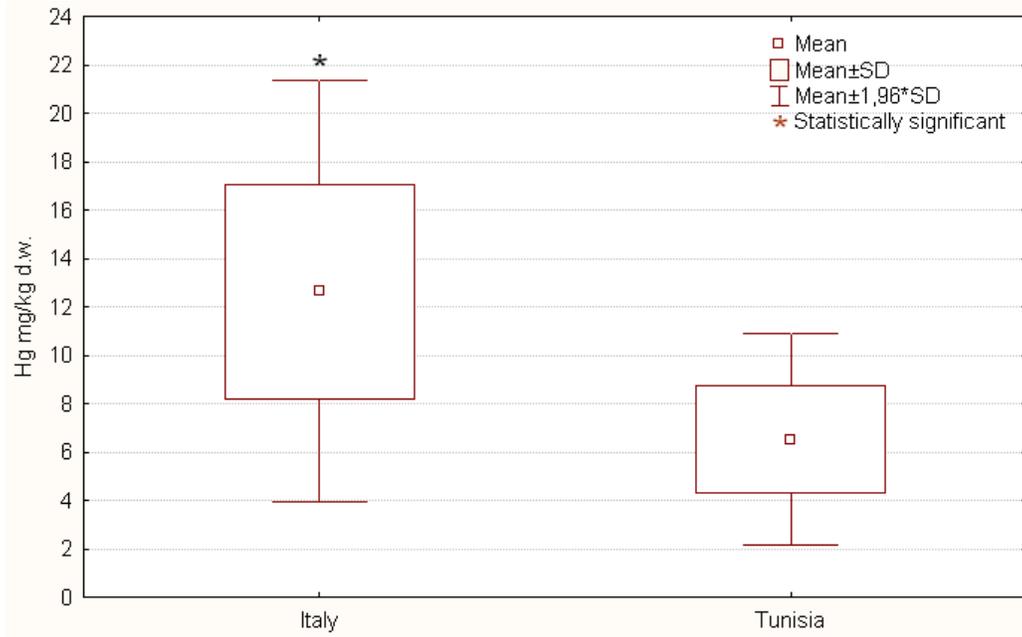


Figure 4A. Mercury levels (mg/kg dry matter) in feathers of scopoli's shearwater sampled in Italy (n=3) and Tunisia (n=3).

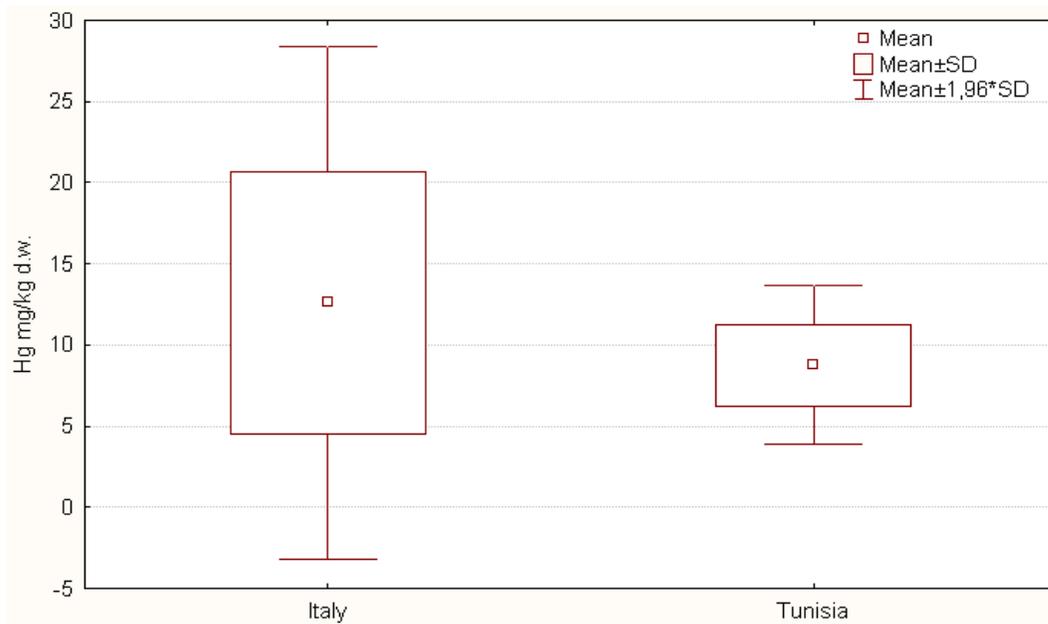


Figure 5A. Mercury levels (mg/kg dry matter) in feathers of audouin's gull (n=3) sampled in Italy and Tunisia.

COMPARISON WITH THE LITERATURE. Studies on mercury in the chick down of yelkouan shearwater are missing in the literature, however the levels determined in this preliminary study on individuals from the Tunisian coast (Figure 1) are comparable with those found by Bourgeois et al., (2011) in the adult's feathers of yelkouan shearwater (2.414 ± 1.068 mg/kg, range from 0,615 to 4,832) in the Hyères archipelago (France) and with those found by Watanuki et al., (2015) in the primaries of congeneric *Puffinus tenuirostris* (2.500 ± 1.400 mg/kg). The concentrations determined in the primary feathers of scopoli's shearwater from the Tunisian coast (Figure 3) are comparable with those observed by Ramos et al., (2009) in individuals of the same species (or of closely related *Calonectris borealis/edwardsi*) from different islands of Mediterranean Sea and Atlantic Ocean, with a range from a minimum of about 2.5 mg/kg in Cape Verde to a maximum of about 6.3 mg/kg in the Chafarinas islands. Instead, the levels measured in Italy in the present study are twice as high. Regarding the Audouin's gull, mercury levels in the down are comparable to levels found in the feathers of adults by Sanpera et al. (2007) in the Ebro Delta (17.88 ± 6.488 mg/kg) and in the Chafarinas islands ($20,798 \pm 7,585$ mg/kg), while they are much higher than those reported by Goutner et al. (2000) for juvenile feathers from the colonies of Paros and Lipsos islands in Greece.

3.2 BIOMARKERS ANALYSIS: ENA ASSAY

COMPARISON BETWEEN SPECIES AND AREAS. The values of frequency of abnormalities in peripheral erythrocytes evaluated in young yelkouan shearwaters from Tunisia are higher than those determined in the blood of audouin's gull and scopoli's shearwaters in the same area. Conversely, in Italy the frequency values of total anomalies are higher in the audouin's gull than in the other two investigated species (Figures 6A-10A).

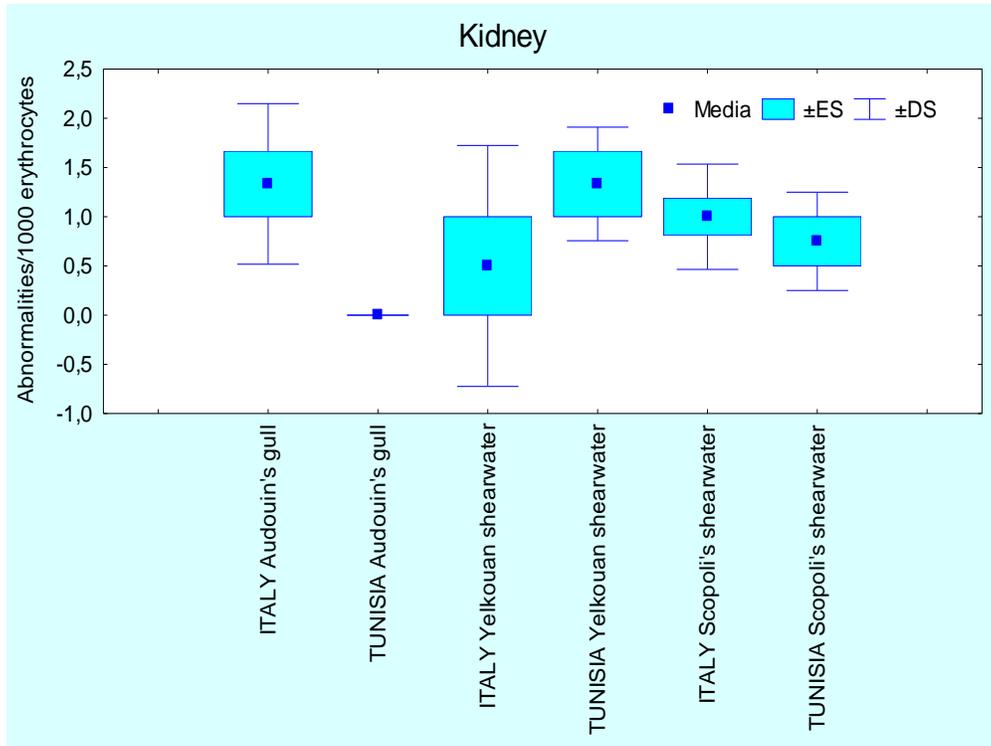


Figure 6A. Frequencies of kidney cells (mean, deviation standard and error standard) in peripheral blood of three different species of birds collected Italy and Tunisia.

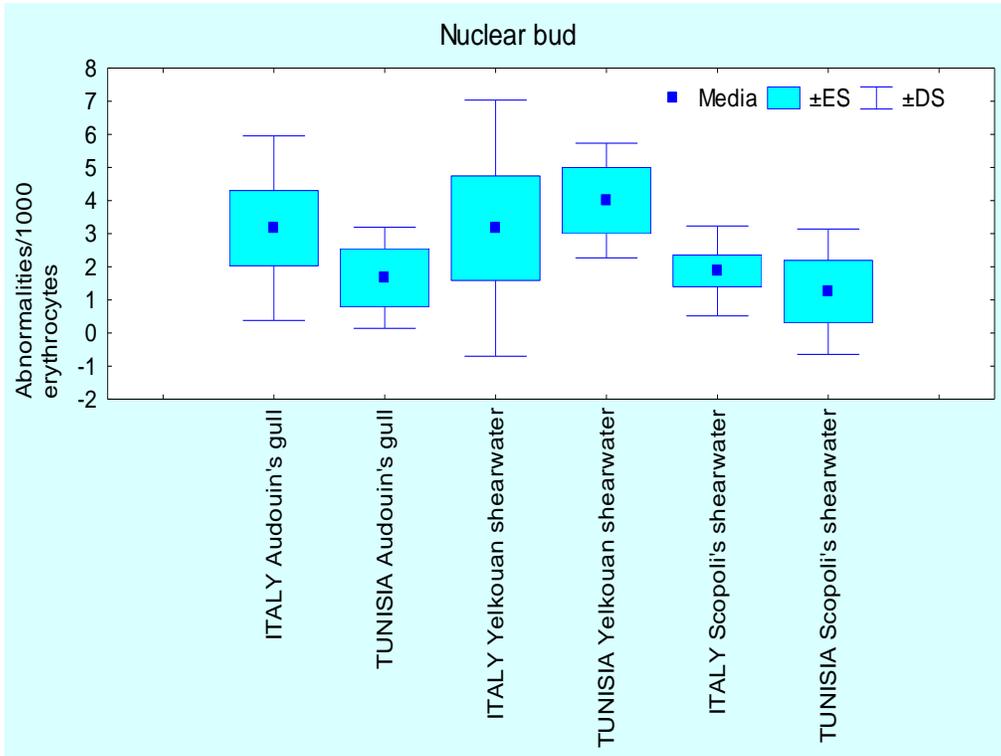


Figure 7A. Frequencies of nuclear bud cells (mean, deviation standard and error standard) in peripheral blood of three different species of birds collected from Italy and Tunisia.

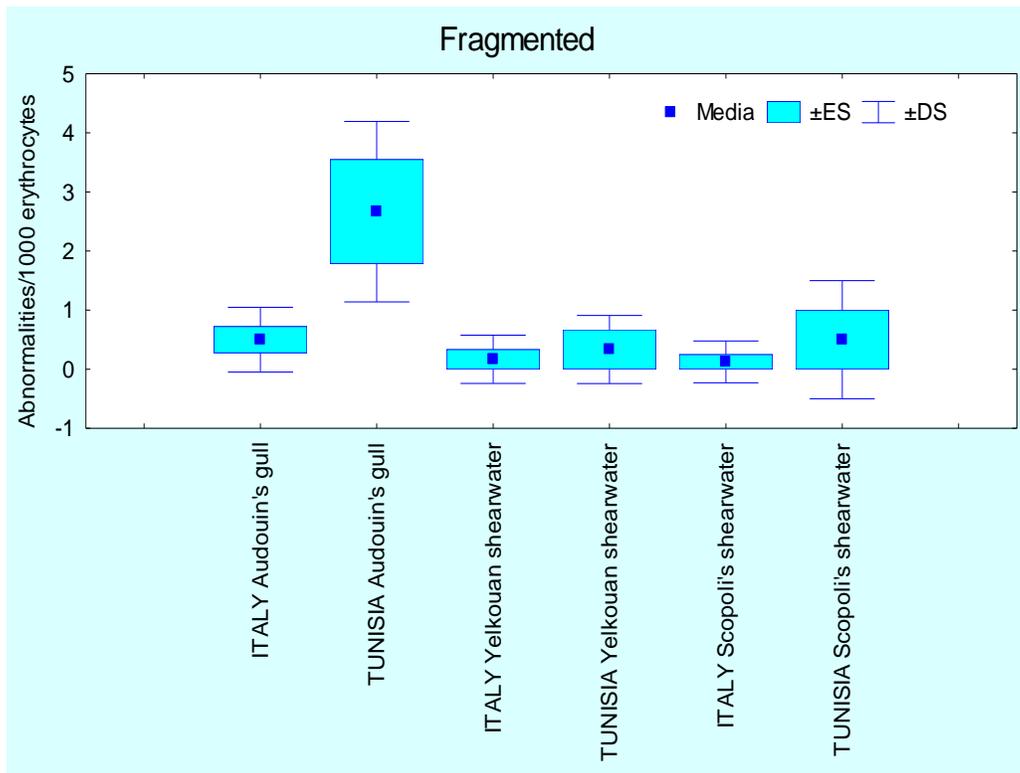


Figure 8A. Frequencies of fragmented cells (mean, deviation standard and error standard) in peripheral blood of three different species of birds collected from Italy and Tunisia.

Figure 9A describes the frequencies of micronuclei measured in Italy and in Tunisia in the three different species. In audouin’s gull and yelkouan shearwater no micronuclei were found. In Scopoli’s shearwater the mean frequency of micronuclei was 0.13 in Italy and 0.25 in Tunisia. No statistically differences were found between the tested groups. The presence of micronuclei only in scopoli could be due to the fact that adults were sampled in this species, while Audouin’s gulls and Yelkouan shearwaters were chicks. Another possibility is that differences between species may be caused by differences in the efficiency of the elimination of micronucleated erythrocytes.

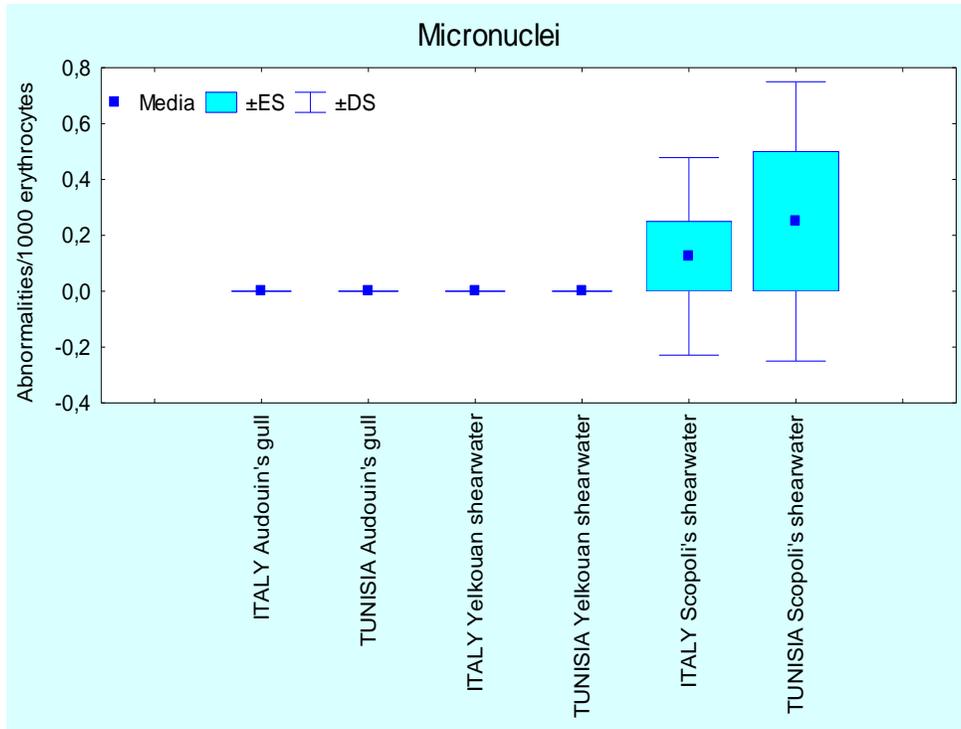


Figure 9A. Frequencies of micronuclei cells (mean, deviation standard and error standard) in peripheral blood of three different species of birds collected from Italy and Tunisia.

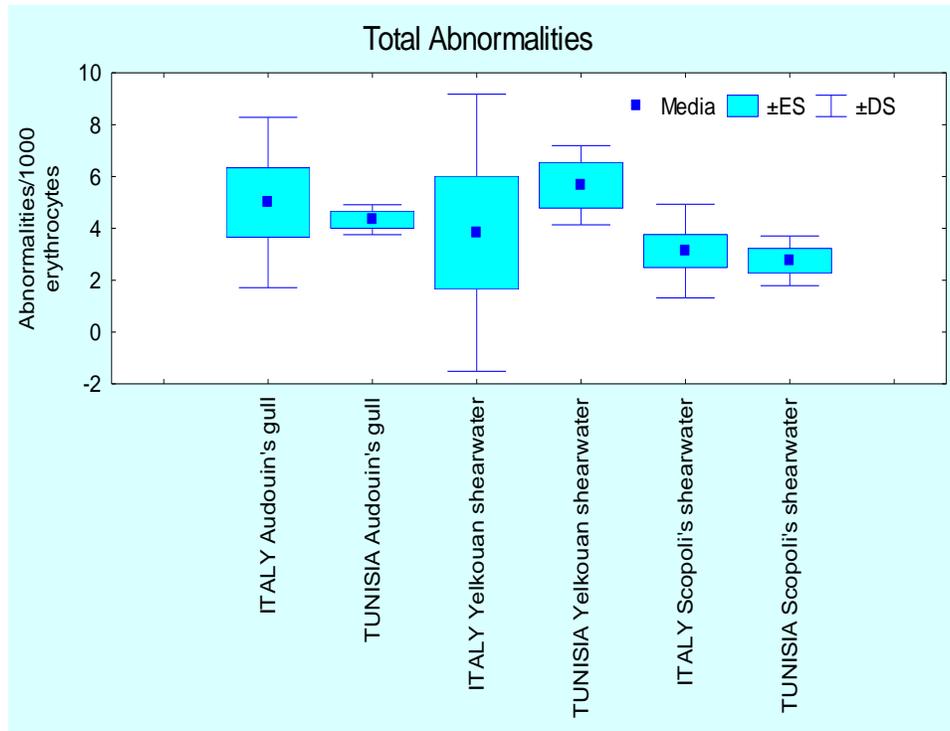


Figure 10A. Frequencies of total abnormalities (mean, deviation standard and error standard) in peripheral blood of three different species of birds collected from Italy and Tunisia.

Tables 1A, 2A and 3A and Figures 11A, 12A and 13A show the main results related to the frequency (%) of the four different types of nuclear abnormalities (kidney, lobed, segmented and micronuclei) observed in erythrocytes of the three species investigated. The tables also indicate, for each species, the total frequency of erythrocyte anomalies.

Table 1. Mean frequencies of kidney, nuclear bud, fragmented micronuclei and total abnormalities in peripheral blood of yelkouan shearwater collected from different sampling areas in Italy (Tavolara in Sardinia north and Cavoli in Sardinia south) and in Tunisia (Zembra island).

	<i>Kidney</i>	<i>Nuclear buds</i>	<i>Fragmented</i>	<i>Micronucleo</i>	<i>Total abnormalities</i>
Tavolara	1	5	0.33	0	6.3
Cavoli	0	1.3	0	0	1.3
Zembra	1.3	4	0.33	0	5.66

No statistically differences were found in specimens collected in Tavolara and Cavoli in comparison with the specimens sampled in Zembra island.

Yelkouan shearwaters. Table 1 shows the results on the frequency abnormalities observed in erythrocytes of yelkouan shearwaters sampled at Tavolara, Cavoli and Zembra. The lowest values for all kinds of abnormalities were found in individuals sampled at Cavoli, as shown in Figure 11A.

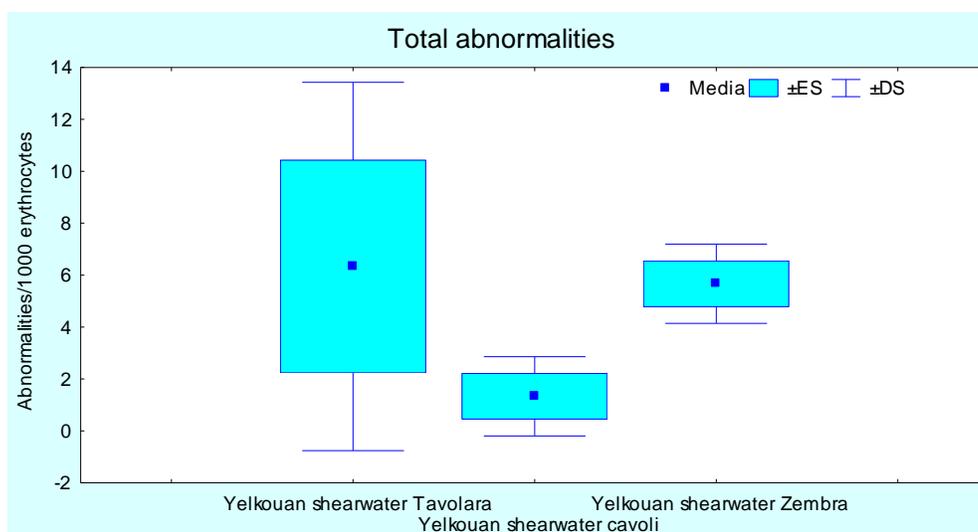


Figure 11A. Frequencies of total abnormalities (mean, deviation standard and error standard) in peripheral blood of Yelkouan shearwater collected from different sampling areas in Italy (Tavolara in Sardinia north and Cavoli in Sardinia south) and in Tunisia (Zembra island).

Scopoli’s shearwaters. Table 2A shows the results on the frequency of abnormalities observed in erythrocytes of scopoli’s shearwaters sampled at Barrettini, Cavoli, Soffi and Zembra. Here the lowest values for all kinds of abnormalities were measured in the specimens sampled at Soffi and the highest of all are those from Cavoli, as can be clearly seen in Figure 12A.

Table 2A. Mean frequencies of kidney, nuclear bud, fragmented micronuclei and total abnormalities in peripheral blood of Scopoli’s shearwater collected from different sampling areas in Italy (Tavolara in Sardinia north and Cavoli in Sardinia south) and in Tunisia (Zembra island).

	<i>Kidney</i>	<i>Nuclear buds</i>	<i>Fragmented</i>	<i>Micronucleo</i>	<i>Total abnormalities</i>
Barrettini	1	1.3	0.33	0	2.66
Cavoli	1	3	0	0.33	4.33
Soffi	1	1	0	0	2
Zembra	0.75	1.25	0.5	0.25	2.75

No statistically significant differences were found in specimens collected in Barrettini, Cavoli and Soffi in comparison with the specimens sampled in Zembra island.

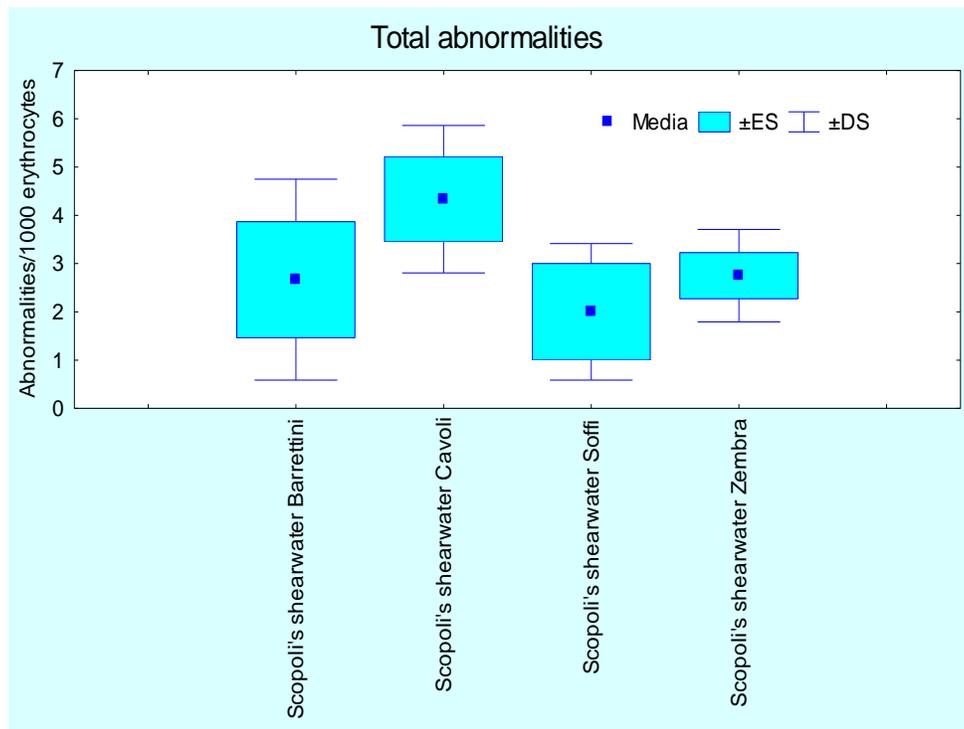


Figure 12A. Frequencies of total abnormalities (mean, deviation standard and error standard) in peripheral blood of Scopoli’s shearwater collected from different sampling areas in Italy (Cavoli in Sardinia south; Soffi and Barrettini in Sardinia north) and in Tunisia (Zembra island).

Table 3A. Mean frequencies of kidney, nuclear bud, fragmented micronuclei and total abnormalities in peripheral blood of Audouin’s gull collected from different sampling areas in Italy (Tavolara in Sardinia north and Cavoli in Sardinia south) and in Tunisia (Zembra island).

	<i>Kidney</i>	<i>Nuclear buds</i>	<i>Fragmented</i>	<i>Micronucleo</i>	<i>Total abnormalities</i>
Procida	1.66	4.66	0.33	0	6.66
Nora	1	1.66	0.66	0	3.33
Zembra	0	1.66	2.66	0	4.33

No statistically differences were found in specimens collected in Procida and Nora in comparison with the specimens sampled in Zembra island.

Audouin's gull. Table 3A shows the results on the mean frequency of abnormalities observed in erythrocytes of audouin’s gull sampled at Procida, Nora and Zembra. The results show that the lowest values for all kinds of abnormalities are those measured in the specimens sampled at Nora. The highest values of all are those measured in Procida, as it is possible to see in Figure 13A.

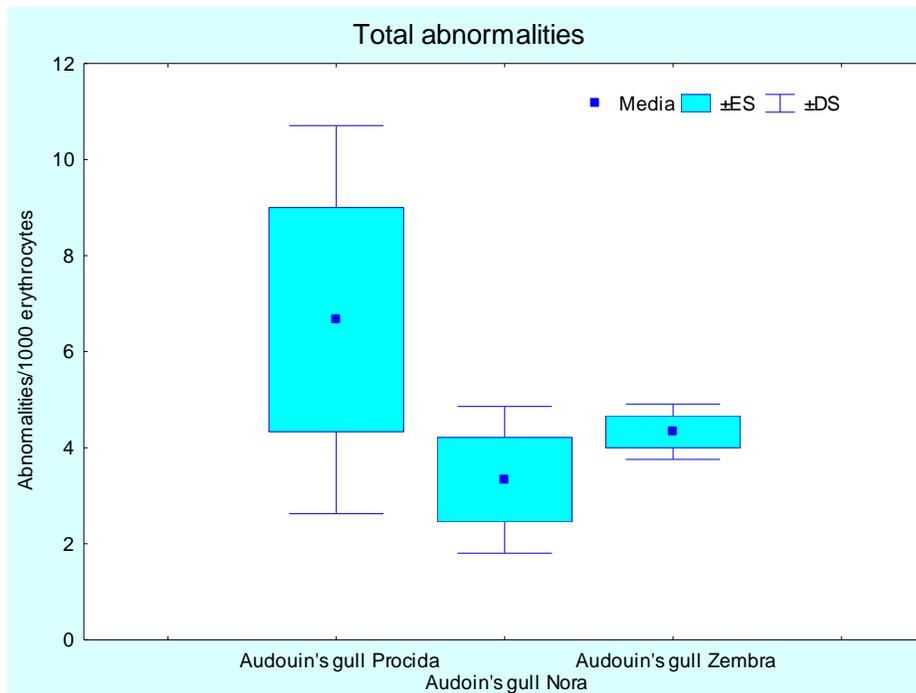


Figure 13. Frequencies of total abnormalities (mean, deviation standard and error standard) in peripheral blood of Audouin’s gull collected from different sampling areas in Italy (Nora in Sardinia and Procida in Naples) and in Tunisia (Zembra island).

COMPARISON WITH THE LITERATURE. To our knowledge, only a few studies on the frequency of micronucleated erythrocytes in birds and no study on the frequency of abnormalities of erythrocytes have been published so far. Skarphedinsdottir et al. (2010) has evaluated the presence of micronuclei in adult herring gulls (*Larus argentatus*), where the individual value of the frequency of micronucleated erythrocytes ranged from 0 to 0.8 ‰. Zúñiga-Gonzales et al. (2000; 2001) measured the frequency of micronucleated erythrocytes in 38 species of captive birds, where the means of the investigated groups ranged from 0 to 1.58‰. The frequency of abnormalities in all the species investigated in this study makes it difficult to assess whether they represent the natural background frequency in each species or they are an effect of exposure to genotoxic agents. However, if we compare the results with the range of the results obtained in other birds or classes of animals (reptiles, fish), we can hypothesize that the frequency of micronuclei and other abnormalities are low and could represent baseline values of the three species investigated.

4. FINAL REMARKS

These preliminary results offer just some indications on the state of the ecotoxicology of the three examined species. Further analyzes are needed to better understand the level of contamination and its effects on organisms. Because the analytical results of this study are very preliminary and hardly significant due to the small number of samples that could be analysed, we stress that it was for economic constraints that it has actually been possible to analyse only a small fraction of the available samples. Adequate resources should be available in future, realistically taking into account the laboratory costs. On the other hand, information was obtained that suggests that the protocol worked properly. Some comments and recommendations for future activities are presented here.

4.1 Amount of collected feathers

The amount of feathers collected for this study proved to be suitable for the spectrophotometric analysis. For the chick down, the amount taken was even excessive for the methodology of mineralization used, as 100 mg would be sufficient. Most of the samples had a weight of 200-300 mg (corresponding to 1-2 Eppendorf tubes stuffed with down material).

4.2 Amount of blood

The amount of blood smeared on slides during the sampling was found to be excessive in some samples. In order to avoid this problem a greater pressure has to be made on the slide which is used to perform the smear. For some samples, only a single smear was taken instead of two as per protocol. Placing a coverslip on the smear was wrong too. However, analysis at the optical microscope allowed the calculation of the frequency of erythrocytic abnormalities in all the samples.

Because most critical points have been found in blood smears, we feel it's important to recall here some points. A smear should be prepared immediately after blood collection to maintain the highest level of cellular integrity. The prolonged exposure to any anticoagulant may cause artefactual morphological changes in blood cells. Some simple steps to maximise the quality of blood smears are detailed below:

1. Homogenise the blood sample. In a syringe (in most birds bleeding could be carried out using a 17-29 mm needle length and an insulin syringe), blood can also be mixed by applying a slow and very gentle up and down pressure to the syringe plunger. Before taking the smear, eliminate the needle to avoid breaking the cells.
2. Blood smear. If a large blood volume is available, discard the first 3–5 drops and pick the next drop up on one end of a clean glass slide. This drop should have less than 5 mm in diameter. If the blood volume is small, the first drop of blood should be wiped away at least to ensure data quality.

It is important to prepare two blood smears for each animal and not applying any slide cover to the slide. In the preparation of a blood smear, researchers can use as guidance the videos uploaded on YouTube by several research centres (e.g. <https://www.youtube.com/watch?v=4NkgEjPKzBA>).

3. Drying. The blood smear should be air-dried as quickly as possible after preparation. Aggressive procedures such as direct heating (e.g. hair drier) or direct sunlight must be avoided since these can distort blood cell morphology.
4. Verifying the quality of the blood smear. A good-quality blood smear is essential to obtain accurate data. At first sight, a well-prepared blood smear should occupy approximately three quarters of the slide surface, with the leading edge of the blood film having a 'feathered' appearance.

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ANNEX B – FIELD SAMPLING CAMPAIGN 2015: ACTIVITY REPORT (in French)

Élaboration de protocoles d'évaluation de la qualité des milieux littoraux méditerranéens par l'étude physiologique de l'avifaune nicheuse des petites îles.

Missions de terrain pour le test des protocoles.

Afin de valider les techniques d'échantillonnage, plusieurs missions de terrain ont été prévues à la fin du printemps 2015.

Le programme préliminaire inclut trois missions de terrain en Méditerranée occidentale :

- une mission sur un site insulaire français
- une mission sur un site insulaire de la côte sud de la Méditerranéenne (Maroc, Algérie ou Tunisie), ensuite concentrée sur la Tunisie (île de Zembra)
- une mission sur un site insulaire de la côte nord de la Méditerranée (Espagne ou Italie), ensuite concentrée sur la Sardaigne

Pour compenser l'absence d'un site insulaire français parmi ceux visités à cause de problèmes administratifs indépendants de notre volonté, dans le plan des missions ils ont été inclus un site en Toscane et un dans le Golfe de Naples.

Les activités d'échantillonnage ont été organisées par Medmaravis, conjointement avec la Délégation Europe international du Conservatoire du littoral en ce qui concerne tous les aspects organisationnels des missions qui variaient selon le site spécifique identifié. Localement, des accords spécifiques ont été pris avec les autorités de gestion des sites.

Le meilleur moment pour collecter des échantillons a été considéré être la fin du printemps (la première moitié de juin), en raison de la phénologie des oiseaux cible. A cette époque, les Goélands d'Audouin et les Puffins yelkouans ont des poussins d'âge approprié, qui peuvent être échantillonnés à moindre risque pour les oiseaux. Les Puffins cendrés éclosent en juin, par conséquent, en ce qui concerne cette espèce, les adultes peuvent être échantillonnés.

En ce qui concerne l'échantillonnage en Sardaigne, le groupe de travail a profité de la mission ISPRA 'Audouin 2015'. La recherche des colonies autour de la Sardaigne a été possible grâce à la disponibilité d'un bateau

appartenant à ISPRA et grâce à la disponibilité de logements mis à disposition par des contacts locaux ou par la Direction des parcs. Pour achever la mission autour de la Sardaigne onze jours ont été nécessaires.

Trois personnes ont visité la plupart des îles Sardes (presque 70) du 12 au 22 juin, parfois avec des collaborateurs locaux, ou en travaillant indépendamment, après avoir obtenu la permission des autorités et avec la collaboration de plusieurs parcs marins ou d'aires marines protégées.

Sur chaque site ont été soigneusement évaluées les conditions de sécurité des oiseaux, quant aux problèmes d'échantillonnage, avant de procéder à la capture. Seule une partie des colonies identifiées était accessible ou avait des caractéristiques appropriées pour l'échantillonnage.

Par conséquent, bien que nous ayons trouvé la colonie, il n'a pas été possible de recueillir des échantillons de Goéland d'Audouin aux Salins de Cagliari (Sardaigne du sud), à Isola di San Pietro (sud-ouest), à Isola Mal di Ventre (ouest), à Isola dell'Asinara (nord-ouest), à Isola Santo Stefano, à Isola Molarotto, à Isola Mortorio à Isola Figarolo (nord-est), et sur la côte d'Orosei (est).

Pour des raisons similaires, les colonies de Puffins à Isola di San Pietro (sud-ouest) et à Isola Molara (nord-est) ont été identifiées mais pas échantillonnées.

L'échantillonnage en Toscane a été fait à Montecristo dans le cadre du Projet LIFE 'Resto con Life' par un collaborateur d'ISPRA, avec la participation active des chercheurs de l'Université de Sienne. Ils ont travaillé quatre jours, en explorant les différentes parties de l'île. Dans le Golfe de Naples, enfin, l'activité a été coordonnée par l'observateur local chargé du suivi d'«Audouin 2015», avec la participation d'un chercheur ISPRA et celle d'un vétérinaire.

La seule colonie de Goélands d'Audouin a été visitée le 18 juin.

De tous les sites inspectés, les suivants sont ceux où nous avons effectué la collecte des échantillons :

- Sardaigne (Fig. 1 et 2)
 - o Peschiera di Nora, sur la côte sud de la Sardaigne (38.99 N – 9.00 E)
 - o Isola dei Cavoli, Villasimius, sud-est de la Sardaigne (38.08 N – 9.53 E)
 - o Isola di Tavolara, Golfe de Olbia, nord-est de la Sardaigne (40.90 N – 9.71 E)
 - o Isola Soffi, Archipel de Maddalena, nord-est de la Sardaigne (41.06 N – 9.57 E)
 - o Isola Barrettini, Archipel de Maddalena, Sardaigne septentrionale (41.28 N – 9.40 E)

- Toscane (Fig. 3)
 - o Isola di Montecristo, Archipel de la Toscane (42.33 N – 10.31 E)

- Golfe de Naples (Fig. 4)
 - o Isola di Vivara, Archipel de la Campanie (40.74 N – 14.00 E)

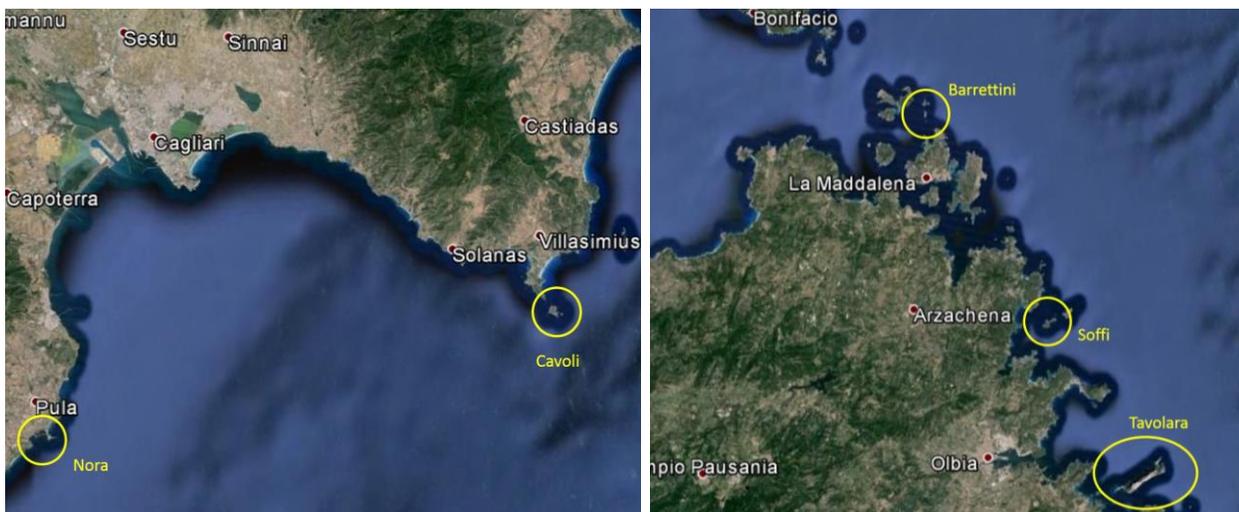


Figure 1B et 2B – A gauche les sites d'échantillonnage au sud de la Sardaigne, à droite les sites au nord de la Sardaigne.



Figure 3B et 4B – A gauche les sites d'échantillonnage en Archipel de la Toscane, à droite le site au Golfe de Naples.

De plus, le résultat global doit inclure l'échantillonnage par le personnel du Conservatoire du littoral. Personnel du CdL et un ornithologue local ont dirigé les opérations d'échantillonnage du 5 au 8 juin dans plusieurs colonies à Zembra et Zembretta, en Tunisie (37.11 N – 10.87 E).

La plupart du matériel énuméré dans le protocole a été acheté par le même personnel du CdL, tandis qu'une petite partie du matériel pour l'échantillonnage, pas facile à trouver, a été envoyé à la fin de mai au Conservatoire du littoral, avant le départ pour la Tunisie.

En ce qui concerne les Goélands d'Audouin de Zembra il a été noté que la colonie est divisée en deux localités différentes, et l'une d'elles a connu un échec de reproduction, par conséquent, il a été difficile de trouver des poussins. Néanmoins, l'objectif a été essentiellement atteint.



Figure 5B – Le deux îles visitées par le personnel du Conservatoire du littoral en Tunisie : Zembra et Zembretta

Grâce à l'effort déployé, on a obtenu les échantillons suivants :

SARDAIGNE

Lagune de Nora (12 juin)

- Goéland d'Audouin
 - o 10 frottis de sang (avec réplique)
 - o 10 échantillons de plumes (avec réplique)
 - o 5 échantillons d'excréments

Isola dei Cavoli (15 juin)

- Puffin cendré
 - o 3 frottis de sang (avec réplique), 3 échantillons de plumes (avec réplique), 1 échantillon d'excréments
- Puffin yelkouan
 - o 3 frottis de sang (avec réplique), 3 échantillons de plumes (avec réplique)

Isola di Tavolara (17 juin)

- Puffin yelkouan
 - o 11 frottis de sang (avec réplique), 11 échantillons de plumes (avec réplique), 1 échantillon d'excréments

Archipel de Maddalena, Isola Soffi (19 juin)

- Puffin cendré

- 2 frottis de sang (avec réplique), 2 échantillons de plumes (avec réplique)

Archipel de Maddalena, Isola Barrettini (22 juin)

- Puffin cendré
 - 8 frottis de sang (avec réplique), 8 échantillons de plumes (avec réplique)

TOSCANE

Isola di Montecristo (16-19 juin)

- Puffin yelkouan
 - 10 frottis de sang (avec réplique), 3 échantillons de plumes (avec réplique), 5 échantillons d'excréments

GOLFE DE NAPLES

Isola di Vivara (18 juin)

- Goéland d'Audouin
 - 10 frottis de sang (sans réplique), 10 échantillons de plumes (sans réplique)

TUNISIE, Archipel de Zembra

- Puffin yelkouan
 - 9 frottis de sang (avec réplique), 9 échantillons de plumes (avec réplique), 9 échantillons d'excréments
- Puffin cendré
 - 10 frottis de sang (avec réplique), 10 échantillons de plumes (avec réplique), 10 échantillons d'excréments
- Goéland d'Audouin
 - 9 frottis de sang (avec réplique), 9 échantillons de plumes (avec réplique), 8 échantillons d'excréments.

Les problèmes majeurs ont concerné l'échantillonnage des excréments en Sardaigne, où il a été très difficile d'obtenir des déjections. En outre, nous avons noté des améliorations possibles de la qualité du matériel et quelques précautions qu'il conviendra d'intégrer dans la version finale du protocole.

Dans tous les sites nous avons pris des échantillons du sol, au cas où à l'avenir, on déciderait d'analyser d'autres oligo-éléments dans les plumes recueillies, en plus du mercure, afin d'évaluer la possibilité d'une contamination externe.

Ci-dessous, un tableau résumant les espèces et les lieux d'échantillonnage (Table 1B).

Espèce	Sardaigne du nord	Sardaigne du sud	Toscane	Naples	Tunisie
Goéland d'Audouin		X		X	X
Puffin yelkouan	X	X	X		X
Puffin cendré	X	X			X

A la fin des missions, tous les échantillons ont été marqués, inventoriés et réfrigérés, avant d'être livrés à l'Université de Sienne où les matériels seront analysés pour tester la réussite de l'échantillonnage.

À la mi-septembre également des échantillons de Zembra sont arrivés à ISPRA, et ont été ajoutés au reste des échantillons. Tout a été contrôlé et remis en mains propres au personnel de l'Université de Sienne début octobre. Deux équipes de chercheurs de l'Université de Sienne a effectué la préparation et l'analyse de 3 échantillons de sang et de plumes pour chaque espèce et pour la plupart des régions. Les résultats préliminaires de cette étude utiles pour comprendre quel niveau d'information peut être obtenu par l'application des protocoles sélectionnés, ont été discutés dans le rapport présenté à l'annexe A.



Photo: F. Borghesi