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NON CONVENTIONAL SPECIES AS MONITOR OF AQUATIC ENVIRONMENTAL CONTAMINATION

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INDEX

ABSTRACT

This thesis collects several ecotoxicological studies focused on the quali-quantitative analysis of several classes of chemical compounds. Our studies have been conducted on different aquatic species occupying different food chain trophic levels and characterized by differences in biology, ethology, and nutrition, but all considered excellent bioindicators. This choice allowed us to have a broad overview of the contamination of aquatic environments. Detrimental effects of several chemical compounds on the species investigated have been discussed, considering the economic and public health implications linked to the pollution of the environment and the exposure to old and emerging xenobiotics. Our studies underline the importance of a multidisciplinary and integrated approach that includes the application of the one health concept to ensure the protection of public health and respect for natural environments. Studies collected in this thesis also aim to overcome some critical limitations of the branch of ecotoxicology, such as the lack of standardization in laboratory methods. Our data also underline the importance of expanding research to a greater number of various biological matrices than those indicated by the literature as target tissues for specific pollutants. This condition enables more detailed information on the kinetics of xenobiotics in animal organisms. Our studies also allow us to expand the knowledge related to the mechanisms of synergy and antagonism of mixtures of pollutants that can simultaneously accumulate in wildlife.

RIASSUNTO

Questa tesi raccoglie diversi studi ecotossicologici, che implicano una analisi quali-quantitativa relativa alla detezione di diverse classi di inquinanti su diverse specie acquatiche, che occupano differenti livellitrofici della catena alimentare, e che sono caratterizzati da differenze biologiche, etologiche e dietetiche, ma tutti considerati eccellenti bioindicatori. Questa scelta ci ha permesso di avere un'ampia panoramica della contaminazione degli ambienti acquatici. Sono stati discussi gli effettidannosi di diversi composti chimici sulle singole specie in esame, considerando anche le implicazioni economiche e relative alla tutela della salute pubblica legate all'inquinamento dell'ambiente e all'esposizione a xenobiotici conosciuti ed emergenti. I nostri studi sottolineano l'importanza di un approccio multidisciplinare e integrato che includa l'applicazione del concetto di One Health per garantire la protezione della salute pubblica e ilrispetto degli ambienti naturali. Gli studi raccolti in questa tesi mirano anchea superare alcuni importanti limiti della branca dell'ecotossicologia, come lamancanza di standardizzazione nelle metodiche laboratoristiche. I nostri dati sottolineano anche l'importanza di espandere la ricerca a un numero maggiore di matrici biologiche diverse rispetto a quelle indicate dalla letteratura come tessuti bersaglio per specifici inquinanti. Questa condizione consente di ottenere informazioni più dettagliate sulla cinetica degli xenobiotici negli organismi animali. I nostri studi permettono anche di ampliare le conoscenze relative ai meccanismi di sinergia e antagonismo

delle miscele di inquinanti che possono accumularsi contemporaneamente nella fauna selvatica.

INTRODUCTION

Ecotoxicology is defined as “the science of contaminants in the biosphere and their effects on constituents of the biosphere, including human beings” (Elliot et al.,2011). Wildlife Ecotoxicology is a relatively new branch of ecotoxicology. Today, it interoperates with different scientific fields such as monitoring, risk assessment, forensic and necroscopic investigations to conduct multidisciplinary studies to obtain as much information as possible. As for other scientific areas, the most important improvements in wildlife ecotoxicology began when scientists noticed unusual natural events and started inquiring about their causes. First, wildlife ecotoxicology studies were conducted in the early twentieth century because of the massive death of birds and marine animals due to natural disasters such as oil spills or accidental poisoning linked, for example, to ingestion of spent lead shot (Mateo et al.,2016). The interest in conducting wildlife ecotoxicology studies is also related to public health. This is an essential aspect of wildlife ecotoxicology research. Public health is involved in ecotoxicology because chemical compounds used for a specific intention on a target species affect other species, including human beings. The first examples of these events occurred during the ‘90s when lethal effects of strychnine and thallium, used as a poison to control rodents, have also affected songbirds. Arsenic intoxication in deer has been described as related to calcium arsenate, used to reduce insect proliferation. Moreover, problems extended also to humans. Probably this is the most relevant and exciting aspect of wildlife ecotoxicology studies. Scientists started to relate ecotoxicology and human beings’ health in the ’50s when several farmworkers in the UK died of dinitro-ortho-cresol intoxication (Rattner B.A., 2009). Ecotoxicology was born and became more and more important in conjunction with the most recent chemical and industrial world development. During the 20th century, the progress of chemistry created and used an impressive number of synthetic substances intended for use in countless human economic activities. A famous example is the discovery of

insecticides in the early twentieth century, such as dichlorodiphenyltrichloroethane (DDT). DDT use spread exponentially after the ’40s. In 1948 traces of DDT were detected in human tissues, and concern also arose for its possible detrimental effects on wildlife worldwide. Moreover, in the same historical period, other chemical compounds with the same purposes were used on a large scale. Therefore, preliminary scientific studies on birds and small mammals were conducted to verify possible adverse repercussions on ecosystems linked to new insecticides’ diffusion (Rattner B.A., 2009). In these years, wildlife ecotoxicology studies focused not only on pesticides but also on effects and damages related to exposure to non-essential trace elements such as lead (Pb), cadmium (Cd), or mercury (Hg). Indeed, heavy metals are still highly exploited in the industrial sector worldwide. As for pesticides, ecotoxicological studies focusing on trace elements have been essential for wildlife and public health. A clear example of how interconnected we are is represented by what happened with fungicides containing mercury. Studies on human beings followed research on birds and mammals that died after using mercury fungicides. This research allowed scientists to prove the danger of mercury in humans too. The syndrome is known as Minamata disease due to the high number of people and animals poisoned by the continuous discharge of methylmercury from factories located in Minamata bay (Rattner B.A. 2009). During the sixties, the international scientific community finally recognized the urgency to investigate ecotoxicological aspects on wildlife, ecosystems, and humans. In 1967, Ratcliffe published an article on Peregrine Falcons (*Falco Peregrinus*) population’s decrease due to the thinning of the eggs. This change in thickness of the eggs has been linked to DDT environmental contamination in the UK (Ratcliffe D.A., 1967). This study represents a symbolic turning point in the history of wildlife ecotoxicology, denouncing for the first time not only the damages caused to a wild species using chemical substances useful for humans’ activities but also how these damages were putting at risk the future survival of an entire species and consequently the balance of the whole ecosystem.

This consideration can seem obvious to us now, but in the late ‘60s, it was innovative and opened the doors to a new way to think and perform wildlife ecotoxicology studies. Therefore, from the sixties, ecotoxicological wildlife research started to consider a new and essential aspect: the need to build predictive models for assuming the evolution in the future years of the population studied. This process is based on data collected, and in consequence, through the years, it highlighted the urgency to establish official protection programs for wildlife. Moreover, the scientific community recognized the fundamental importance of the long-term monitoring of species and populations. This new awareness, united with the advent of new diagnostic technologies, has enabled wildlife studies to be expanded. First, it allowed scientists to investigate a more comprehensive number of xenobiotics. Furthermore, it allowed them to examine the relationships between xenobiotics, to investigate effective doses able to induce alterations, the way of exposure, and the body’s response. This helped scientists differentiate pathological findings related to acute intoxications or chronic exposure to dangerous xenobiotics. Especially regarding chronic cases of intoxications, wildlife ecotoxicological studies allowed scientists to develop a new branch of ecotoxicology science that is still growing and deserve to be widely deepened: immunotoxicology. From 1969 on, the size and heterogeneity of wildlife ecotoxicological research and scientific publications grew. The evidence that resulted from these studies encouraged governments worldwide to establish restrictions on the use of specific substances or to ban their use completely (Rattner B.A. 2009). Nowadays, we know legal and official restrictions are essential to protect wildlife and human beings exposed to threatening xenobiotics in numerous ways. Exposure to pollutants can occur through dermic contact, breathing, or ingestion. Today in the European Union, any compound with a wet Bio Accumulation Factor (BAF) > 100 is considered dangerous for the environment because of its potential for bioaccumulation and its possibility to damage aquatic wildlife and predators feeding on it. For the USA, the limit for wet BAF is set at 1000; in Canada, the limit is set to 5000. These data highlight the lack of

homogeneity among countries in the perception of risk and protection of the environment and humans.

The extension of ecotoxicological studies and the availability of more technological resources also led scientists to acquire information on different species widening the scientific community's interest, which initially focused mainly on birds and small mammals. This meant the first studies on species like wolves, big African species, and marine mammals began, implementing enormously scientific knowledge on these species and their health status. Through the years, wildlife ecotoxicology has further expanded its research field, and due to numerous factors, studies on fresh water and marine wildlife have acquired significant importance. Nowadays, it is well known that many different pollutants such as trace elements, PCBs (polychlorinated biphenyls), OCPs (organochlorine pesticides), PAH (polycyclic aromatic hydrocarbons), PCDFs (polychlorinated dibenzofurans), and PCDDs (dibenzo-p-dioxins) enter the watercycle, released by human communities and industries. In most cases, the final destination of these contaminants is represented by the aquatic environment for direct release or through atmospheric and geological events. Anyway, detecting pollutants in the environment is not enough to understand if they are dangerous for wildlife. In wildlife ecotoxicology studies, one of the ultimate goals is to find a reliable connectionbetween external levels of exposure, levels of pollutants detected in internal organs andtissues, and, whenever possible, levels of contaminants excreted and adverse effects on the organism.

AQUATIC WILDLIFE ECOTOXICOLOGY

Wildlife ecotoxicology studies on fresh water and marine species have gained great importance because oceans represent an essential supply source and economic resource worldwide for humans. As a result of scientific community awareness about oceans' progressive and widespread pollution, the concept of One Health and ERA (Environmental risk assessment) was established. One Health is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals, and ecosystems. It recognizes the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and interdependent. The One Health concept is a collaborative, multisectoral, and transdisciplinary approach to designing and implementing programs, policies, legislation, and research in which multiple sectors communicate and work together to achieve better public health outcomes. It is particularly relevant in food safety, in controlling zoonoses (diseases that can spread between animals and humans, such as flu, rabies, and Rift Valley Fever), and antibiotic resistance (https://www.who.int/news-room/questions-and-answers/item/one-health). An ERA is a process that encloses all the scientific steps necessary to clearly define the adverse effects of substances on the ecosystem (Van der Oost et al.,2003). The risk assessment can be separated into two different moments: risk analysis and management. Risk analysis is the more scientific part of the process, and it is composed of hazard identification, effect and exposure assessment, and risk characterization. Following data gained by risk analysis, it is possible to develop risk management which includes all the possible actions that we can pursue to mitigate the detrimental effects of pollutants and establish regulatory measures to reduce the dangerous effects of the chemicals on the ecosystem (Van der Oost et al.,2003). Due to their chemistry and toxicokinetic, many compounds can bioaccumulate and biomagnify. Hence, animals on the top of the food chain represent good subjects to obtain information. These species are useful to gain data on the health status of the population and species considered and the health of the entire ecosystem, acting as reliable environmental

sentinels, so they are called bioindicators. Moreover, many different fish species represent excellent biomarkers for monitoring pollutants in the aquatic ecosystem. Bioaccumulation is defined as the process that leads aquatic organisms to concentrate a greater amount of chemicals than that detected in water (Mackay, D., & Fraser, A., 2000). An organism can accumulate xenobiotics in different ways. The process can occur via direct uptake from surrounding medium (es: sediments, soil, or water) by animals' respiratory system or skin or indirectly by the gastrointestinal system through ingestion (Franke et al., 1994). The BAF is described as a mathematical formula expressed by the ratio of the concentration of pollutants in the animal to that in the surrounding water (Mackay, D., & Fraser, A., 2000). Furthermore, chemical compounds released in oceans primarily by anthropogenic activities can also biomagnify. Biomagnification is a process strictly related to the concept of trophic chain, representing the condition in which, due to dietary absorption, pollutants' concentration in an animal exceeds that in the animal's diet. A biomagnification factor (BMF) exists, expressed by the ratio of the concentration of pollutants in the animal to that in the animal's diet. The problem in calculating a BMF emerges when the animal has a varied diet and feeds on different food sources. Not all bioaccumulative chemicals can also biomagnify, so every pollutant should be deeply analyzed individually (Mackay, D., & Fraser, A., 2000). Moreover, the capacity of xenobiotics to bioaccumulate and/or biomagnify is dependent on their chemical formulation, which is strongly linked to their bioavailability. So, when an ecotoxicology study is conducted, it is essential to know the chemical composition and formulation of the xenobiotic deeply and gain information on its kinetic and chemical behavior in the water environment. The chemical composition of pollutants provides information also on their kinetics in animals organisms. For example, hydrophobic compounds are primarily stocked in anatomical districts rich in lipids. Therefore, this kind of chemical research should be focused on fatty tissues (Mackay, D., & Fraser, A., 2000). In addition to the uptake routes, animals use clearance mechanisms to eliminate pollutants or inhibit their toxicity. The respiratory system, for example, works both as an uptake

and loss system through the passive diffusion process. Skin also can act by a clearance mechanism for detoxification. Besides these two defensive mechanisms, xenobiotics can also be eliminated through feces and urine or converted by metabolic processes into more hydrophilic and oxygenated compounds. This event can also lead to the synthesis of more reactive and more toxic chemical species. We must consider the animals' chemical characteristics and kinetic and some intrinsic parameters about clearance mechanisms. Species, age, and sex are variables that must be considered when analyzing data. For example, females can eliminate part of contaminants accumulated in their organism through pregnancy, egg deposition, or lactation. Unfortunately, these maternal losses usually represent an uptake for the progeny (Mackay, D., & Fraser, A., 2000). It means that xenobiotics 'exposure and contamination of animals don't involve just the individuals considered but involve the next generation, putting the entire population or species at risk. This consideration highlights the long-term effects of wildlife exposure to contaminants. Therefore, even though ERA is usually built on predictive methods, there is an increasing interest in deepening the assessment of pollution that began in the past but continues to show adverse effects. This evidence is leading to conducting studies based on retrospective ERAs. Retrospective ERA studies aim to identify the connection between the source of contamination and negative ecological effects over time. These types of research are complex as they require a high amount of funding, specialized teams, many years of monitoring, and a high number of data to be analyzed. Moreover, to further complicate wildlife ecotoxicology studies, we must consider that animals included in the research are commonly exposed to mixtures of pollutants. So, it is essential to consider the possible synergy or antagonism of compounds and how these chemical interactions can influence the outcome, the degree, and severity of intoxications (Van der Oost et al.,2003). This is complicated to be evaluated in laboratory and domestic animals but is even more difficult in wildlife. Wild animals can be difficult to catch. They can become easily stressed during medical manipulations. It can be challenging to collect biological samples or hide clinical signs of illness for long periods and show discomfort

only when irreversible pathological conditions occur. For these reasons, scientists are trying to establish early-warning signals called biomarkers (Van der Oost et al.,2003). Biomarkers are represented by measurements in cells, fluids, or tissues that reveal alteration linked to the presence and the concentration of pollutants in the organism and the potential immune response of the animal. Marine wildlife, especially fish, aroused considerable interest as biomarkers. Indeed, fish are distributed worldwide, playing an essential role as energy carriers in the trophic chain from lowest to highest levels. Therefore, the scientific understanding of mechanisms regulating exposure, uptake, accumulation, elimination, and effects of pollutants in fish acquires great significance.

IMMUNOTOXICOLOGY

Immunotoxicology is defined as immune perturbation resulting from exposure to exogenous substances. It had its origin in the ’70s when scientists realized the correlation between xenobiotics exposure and alterations in the immune system response of different animal species (Germolec et al.,2017). The effect of infections on the immune system, with primary and acquired immunity depletion, was already known. Still, the implication of chemical substances in variations of immune system functions was new. Initially, the awareness that pollutants can interfere with the host’s immune system was born with observations of industrial workers showing respiratory distress. These conditions were linked to exposure to chemicals and diagnosed as immune-mediated lung diseases (Rehberger et al., 2017). The immune system is a complex structure formed by different cells, tissues, and organs. It is susceptible to immunotoxic compounds because of the intense vascularization of its components as peripheral immune cells in the bloodstream and resident immune elements in organs like the liver (Rehberger et al., 2017). Alterations observed were not only detected in the sense of a lowering of the immune system but included hypersensitivity reactions (Germolec et al.,2017; Grasman K.A., 2002). First, studies showed how low-weight

molecules could act as antigens and stimulate allergic responses. Although many studies have been conducted since the late 70s, and the evidence that immunosuppression, autoimmunity, and hypersensitivity are involved in pathological mechanisms linked to exposure to xenobiotics, lots of information still needs to be acquired. It is also important to notice that damages induced by chemical compounds on the immune system's functions usually occur at lower concentrations than those able to induce mortality or evident clinical signs in a brief time. This can be misleading since alterations in the health status cannot be promptly noticeable, delaying a diagnosis's issuance. Moreover, it implies that immunotoxicity is not a consequence of a general, systemic intoxication process but a proper toxic modality of action (Rehberger et al., 2017). First studies were applied to rodents' models and performed in laboratories. Later, immunotoxicology has also been applied to wildlife. Studies performed on amphibians highlighted the role of immunotoxic damages in the proliferation of parasitic infestations. Distemper virus outbreak in harbor porpoises (*Phocoena phocoena*) has been linked to bioaccumulation of PCBs. For birds, changes in non-specific response contextual to exposure to chemicals have been observed; adverse effects can be so severe in altering the host's fitness to lead them to death (Rehberger et al., 2017). Primarily, wildlife immunotoxicology research has foreseen the use of in *vitro techniques*, which provides many advantages, like investigating multiple mechanisms of immune alterations linked to one or various chemical compounds and reducing the cost-of-living animal testing. Two main tests are exploited to define basic immunotoxicology principles. One regards the assessment of myelotoxicity, which provides a first measure of the impact of chemical compounds on white blood cells, their growth process, and differentiation. The other basic principle of immunotoxicology *in vitro* techniques is linked to lymph toxicity (Germolec et al.,2017). Talking specifically of wildlife immunotoxicology assays, the first successful study was conducted by Friend and Trainer in 1970. They demonstrated the increased susceptibility of mallards (*Anas plathyrynchos*) to hepatitis virus consequently to PCBs exposure. One of the major limits of the application of

immunotoxicology to wildlife ecotoxicology study is the lack of knowledge that often we have on the physiology, immunology, and physiopathology of certain wild species. Few methods have been developed for wild fauna because of the impossibility of synthesizing species-specific antibodies directed against immunoglobulins or WBC (white blood cells) receptors (Grasman K.A. 2002). Following the first research, other immunotoxicology studies were conducted on marine wildlife, demonstrating severe damage to the immune system of California sea lions (*Zalophus californianus*), beluga whales (*Delphinapterus leucas*), common seals (*Phoca vitulina*), and marine birds linked to organochlorines, opening the doors to an important line of research concerning marine ecosystem and pollution due to anthropogenic activities (Grasman K.A. 2002). Differences have been observed between marine and terrestrial mammals' immune systems. Marine mammals' phagocytic cells have been identified through reactions among cell surface antigens, such as major histocompatibility processes and species-specific monoclonal and polyclonal antibodies. In harbor seals (*Phoca vitulina)* and beluga*,* scientists described natural killer cells to destroy viruses and cancerous cells (Desforges et al., 2016). Mass mortality events involving marine mammals usually occur in heavily urbanized coastal areas; this evidence led scientists to hypothesize the existence of a link between these anomalous events and the presenceof anthropogenic activities in the same area. Morphological modifications in lymphoid tissues secondary to exposure to contaminants have been reported in polar bears (*Ursus maritimus*) and harbor porpoises. In these animals, spleen disfunction, fibrosis, and splenic lymphocytes depletion have been observed in connection with blubber concentration of POPs between 1 and 26 μg/g lw sample. Moreover, hematological aberrations in harbor seals and northern fur seals (*Callorhinus ursinus*) were linked to POPs exposure, evolving in depletion of circulating neutrophils and basophils. In bottlenose dolphins (*Tursiops truncatus*), a correlation between numeric depletion in neutrophils and band cells and mercury exposure has been established (Desforges et al., 2016). Laboratory assays have been developed to evaluate lymphocytes characteristics. Mitogens are proteins that can induce the proliferation of lymphocytes

and can be used in marine mammals to measure lymphocytes activity. Out of about thirty studies conducted on marine mammals about lymphocytes activity and contaminants, the majority reported reduced T cell proliferation and no effects on B cells activity. The positive correlation between POPs or metals and immune system alterations in marine mammals was highlighted by *in vitro* studies conducted on the same specimens. According to the literature, toxic levels of MeHg (methylmercury) from 4 to 30 μg/g w.w. (wet weight) can induce immunotoxic effects on marine mammals, while for other metals, toxicity levels have been established ranging from 1 to 100 μg/g w.w. Regarding POPs, this class includes a high number of different chemicals and congeners. Although the active mechanism of xenobiotics on the physiological phagocytosis process is not completely understood yet, a different sensitivity of distinct species to singular congeners is well demonstrated, suggesting the involvement of a receptor-mediated pathway (Desforges et al., 2016). A few studies were conducted on antibody production and activity related to exposure to xenobiotics in marine mammals. The research focused primarily on IgG, the main immunoglobulin isomer present in the bloodstream of marine mammals. Results demonstrated a reduced amount of total circulating IgG following the exposure to POPs. Following studies discovered that exposure to POPs could reduce specific immunoglobulins after exposure to viral antigens through vaccination. This condition was observed in polar bears recaptured after administration of vaccine against tetanus, influenza, reovirus, and herpes virus. Those animals who received the immunization and presented important levels of POPs in the organism could not produce an adequate number of antibodies. The same condition was not observed in harbor seals (Desforges et al., 2016). A new relevant application of immunotoxicology on marine mammals regards the study of cytokines and the effects of pollutants on them. These studies exploited a genomic approach to understand the synthesis process of cytokines. Several interleukins and their products were correlated to exposure to xenobiotics such as POPs. For example, POPs stimulate the production of IL-4 and to suppress IL-2. This condition is due to the essential roles of interleukins in the cascade of the immune

system and the synthesis of pro-inflammatory proteins and immunoglobulins. Nevertheless, studies are scarce, and data are variable and difficult to compare. So, more studies are necessary to understand immunotoxicology in marine mammals fully.

These studies focused on those marine species due to their accepted role as bioindicators as predators at the top of the food chain. This is an essential topic of wildlife ecotoxicology because, since ancient times, humans have exploited the sea and its resources. Today, the problem of water pollution is widely recognized and very serious. So, the importance of ecotoxicology studies focused on marine fauna is linked to human consumption of numerous fishing products, including mussels, crustaceans, and fishes of all sizes, which occupy different positions in the food chain. As for mammals, fish's immune system is a complex structure involving cellular and humoral responses. Lymphocytes, granulocytes, and monocytes are present in fish in addition to cytokines, immunoglobulins, and innate proteins such as lysozyme and complement. Nevertheless, in fish, innate response is much more competent than acquired immunity, with numerous C3 isoforms and non-specific cytotoxic cells (Milla et al., 2011). Some of the same criteria as immunopathology or innate and acquired immune response alterations used to define immunotoxicity in mammals have been used to assess immunotoxicology damages in fish. Numerous immune endpoints exploited to reveal immunotoxicity in laboratory fish have also successfully assessed detrimental effects in feral fish populations (Zelikoff et al.,2000). Laboratory investigations are a fundamental preliminary step before an immune assay can be applied to a wild population. Working in the laboratory allows establishing specific protocols of action. It will enable scientists to correctly validate the method, using the repeatability of tests and setting specific parameters for the assay. For example, laboratory tests could define and measure T and B lymphocyte proliferation in fish, host resistance against bacteria, macrophage activity, and antibody-forming cell response to T-dependent antigens related to fish exposure to pesticides, trace elements, and PCB. Moreover, these studies allowed scientists to set baseline parameters clearly defining which concentration levels of pollutants could induce damage to the host's immune system. For example,

fish exposed to permethrin at a non-lethal concentration (0.05 ppb) and then injected with LD_{40} of *Yersinia ruckerii* showed greater mortality than the control group injected with the same dosage of *Yersinia* but was not exposed to the insecticide (Zelikoff et al., 2000). Studies performed on smallmouth bass (*Micropterus dolomieu*) living in a site contaminated by PCB showed a reduction in the synthesis of kidney phagocyte mediate superoxide anion and superoxide dismutase activity. Other research demonstrated that salmonids exposed to PAHs were more sensitive to bacterial infections. However, immunotoxicology studies face numerous challenges. It is essential to distinguish direct adverse effects due to the interaction between the toxic substances and the immune system's elements, from stress responses induced by the pollutant and the modulating activity of the stress endocrine pathway. Contaminants may show no effects on the resting immune system. Still, they may alter the ability ofthe host to resist infectious diseases, demonstrating their harmful action only during the activation of the immune response. Additionally, these perturbations on immune system activity can be transient or permanent, taking place in the host's mature or developing immune system (Rehberger et al., 2017). Unbalance in uptake and excretion of trace elements may also lead to immunotoxicity. Especially non-essentialtrace elements such as Cd, Hg, and Pb can induce immunosuppression in aquatic species. An excess of metals that usually have a biological role in animals can cause alteration in the physiological function of the immune system. Recent research showed how rainbow trout (*Oncorhynchus mykiss*) exposed to cadmium, mercuric, and zinc chloride suffered a decreased synthesis of Immunoglobulin M, lysozyme, and phagocytic activity. Moreover, today the wide use of metal-nanoparticles in cosmetics,drugs, and hygiene products raises concern. These small particles can act differently from the original metal compound, and their effects on the immune system are still largely unknown (Rehberger et al., 2017).

ENDOCRINE DISRUPTORS

Endocrine disruptors (EDs) are defined as *"an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations*" (IPCS, 2002. Global assessment of the state-of-the-science of endocrine disruptors. Geneva, Switzerland, World Health Organization, International Programme on Chemical Safety). EDs can have natural or synthetic sources. They derive from anthropogenic products, including plastics, detergents, and medicines (i.e., oral contraceptives). They can also be found in cosmetics, pesticides, and flame retardants. EDs easily enter the aquatic environment through industrial and sewage discharges (Casals-Casas, C., & Desvergne, B. 2011; Goksøyr, A. 2006). EDs may act on synthesis, release, metabolism, and elimination of hormones or imitate natural hormones' behavior. Reproductive and development problems linked to EDs have been demonstrated in animals and humans. Nowadays, global concern is arising about the potential effects of EDs also on metabolic disorders affecting animals and humans as obesity and diabetes (Casals-Casas, C., & Desvergne, B. 2011). The mechanism of action of EDs is related to their activity on hormone receptors. The endocrine system is a complex system in which specific receptors allow the normal function of hormones. Receptors are classified into two main groups: membrane-bound receptors and nuclear receptors. The majority of EDs are lipophilic compounds that interact with nuclear receptors. EDs can act as agonists compared to natural hormones, binding with a higher affinity to the receptor, or act as antagonists blocking the activation of receptors (Milla et al., 2011). So, the action of EDs is influenced by their affinity for the receptors, which is different between species. This will lead to a greater sensitivity of some species than others even if exposed to the same EDs concentration. Binding to the receptor is necessary but insufficient for EDs to perform their activity. Indeed, EDs activity is influenced by exposure, metabolism, absorption, distribution between aqueous and lipidic body areas, binding to plasma and tissues, and the concentration at the target organ or receptor. Many of these pollutants explicit their activity, interfering primarily with estrogens. 0estrogens are female sex

hormones. They act on many body sites, with important functions on the reproductive cycle, neuroendocrine system, and mammary glands. The synthesis of estrogens begins with cholesterol metabolized to progestogens and androgens. Sexual hormones play a fundamental role in the development of embryos and during the post-natal period. During these stages, embryos are susceptible to exogenous hormones that interfere with physiological development (Gregoraszczuk, E. L., & Ptak, A.; 2013). In the fresh water and marine ecosystem, environmental pollutants such as pesticides, trace elements, and organic compounds can seriously affect the health of marine wildlife, acting as endocrine disruptors. Fish are widely used as biomarkers for EDs. For example, one process used as a biomarker for monitoring the aquatic environment is linked to the fact that in fish, primarily sites of xenobiotics biotransformation involve several enzymes. Primarily the cytochrome P450 monooxygenases are inducible by many different chemicals. Moreover, hepatocytes' role in fish reproduction makes them a critical target for compounds with endocrine interference activity (Zezza et al.,2020). A well-known example regarding marine species is represented by the alteration of VTG (vitellogenin) concentration under exposure to EDs. This condition is considered a biomarker for estrogenic contamination. VTG is a precursor of egg yolk protein providing energy for embryonic development in oviparous species. VTG is produced in the liver under estrogenic control and stored in oocytes. VTG is produced in males too, usually in small amounts, but it can increase consequently to exposure to exogenous estrogens, reaching concentrations comparable to those found in sexually mature females; this was observed when the aquatic environment is contaminated by non-essential trace elements like Cd (Merola et al., 2021) or pesticides like DDT. This can be associated with the feminization of males, although the increase in VTG levels should not be considered the leading mechanism of feminization. Moreover, these chemicals can bind to more than one type of receptor. For example, o-p’DDT can bind both estrogens and progesterone receptors, interfering with more than one endocrine pathway. Sex steroid receptors normally act to control the transcription of specific genes. So, any interruption in their signaling may impair the genomic pathways. One

example is the interference with the aromatase CYP19 system. This condition leads to the inhibition of estrogens production (Milla et al., 2011). Several studies report that exposure to EDs such as 17α-ethynylestradiol (EE2) or DDT induced a decrease in circulating steroid hormones concentration and affected P-450 aromatase activity, which role is to catalyze the final transformation of testosterone to estrogens. Other research focused on exposure to PAHs and PCBs, known to act as antioestrogens. In 2004 a study demonstrated the action of TCDD (tetrachlorodibenzo-p-dioxin) on the expression of genes regulating estrogens production in salmon hepatocytes, depressing their synthesis (Goksøyr, A.2006). Furthermore, there is evidence that EDs can increase the sensitivity of fish to diseases, interfering with hormonal activity and consequently altering immune responses.

POPS (PERSISTENT ORGANIC POLLUTANTS)

Among chemicals able to act as EDs, POPs (persistent organic pollutants) and heavy metals are those causing great concern for wildlife preservation. POPs represent a wide class of compounds, including PCDDS (polychlorinated dibenzo-p-dioxins), pesticides such as DDT, PCBs, PBDEs (polybrominated ethers), PCDFs (dibenzofurans), and bisphenol A (BPA), OCPs organochlorine pesticides. Adverse effects of these chemicals have been known since the 60s, but just in 2001, during the Stockholm Convention, an international agreement stated the ban or regulation of the use of this large group of compounds (Krasnobaev et al., 2018). They are stable and lipophilic, and most of them mimic the estrogen's behavior. Some common aspects characterize POPs: they have a long-range atmospheric transport; indeed, they have also been detected in the Antarctic region (Krasnobaev et al., 2018). They can evaporate and condense at environmental temperatures; they have a high lipophilicity, persistence, and bioaccumulation potential in organisms. They also have detrimental effects on reproduction, immune system, and development, and some of them are recognized as carcinogenic (Gregoraszczuk, E. L., & Ptak, A. 2013). Among POPs, DDT and its metabolites o,p'-DDE, o,p'-DDD, methoxychlor, chlordecone, dieldrin, toxaphene, and endosulfan are chemicals that can act as EDs. POPs have been restricted in many civilized states, but they remain widely used in developing countries. Moreover, these chemicals can bind to more than one type of receptor. For example, o,p'-DDT can bind both estrogens and progesterone receptors, interfering with more than one endocrine pathway (Gregoraszczuk, E. L., & Ptak, A.; 2013). A great concern in the scientific community involves 12 compounds called the "dirty dozen" represented by Aldrin, Chlordane, DDT, Dieldrin, Endrin, heptachlor, hexachlorobenzene, mirex, PCBs, PCDDs, PCDFs, toxaphene. DDT, probably the most famous organochlorine insecticide, was synthesized in 1874, but its insecticidal properties were discovered only in 1939. After World War II, it was utilized primarily to defeat malaria and typhoid, and only later was it used in agriculture. The soil has a strong absorptive capacity for DDT related to its organic content. DDT can cause intoxication in all fresh

and marine microorganisms and predators at the apex of the trophic chain like marine mammals and marine birds. DDT usually does not induce acute intoxications, but it is strongly persistent and accumulates in the food web and host tissues. Many studies detected DDT in the different biological types of samples, but the highest accumulation is generally found in lipidic tissues. Great values have been recorded even if the accumulation was probably due to repeated exposure at low environmental concentrations. DDT was banned in most civilized countries; despite this, its residues and metabolites are still detected in numerous wild species. In the USA, total DDT in body fat reached a peak in 1956 and then progressively declined until the 80s. In Italy, the maximum concentration of DDT in human fat was reached in the 70s, then slowly declined in the next decades. Unfortunately, the trend of DDT metabolites is not so encouraging. Indeed, the concentration of DDE (di-chlorodiphenyltrichloroethyle), one of the most common metabolites of DDT, ingested with food, especially through the consumption of seafood products, remained constant through time (Turusov et al.,2002). DDE is also considered the principal responsible for adverse effects recorded in animals. DDE is called the "chemical of extinction". The main adverse effect of DDE is observed in avian species, causing eggshell thinning making hatching difficult. The pathogenic mechanism that leads to the increased fragility of eggshells still needs to be clarified. In the late 70s, numerous attempts have been made to understand the chemical and biological mechanism behind this process by exposing sexually mature females to various chemical species. Reduced prostaglandin synthetase activity, reduced prostaglandin E2, and reduced uptake of Ca by the eggshell gland mucosa were associated with p.p'-DDE exposure (Lundholm, C.E.1997). Hexachlorocycloexane (HCH) was synthesized in 1825 and discovered to have pesticide properties in 1942. HCH was used worldwide on a large scale until the 70s when the first ecotoxicology study demonstrated its detrimental effects on the environment leading many countries to restrict its application in agriculture. HCH is a scientific definition including eight isomers 1,2,3,4,5,6 - hexachlorohexan. The eight isomers are distinguished by Greek letters in α, β, γ, δ, ε, η, and θ differing in

their axial equatorial substitution groups around the ring. They are stable to light, elevated temperatures, and acid systems (Nayar et al., 2014). The γ isomer known as lindane has the most efficient pesticide activity. The β isomer is one of the most persistent and stable. These characteristics make this compound one of the most polluting chemicals. Due to the molecular composition, diverse environmental behavior and persistence are linked to different chemical properties, especially the chlorine atoms' axial and equatorial positions (Nayar et al., 2014). The β isomer characteristics of low vapor pressure but high bioaccumulation factor is a consequence of all the chlorines atoms sited in an equatorial location. The toxic effects of HCH include dysfunction of the central nervous system. For example, Lindane has the power to inhibit the GABA system in rats acting as an agonist towards neuroreceptors, determining a central excitatory status. In opposition to lindane, the β isomer act as a depressant of the CNS. Other toxic effects of HCH were recorded in the renal tubular district of rats complemented by glycosuria without alterations in blood glucose concentration. Besides that, liver dysfunctions have been observed leading to hepatic lipidosis and were also demonstrated by serum increase of transaminases. On the contrary, haematologic parameters showed a reduction of hematocrit and white blood cells (Prasad et al., 2021). HCH also acts as ED, causing reproductive problems observed in mammals. In rodents, HCH is responsible for a reduction in ovulatory capacity in females and atrophy of testes and size of seminiferous tubules in males. Concerning their chemical characteristics, HCH easily enters and persists in the aquatic environment, affecting the health of different organisms. HCH compounds may have adverse effects like those induced by DDT in fish. HCH may interfere with vitellogenin production leading to hermaphroditism and feminization in male specimens of numerous species. HCH diffusion and accumulation in terrestrial and aquatic ecosystems are important parameters for public health since humans can be exposed to and intoxicated by HCH through different routes. Several acute intoxications occurred in people working at the production of these chemicals, mainly expressed through the appearance of neurological disorders. Humans' chronic contamination is more

commonly related to exposure with food (Vasseghian et al., 2021). Moreover, these compounds still represent a danger also for terrestrial wildlife worldwide. Despite the restrictions applied by the governments of many European countries on the use and marketing of these chemicals, a recent study conducted in Italy shows that this class of compounds is still detected in the tissues of several terrestrial wild and domestic species in concentrations that can induce harmful effects (Bertero et al.,2020).

PBDEs are chemical compounds with endocrine disruptive activity. The chemistry of PBDEs is composed of two phenyl rings connected by an oxygen atom and a different number of bromine atoms binding on the two rings. The position and number of bromine atoms result in the existence of 209 congeners (Zhang et al., 2020). PBDEs exhibit low water solubility and bioaccumulation power. Vapour pressure varies among congeners influencing their dispersion in the environment. Once PBDEs enter the environment, they may undergo a debromination reaction. Debromination often may lead to the synthesis of lower brominated congeners, which are more toxic and possess a higher ability to bioaccumulate. Worldwide, PBDEs are used as flame retardants in textiles, polyurethane foams for furniture, construction materials, and electronics products. The danger arising from PBDEs is due to their environmental persistence and ability to accumulate through the food chain affecting the health of multiple animal species and human beings. In humans, PBDEs exposure has been linked to thyroid dysfunctions which usually lead to hypothyroidism. Moreover, PBDEs are implicated in reproductive failure with poor sperm quality, decreased testosterone concentration in blood, and cryptorchidism. Some studies also related the exposure to PBDEs with the insurgence of testicular neoplasia. It is important to underline that every congener can act differently and have different adverse effects. The most detected congener in humans is BDE-47; recent data demonstrate traces of PBDEs also in placentas and breast milk. PBDEs have also been associated with endocrine disorders, carcinogenicity, and neurotoxicity (Siddiqi et al.,.2003; Wu et al., 2020). Human exposure to PBDEs primarily occurs by ingestion, inhalation of dust, and dermal contact. The principal problem for public health is linked to human

consumption of marine products because of the chemistry of PBDEs, which can persist in the marine environment for many years and bioaccumulate in the food web, reaching high concentrations in apex predators. Fish and fish products are among the food products most polluted by PBDEs (Wu et al.,2020). There is an increasing awareness and scientific evidence of endocrine damages caused by PBDEs contamination on thyroid function, sexual hormones balance, and reproductive success. Those conditions may lead to permanent alteration in cognitive functions and motor skills. The pathogenetic mechanism of action of PBDEs involves altering mRNA expression in estrogenic, progestin, and androgenic receptors, acting as agonists with a major affinity for hormonal receptors. This modification influences the normal physiological conversion of testosterone and oestradiol (Siddiqi et al., 2003; Wu et al., 2020). Moreover, this agonistic behavior destroys thyroid homeostasis, affects transport proteins' function, and speeds up the metabolism of the hormones in the liver and brain. These conditions are also observed consequently to low dosage PBDEs exposure. Prenatal exposure has also been observed and recognized as the cause of behavioral modifications in children, such as attention disorders and hyperactivity (Wu et al., 2020). Another important aspect to be considered about the detrimental effects of PBDEs on humans and animals is their carcinogenetic potential. Congeners 47, 100, and 153 are implicated in breast, ovarian, and colorectal cancer onset. In animals, acute intoxications have been connected to neurological clinical signs and loss of learning and memory in rodents (Mikula, P., & Svobodova, 2006). Another effect recorded in mice exposed to PBDEs mixture was an alteration in spermatogenesis also at low dosage, underling the necessity not to underestimate the importance of investigating repercussions of persistent chronic exposure at low concentration. As for humans, PBDEs in aquatic and terrestrial wildlife are responsible for severe endocrine dysfunctions and carcinogenetic activity. The first laboratory studies conducted on rodents were fundamental to investigating PBDEs' toxicokinetics. In rats orally exposed to BDE-47, 93% of the dosage administered was absorbed by the gastrointestinal tract and then redistributed to tissues with an affinity for lipidic

anatomic compartments (Mikula, P., & Svobodova, 2006). The metabolism of PBDEs has also been studied in fish. Studies on the common carp (*Cyprinus carpio*) orally exposed to different PBDEs congeners helped scientists to discover and understand diversities in excretion and absorption based on the chemistry of the compound. Scientists observed how the debromination process of highly brominated PBDEs into low brominated compounds is the leading reaction to the accumulation of the pollutant in the fish organism. Another important aspect of studies focused on common carp and PBDEs is connected to BDE-99 metabolism. BDE-99 is one of the most common PBDEs produced. Therefore, it is one of the most detected in wildlife. In common carps exposed to BDE-99, no conger traces have been identified in the fish organism, except for the gut. The scientific explanation is linked to the fact that debromination occurs in the intestine, operated by bacterial resident population and enzymes, conducing to an efficient capacity to metabolize the BDE-99 congener (Mikula, P., & Svobodova, 2006).

POPS EFFECTS ON AVIAN SPECIES

Breeding failure in avian species is one of the most characteristic damages of DDT environmental pollution. Research to understand the pathogenesis of this condition is still ongoing. The eggshell is composed mainly of calcium carbonate. Calcium carbonate is a fundamental constituent of the eggshell. The carbonic anhydrase enzyme catalyzes the reaction that leads to carbonate ion formation, essential for eggshell formation. Some studies confirmed that quail embryonic exposure to synthetic estrogens leads to disrupted CA (Calcium) localization in the shell glands of adult specimens. So, the pathogenic mechanism behind the DDE action may be related to an alteration of CA expression (Holm et al., 2006). A diminished quantity of capillaries involved in CA activity was highlighted regarding CA expression. The formation of carbonate ions is linked to CA catalyzed hydration of CO_2 to HCO_3^-. Chemical elements needed for this reaction are located in membrane cells, capillaries, and plasma blood. The reduction of capillaries with CA activity might reduce CO_2 diffusion leading to a reduction of HCO_3^- synthesis, including reducing the bioavailability of Ca_2^+ ions (Holm et al., 2006). In addition, more recent studies on birds reveal that adults' exposure to DDT metabolites such as DDE may inhibit prostaglandins production in the shell gland mucosa. Moreover, later studies showed how embryonic exposure of chickens to high doses of DDT was linked to gross deformities and malformation of both the oviducts. Necroscopic examinations highlighted adherences among oviducts, intestine, and abdominal walls in subjects included. In many specimens analyzed, oviducts did not open into the cloaca. Several avian species exposed to DDT showed no regression of the Mullerian ducts. Mullerian ducts are embryonic structures belonging both to males and females. The right duct becomes atrophic during embryonic development in most bird species, appearing rudimental by hatching. The left Mullerian duct will differentiate later as well as the shell gland. The absence of regression of the right duct in birds exposed to DDT was observed in domestic and wild avian species such as gulls and Baltic white-tailed sea eagles (*Haliaeetus albicilla*) (Holm et al., 2006). Besides these anatomic and functional alterations,

birds contaminated by DDT also showed an impairment of the length of the left oviduct. In these subjects, the left tract of the reproductive system was shortened. The length reduction was also DDT dose-dependent (Holm et al., 2006). Further studies discovered a reduced quantity of m-RNA deputed in the shortened left oviduct to transcribe genes encoding proteins such as calbindin-D28k (CALBI) and osteopontin (SPPI). CALBI and SPPI are two essential calcium-binding proteins. Reducing these proteins indicates the interruption of calcium mobilization for eggshell production (Kamata et al.,2020). Since anatomic alterations of the reproductive tract are permanent, numerous avian species exposed to DDT continue to produce fragile eggs even if an important decreased concentration of DDT has been recorded in samples collected over the following years. As for mammals, females can eliminate DDT through oviposition. Via the yolk, DDT passes into eggs. Therefore, embryos are immediately exposed to the pollutant. This event may be particularly harmful to the developing organism because it may interfere with organogenesis and hormonal organization (Holm etal., 2006). These conditions explain the reproductive failure. Interestingly, anatomic malformations were present only in birds exposed to the high concentration of DDT. At the same time, negative effects on shell gland function were evident in all subjects, including those exposed to low DDT dosage. This means the two pathogenic mechanisms act independently. Furthermore, this implies that minimal pollutant concentration is sufficient to induce severe damage for the species' future survival. Recent studies on HCH's detection in avian species have been carried out in developing countries where these pesticides are often widely used and poorly regulated. Avian species are excellent environmental sentinels due totheir position in the trophic chain and often because many avian species have a varied generalist diet. Different biological samples (eggs, feathers, blood, and tissues) from different birds were used to evaluate the presence and concentration of HCH's class. In Brazil, HCH was banned in 1985; nevertheless, research conducted in 2019 showed a high concentration of both HCH and DDT in different biological matrices collected

from three raptors species (Aver et al., 2019). In recent years, India faced a drastic reduction of colonial nesting birds. Ecotoxicological studies on these avian species highlighted the detrimental role of HCH isomers on the survival of India's nesting birds, showing dangerous levels of contaminants detected in the internal organs of birds included in the study (Jayakumar et al., 2020). Similar results have been obtained from research carried out in Japan, including iconic bird species such as the red-crowned cranes (*Grus japonensis*), officially declared "endangered" in 2020. Muscles 'samples analyzed showed high PCB content, PBDEs, and HCH, providing evidence of potential negative effects of these chemicals on the species (Kakimoto et al.,2018). Although the economic, legislative, and sanitary conditions of developing countries attract the attention of the modern scientific community, current studies conducted on European birds also raise concerns. González-Gomez et al., in 2020, analyzed biological samples of feral pigeons (*Columba livia domestica*) in Spain. Pigeons are a sedentary species, sympatric with humans. These characteristics are important to assess in terms of acquiring public health information. Pigeons share with human beings the urban environment and have a varied diet. These features make it an exemplary candidate for monitoring the quality of the environment in which humans live. González-Gomez et al. discovered the presence of HCH in all the samples included in the study (González-Gomez et al., 2020). Chemical characteristics of HCH allow these contaminants to be found in alarming concentrations also in isolated geographic areas, usually not directly impacted by anthropic activities such as the Antarctic ecosystem. Studies on two migratory birds species that spend part of the solar year in the Antarctic demonstrated threatening concentration of HCH in fat, muscles, and eggs analyzed (Krasnobaev et al., 2018). First, laboratory experiments conducted on birds in the 80s allowed scientists to understand the mechanism of action of HCH on birds' organisms and linked it to HCH administration's recordable detrimental effects. Moreover, this research helped set a baseline of valid values to discriminate which concentration can be dangerous for specific avian species. Six avian species were orally exposed to ϒ-hexachlorocyclohexane at 5mg/kg BID (Bis in die) for seven days; then, hematological

parameters were evaluated. All the animals showed anemia, prolonged bleeding and clotting time, and leucocytosis with decreased number of total lymphocytes and monocytes. Spleen showed a reduction of cell number, indicating immunosuppression because of the spleen's physiologic hematopoietic and immunological role in birds. In addition to this finding peripheral blood reduction of WBC confirmed the immunosuppressive effect (Mandal et al., 1986). The same results were recorded by Whitehead et al. (Whitehead, C. C., Downie, J. N., & Phillips, J. A. 1974) working on Japanese quails (*Coturnix coturnix*) treated with 200 mg/kg of γ-HCH. Animals showed anemia, hematocrit, and hemoglobin reduction (Jackovitz, A. M., & Hebert, R. M. 2015). Besides these hematological alterations, also reproductive changes have been observed in birds exposed to OCs. OCs can interfere with physiological sexual hormones balance and vary mating behavior. Birds exposed to a sublethal dosage of OCs are less careful in parental care, less interested in proper courtship rituals, and more prone to ignore territorial boundaries (Mitra et al., 2011). Anomalies due to chronic OCs intoxications include hydrocephalus, hermaphroditism, and deformities of chicken (Mitra et al., 2011). The detrimental effect of POPs on birds can also lead to severe consequences for the species future survival. Ecotoxicological research is fundamental to monitoring the trend of populations and the health of wildlife and ecosystems.

PBDEs IN MARINE MAMMALS

In ecotoxicology, marine mammals are considered optimal bioindicators for investigating the contamination of the environment by persistent pollutants such as PBDEs because of their apex position in the food chain, long-life span, late reproduction, and high content of lipidic tissue. Due to their chemical composition, PBDEs have a high affinity with fatty tissue, so the target organ in which PBDEs are usually researched and detected in marine mammals is the blubber. Blubber is a dense vascularized layer of lipidic tissue located under the skin. Blubber has numerous functions; it intervenes in thermoregulation, locomotion, and metabolic energy storage.it is defined as a "dynamic tissue", reflecting the health status, nutritional condition, and life history of a specimen. Blubber is a common and unique characteristic of all marine mammals and an important biological source of information for toxicology studies (Iverson, S. J., 2009; Struntz et al., 2004). Moreover, most marine mammal species worldwide have shown dramatic numerical reduction over the last decades, so studies are needed to understand the causes of this trend and find potential solutions to protect the future of these species and the balance of ecosystems. Despite official governmental restrictions and regulations on the use of PBDEs at the beginning of the 2000s, later studies demonstrated a high concentration of this class of compounds in marine mammal tissues worldwide. As for humans, PBDEs' effects on marine mammals' health can involve reproductive success, survival of offspring, immune system susceptibility to the onset of infectious and parasitic disease, and the balance of thyroid hormones. The primary route of exposure for marine mammals is represented by ingestion. Contaminants enter the organism through the diet, and once ingested, each marine mammal species shows a peculiar ability to metabolize and eliminate the compounds (Bartalini et al., 2019). The pollutants undergo chemical reactions in animal organisms that modify functional chemical groups. After that, the chemicals undergo conjugation or other synthetic reactions, leading to higher pollutant solubility. Unfortunately, we have little information about these processes in marine mammals, but early studies showed that biotransformation of pollutants in these

animals is scarce. This conduces to the low capability of pollutants 'metabolization (Bartalini et al., 2009). As far as we know, after metabolic reactions, compounds with new chemical characteristics can be eliminated from the body or accumulated anyway. In marine mammals, the elimination of pollutants can occur primarily through the digestive system with feces and urines and skin and reproduction. Skin represents a valid system of excretion for pollutants because of the regular molting of many species and the epidermis's rapid and frequent cell renewal (Wejis et al.,2014). Studies have been conducted on different marine mammal species worldwide, monitoring populations for decades to evaluate the trend of contamination over time. Rotander et al., for example, monitored distinct species from the Arctic and North Atlantic region for over twenty years. biological samples included only blubber from pilot whales (*Globicephala melas*), ringed seal (*Phoca hispida*), hooded seal (*Cystophora cristata*), Harbour porpoise (*Phocoena phocoena*), minke whale (*Balaenoptera acutorostrata*), and fin whale (*Balaenoptera physalus*). Congeners 'concentration varied from species to species in relation to their metabolic abilities, other than intrinsic parameters such as sex, age, and diet. Surprisingly, while pilot whales' concentration of BDE 153 and 154 seems to decrease through 20 years of research, BDE 153 and 154 concentrations increased in minke and fin whales in the most recent blubber samples analyzed. Differences are remarkable also evaluating the gender of subjects included. Mature females show lower concentrations than sexually mature males, confirming the hypothesis that females can reduce their accumulation of toxic substances through pregnancy and lactation and potentially transfer part of the chemicals to the offspring (Rotander et al., 2012; Weijs et al., 2012). Comparable results have been obtained in other research, which underlined the increasing concentration of PBDEs in the blubber of seals and beluga whales (*Delphinapterus leucas*) in the last 20 years despite official protective measures implemented by governments all over the world (McDonald, T.A. 2002). What is worrying is that the same increasing trend in PBDEs' concentration has also been reported for human biological samples analyzed, such as breast milk (McDonald, T.A. 2002). Because of the extreme variability of habitats, behavioral

habits, and diets, specific models to understand the kinetic of PBDEs in marine mammals are mandatory. Physiologically based pharmacokinetic models (PBPK) try to answer this necessity. PBPK is a mass balanced system of compartments in which the chemicals' distribution is regulated by the anatomic compartments' physiological properties and the toxic chemistry. These models are essential to investigate PBDEs kinetic in marine mammals since they are part of the protected wildlife worldwide and *in vitro* research is rare. From blubber samples and PBPK applied to this tissue, it is possible to predict the pollutants' kinetic in other biological compartments such as blood or liver (Weijs et al., 2012). Although it is well known the diet represents the principal way of exposure to PBDEs for marine mammals, little is yet known about levels of these contaminants accumulated in species at the base of the food chain, such as mussels and fish. A study focused on the detection of PBDEs in blue mussels (*Mytilus edulis*), shorthorn sculpin (*Myoxocephalus Scorpius),* and starry ray (*Amblyraja radiata*) in Greenland recorded a concentration of BDE-47 above the detection limit in all the samples. In Danish research, the levels detected in mussels increased by 90- 95% in tissues of marine mammals at the apex of the trophic chain for BDE-47,99,100, and 153. Data emphasizes the danger of these compounds for human beings as well. A mixture of PBDEs has also been identified in herrings (*Clupea harengus*), salmons (*Salmo salar*), and fish oil, demonstrating further evidence of extensive and widespread contamination of the marine environment and the process of bioaccumulation and biomagnification operated by these toxics (Christensen et al., 2002).To investigate the behavior of PBDEs in the trophic chain, analyses were performed both on herrings, gadoid species, and marine mammals, including in the study liver and filet of fish and liver and blubber of marine mammals. The physiology of lipidic metabolism is quite different between herrings and gadoids. Herring's store lipids primarily in filet, while gadoids accumulate fats mostly in the liver. In support of this thesis, results of the investigation showed in gadoid species higher content of lipids in the liver than in the muscle. This main affinity for the liver was not detected in herrings, where PBDEs were distributed in the liver and the filet with slight

differences in concentration (Boon et al., 2002). The same condition was recorded in marine mammals' samples analyzed, and this highlighted the urgency to extend the type of biological samples to be included in ecotoxicology studies. Indeed, even if the target sample for detecting chemicals with a high affinity for lipidic tissues has always been considered the blubber, new and valuable information may be gained from the analyses of other tissues. This research confirmed the process of biomagnification of PBDEs, for which the highest concentrations were recorded in marine mammals. Concentrations were more than one order of magnitude higher in marine mammals than in fish. Moreover, relevant differences were also observed between concentrations detected in zooplankton and large herrings, with the latter showing superior values of PBDEs accumulated. Furthermore, an even higher concentration was recorded in salmons than in large herrings, following the positions occupied by these fish in the trophic chain (Boon et al., 2002). Only limited information is available regarding the effects of PBDEs on aquatic species. PBDEs seem to affect mammals and fish with comparable repercussions. Neurobehavioral alterations have been demonstrated in two diverse species. Embryos of *Fundulus heteroclitus* exposed to PBDEs reacted with behavioral changes at a dosage of 0.001 μg/L. In zebrafish (*Danio rerio*), abnormal cardiac function and retarded growth have been recorded following the experimental exposure to PBDEs' mixture at a dosage of 500 μg/L. Delayed hatching and locomotion problems such as scoliosis were recorded at 2000 μg/L and 5000 μg/L (Ross et al., 2008). In fathead minnows (*Pimephales promelas*), cessation of spawning occurred at an organic concentration of 15 μg/g in males and 50 μg/g in females. In rainbow trout, thyroxine in the bloodstream decreased progressively during 56 days of exposure, and it was still reduced after 112 days of depuration (Ross et al., 2008). Detrimental effects in marine mammals involve both the endocrine and immune system.

PYRETHROIDS IN WILDLIFE

Pyrethroids are a class of synthetic chemicals used as insecticides and derived from pyrethrins, which are natural compounds of *chrysanthemum* flowers. Pyrethrins are esters of cyclopropane carboxylic acid and a cyclopentenolone alcohol modified to enhance insecticidal power and lengthen their life in the presence of atmospheric agents such as sunlight and water (Burns, C. J., & Pastoor, 2018). The chemistry of pyrethroids is similar among the entire class; indeed, all the compounds conserve the acid-alcoholic composition of pyrethrins. The Pyrethroids class includes around 18 compounds and comprises some of the most widely used pesticides, such as cypermethrin and deltamethrin (Burns, C. J., & Pastoor, 2018). Pyrethroids disrupt sodium channels in nervous cells without interfering with acetylcholinesterase release. Pyrethroids have been used since 1980, progressively replacing organophosphorus and carbamate compounds because of their equal efficacy but lower toxicity. Although they have less power to pollute the environment, they may enter the food chain and contaminate wildlife. Moreover, even if pyrethroids have lower toxicity than other pesticides, they are intensively used worldwide. Pyrethroids have a high lipophilic nature; therefore, removing them from the organism is difficult. Pyrethroids are not particularly toxic for avian species because of birds' higher metabolic rates than mammals. Pyrethroids' traces have also been found in terrestrial animals such as livestock, probably due to contamination of livestock feed. Residues of pyrethroids have also been detected in pets. In Brazil, pyrethroids have been associated with the onset of mammary glands' malignant neoplasia by detecting these pesticides in the adipose tissue adjacent to the neoformation (Tang et al.,2018). Nevertheless, pyrethroids are highly toxic to aquatic wildlife. Especially long-term, low-dose exposure is dangerous for aquatic fauna, potentially leading to harmful effects on animals' genetic, immune, vascular, nervous, and endocrine systems (Tang et al.,2018). These pollutants enter the water cycle, transported from agricultural and urban areas through surface runoff and drainage channels arriving at the sea. Once they reached the aquatic environment, pyrethroids are metabolized by bacteria, fish, and other

organisms. For this reason, fish are exposed at the same time to primary chemical compounds, to metabolites generated inside the organism and outside in the environment through hydrolysis or bacteria metabolism (Brander et al., 2016). Due to their lipophilic characteristic, pyrethroids can easily enter aquatic organisms through the respiratory system. In fish, these pollutants may enter through the gills, and then they can be transported into the circulatory stream, and from blood, they can reach all anatomical compartments. In China, high levels of pyrethroids in fish were detected subsequently to a massive die-off that occurred after an involuntary release of pesticides into water. The concentration of fenpropathrin recorded in the fish that died in this natural disaster was 22.7 times higher in internal organs than in gills and in the external environment. These data corroborate the theory of the ability of bioaccumulation and the potential damages that pyrethroids can have on the aquatic ecosystem. Moreover, another study in China highlighted the power of bioaccumulation of pyrethroids recording higher concentrations of contaminants in shrimps and shellfish biological samples than in water (Tang et al.,2018). In fish, pyrethroids may interfere with dopamine release, which regulates the final synthesis of steroids. Alterations of brain enzymes such as aromatase have been reported following pyrethroids exposure, leading to variations in the circulating level of oestradiol and testosterone. Decreased expression of genes responsible for steroidogenesis has been recorded both in female and male fish, resulting in difficulties in reproduction (Brander et al., 2016). Due to the extreme variability of aquatic species, it isn't easy to assess single species' sensitivity to exposure to these chemicals. In this regard, it is useful to build a "species sensitivity distribution". The species sensitivity distribution makes it possible to determine the exposure concentration at which a specific proportion of the species is disturbed. In this way, it is possible to assess the environmental risk secondary to the spread of these pollutants (Maund et al.,2011). Over the last decades, studies on pyrethroids' effects on marine mammals have been conducted. In Brazil, livers from franciscana dolphins (*Plontoporia blainvillei*) were analyzed to detect different congeners of pyrethroids. The range of total concentrations runs from 7.4 ng/g

l.w. detected in the adult specimen to 68.4 ng/g l.w. (lipid weight) recorded in the liver of the calf. The most relevant pyrethroid identified was permethrin, followed by cypermethrin (Alonso et al.,2012). In this study, a link between concentrations and the age of animals has been established. The highest concentration was present in the youngest exemplar and decreased progressively until the subjects reached sexual maturity. Then, pyrethroids' concentration grew again. The explanation for this condition can be related to an initial accumulation of contaminants during fetal life and lactation, which then gradually dilutes and increases following dietary change and predation on fish and cephalopods. In support of this argument, relevant traces of pyrethroids were found in placenta e milk samples. Research on pyrethroids has also been conducted on livers of striped dolphins (Stenella coeruleoalba) in the Mediterranean Sea, reporting concentrations of contaminants in the same order of magnitude as those detected in Brazilian dolphins (Aznar-Alemany et al., 2017). Furthermore, a study published in 2020 on pyrethroids' presence in hepatic samples of Guiana dolphins (*Sotalia guianensis*) revealed a worrying situation. Data collected reflected a dramatic accumulation of these pollutants in dolphin's tissues, reaching medium concentrations of 1166 ng/g $^{-1}$ l.w. (Vidal et al.,2020). Pyrethroids are also toxic for human beings. Recent studies on humans demonstrated the link between urinary excretion of pyrethroid metabolites and sperm aneuploidy, confirming the role of pyrethroids as endocrine disruptors (Tang et al., 2018). Furthermore, high levels of pyrethroid have also been linked to the onset of oncologic disease in children. In China, scientific research has linked exposure to pyrethroids with a juvenile form of brain tumors, acute lymphocytic leukemia, and heart anomalies (Tang et al., 2018). Human exposure to pyrethroids can occur in numerous ways; among them, it is interesting to notice that consumers may encounter pyrethroids through the consumption of fishery products. It is important to mention that pyrethroids are the most common product used against sea lice in salmon farming. A recent study detected pyrethroids in 100% of farmed salmons included in the research with a mean concentration of 1.31 ± 1.39 ng g^{-1} w.w. Half of the wild salmons analyzed showed a mean concentration of 0.02 ± 0.03

ng g^{-1} w.w. (Aznar-Alemany et al.,2017). Although these data may give rise to public health concerns, levels detected are several orders of magnitude below the accepted daily intake. On the one hand, this gives important reassurances on the consumption of this fish species and, on the other, underlines the importance of continuing ecotoxicological studies (Aznar-Alemany et al.,2017).

ATRAZINE

Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) is a synthetic compound classified as chloro-triazine. It has been used worldwide for decades as an efficient and low-cost herbicide. Atrazine exerts its action by interfering with the normal process of chlorophyll photosynthesis. It is highly persistent in icy and dry environments, stable in all pH environments, and readily found in the subsurface and aquatic ecosystems. Atrazine is most effective when applied to wet soil; therefore, it is usually used after heavy rainfall, when the ground is most receptive. For this same reason, atrazine can more easily enter the soil substrate and water systems, eventually flowing into rivers and streams and thus into the marine environment. Since the mode of action of atrazine is to inhibit the process of chlorophyll photosynthesis, the first harmful impact suffered by the aquatic ecosystem concerns the algal population; the reduction in algal growth leads to a subsequent alteration in the aquatic fauna, and the adverse effects are already appreciable at very low concentrations of atrazine, between 1 and 10 μg/L (Graymore et al.; 2001). Even if its use has been restricted in Europe since 1986 and was officially banned in Italy in 1990, recent national and international reports highlighted Atrazine persistence in marine and river waters of the Italian hydric system and Mediterranean basin (Nodler et al.,2013; Ispra,2005). This evidence can be explained by the compound's intrinsic characteristics, as it is highly persistent in water. Moreover, atrazine is still legal in many developing countries, posing a real danger to the natural aquatic ecosystem in the present and the future. Concern about detecting atrazine in the aquatic ecosystem is due to its proprieties as an endocrine disruptor and immunotoxic chemical. Nowadays, Atrazine is included in the list of priority substances within the EU Strategy as an endocrine disruptor in category 1. One of the earliest significant scientific achievements in detecting Atrazine was in 1992 by Christopher and Bird. They linked the die-off of wild fish and avian species in the Chesapeake Bay to high levels of Atrazine in its waters and the subsequent disappearance of aquatic plants in the Bay. Despite these persistent and environmentally resistant characteristics and its widespread use worldwide, it is

surprising that only a few ecotoxicological studies have been carried out to detect its presence and estimate its amount in natural ecosystems and wild species. Studies on atrazine's effects on aquatic fauna showed how this chemical could alter the antioxidant activity in fish and crustaceans, leading to ROS formation and unbalancing the immune system's function (De Albuquerque et al., 2020). Histopathological findings in freshwater fishes demonstrated liver alterations compatible with leukocytosis and hepatocyte modifications, including vacuolization and necrosis (Singh et al., 2018). Adverse effects of Atrazine have been recently reported in *Procambarus clarkia,* which showed hepatopancreas damages, reduced ability to resist infectious diseases, and decreased efficiency of antioxidant enzymes (Yang et al., 2021). The power of atrazine to interfere with the physiological functions of the liver also leads to an impairment in the normal ability of the organism to metabolize other toxic compounds. This condition may exacerbate the detrimental effects of exposure also to other pollutants. Immunotoxic effects of Atrazine's exposure of reptiles include a reduction of serum complement system activity and lysozyme function. Hematological parameters have been affected in different aquatic species by chronic exposure to low levels of Atrazine. In these subjects, hematocrit, hemoglobin, and erythrocytes values showed a consistent decrease (Singh et al., 2018). Atrazine can also affect mammals, acting as an endocrine disruptor altering the normal feedback system of pituitary hormones. Modification in the LH (luteinizing hormone) secretion translates into a prolonged release of prolactin, which can induce changes in mammary glands' tissues, increasing the chances of developing adenocarcinomas (Singh et al. 2018). Furthermore, the endocrine activity of Atrazine can cause reproductive problems with poor quality of sperm and oocytes deformities discovered in rats (Singh et al., 2018).

TRACE ELEMENTS

Trace elements include all the natural elements present in the periodic system apart from macro-elements and Carbon, Hydrogen, Nitrogen, Oxygen, and Sulphur. On Earth, 118 trace elements are naturally present in the environment. Among them, just a few are essential to animal life. Hence, an element is essential when its severe deficiency leads to an impairment of a function that can be resolved by adequate supplementation of that element (Kalisińska, E., 2019). The recognition of the essential activity of trace elements was made possible by the artificial building of an "ultra-clear environment" which allowed to exclude most contaminants. The severity of symptoms and effects of supplementation linked to the degree of the deficiency is summarized in a mathematical formula identified by Bertrand. The rule asserts that a function for which an element is necessary is absent in a total deficiency but increases with increasing exposure to the element. This phase precedes a plateau corresponding to the maintenance of optimal function, followed by a decrescent step down to zero when physiologic regulation mechanisms are overlaid by the toxic concentration of the element (Kalisińska, E., 2019). This principle can be applied to all trace elements. Furthermore, this consideration clarifies some aspects of trace elements' ecotoxicology: a range of safe exposure within which homeostasis can maintain an optimal balance for each element. Despite this, every element can become toxic, exceeding a specific concentration value. Cobalt (Co), Copper (Cu), Chromium (Ch), Iron (Fe), Manganese (Mn), Selenium (Se), and Zinc (Zn). Some other elements, such as Arsenic (As), Fluorine (Fl), and Nickel (Ni), are considered helpful to animals but potentially damaging if accumulated in too high a dosage for the species. Moreover, part of trace elements such as Cadmium (Cd), Lead (Pb), and Mercury (Hg) are well known to be non-essentials for animals and have detrimental effects on wildlife and human beings. Cobalt is a hard metal found in two principal valence states: (Co^{2+}) and (CO^{3+}). Co is used as a drying agent in paintings, additive in fertilizers, and catalyzer in the rubber industry (Lock et al., 2004). Co and its compounds are extensively diffused in nature and present in numerous anthropogenic activities. Co has a

fundamental role in animals entering the cycle that leads to the synthesis of cyanocobalamin (vitamin B_{12}). Nevertheless, excessive cobalt may induce detrimental effects (Leyssens et al., 2017). Adverse effects have been investigated primarily on fish. Studies revealed that high doses of Cobalt induce cytotoxicity, apoptosis, and inflammation. Co can also determine DNA damages due to reactive oxygen species action (Simonsen et al., 2012). In the liver and brain of goldfish (*Carassius auratus*), the activity of catalase enzymes is reduced by Co activity. Lipid oxidation in fish is a standard parameter for information on alterations secondary to reactive oxygen species. This is due to the high polyunsaturated fatty acids being easily modified by peroxidation. Alterations in lipids have been recorded in the brain and liver associated with increasing lipid peroxides (Kubrak et al., 2011).

Copper (Cu) is an essential trace element fundamental for the function of cellular enzymes. It is found in animals in two different forms, Cu II, the oxidized state, and Cu I, the reduced one. Copper is an essential cofactor for redox reactions for proteins intervening in the growth and development of the organism. Cu can become toxic in massive quantities, inducing cellular damages that lead to reactive oxygen species production. Part of the copper is absorbed and reused in the gastroenteric system and body fluids in humans. Especially glutathione has a recognized role in protecting organisms against copper toxicity, helping bind the metal with metallothioneins. Metallothioneins synthesis and induction are mediated by metal responsive transcription factor 1 and metal responsive elements. In mammals, the predominant protein in serum that binds copper is ceruloplasmin, followed by transcuprein and albumin. These three proteins have a role in transporting the excess copper to the liver, which intervenes in the final excretion of copper (Tapieroe t al., 2003).

Chromium exists in Cr (III) and Cr (VI) oxidation states. Instead, the hexavalent form is considered more toxic because of its greater solubility and mobility (Abedi et al., 2013). Hexavalent Chromium is a strong oxidizing form that can cross biological barriers and react with proteins and nucleic acids (Bojarski et al., 2021). Excess Cr in the organism is not reduced to trivalent non-toxic forms and can enter erythrocytes and

white blood cells, inducing anemia (Bojarski et al., 2021). Experiments conducted on rats demonstrated the important function of Cr in maintaining physiological glucose tolerance. This research helped understand which micrograms' doses of Chromium are necessary to preserve glucose tolerance and in which chemical form Chromium shall be administrated. It is important to notice that the inorganic or organic form of trace elements can heavily change the bioavailability of elements. Chromium must be present in an inorganic trivalent form to function. These data testify to the Chromium's role in sugar metabolism, cells, and insulin functions. *In vivo* ed *in vitro* studies later demonstrated general insulin resistance related to Chromium deficiency. Chromium deficiency related to glucose intolerance has also been reported in malnourished African children. Daily adequate intake for humans has been reported to be between 5-200 µg. Chronic exposure to Cr indicated increased glucose, neutrophiles, and albumin in fish. Studies on common carp exposed to trivalent Chromium for 28 days showed hyperglycemia probably linked to enhanced glycogenolysis and glucose production by extrahepatic tissues. Research on the glucose cycle and its absorption in the gut of trout exposed to Cr underlined a reduction in glucose uptake. This condition can lead fish to deterioration of processes necessary to maintain the adequate energy for physiological processes. Furthermore, a decrement of white blood cells was observed, helping the onset of infectious diseases (Abedi et al., 2013). Chromium can also affect terrestrial and avian species. In 2021 a study focused on the effects of hexavalent Cr ondeveloping chickens' embryos. Results showed dose-dependent hatchability alterations (Bojarski et al., 2021). In Japanese quail (*Coturnix japonica*), exposure to Cr VII led to brain, heart, kidney, and liver accumulation. Moreover, birds developed anemia and lowered immune defenses (Suljevic et al., 2021). In humans and marine mammals, inhalation is one of the most dangerous exposures to Chromium. In humanbeings, inhalation of Cr has been linked to the onset of lung cancer, and people who died of this disease have been found with high levels of Cr accumulated both in the bronchi and in the skin (Li Chen et al., 2009). These data connected the airway exposure with the following distribution and excretion of the metal through the dermal

tissue. The same condition has also been described in right whales (*Eubalaena glacialis*). In the marine environment, the toxic hexavalent form is the predominant one. Data recorded for this animal species are worrying because Cr mean skin concentration detected was 7 μg/g, a level 23 times higher than the mean concentration registered for humans not exposed for occupational reasons but were in the same order of magnitude as people daily exposed to Chromium on the workplace and died for lungs' disease (Li Chen et al., 2009). Right whales' fibroblasts and skin cells showed cytotoxic and genotoxic signs, including chromosomes damages. The same results have been obtained by analyzing fibroblasts and lungs cells of Steller sea lions (*Eumetopias jubatus*), Sperm whales (*Physeter macrocephalus*), and Indo pacific dolphins (*Tursiops aduncus*) (Wise et al., 2009; Chen et al., 2012; Yu et al., 2018). These data confirm the urgency to investigate more biological samples to gain deeper information on marine mammals' health status and obtain a wider knowledge of kinetics and the effects of trace elements on wildlife. Iron (Fe) is an essential trace element, abundant on earth, and fundamental for numerous vital functions. Fe bioavailability in nature can be scarce because it reacts with oxygen-forming oxides, which are insoluble and unavailable for the animal organism. In mammals, almost 70% of the iron in the body is detected as the ferrous form (Fe^{2+}) entering the synthesis of hemoglobin and myoglobin, creating the heme compound. Furthermore, iron is stored in different anatomic districts such as the liver, spleen, and bone marrow in the ferric form (Fe^{3+}) as hemosiderin, ferritin, and transferrin. The storage of ferric form is an important part of many enzymes like catalase, cytochrome-c, and peroxidase (Albretsen, J. 2006). Iron is recaptured by the body and delivered to tissues through the action of ferritin protein that catches iron released in the bloodstream and allows the metal entrance into cells where mitochondria use it for the constitution of metalloproteins and heme groups (Abbaspour et al., 2014). In more detail, we can distinguish three major classes of proteins present in animals depending on iron: iron-sulfur cluster containing proteins, heme-containing proteins, and iron-containing enzymes. Iron-sulfur-containing proteins and autonomous iron-containing enzymes

catalyze important chemical reactions. In contrast, proteins binding heme group are an essential part of the complex oxygen transportation process in the body (Kaplan, J., & Ward, D. M. 2013). Despite iron's crucial role in animals' biological processes, its deficiency or excess can lead to severe damages that interfere with physiological immune response. Fe overload reduces phagocytic activities, variation in T lymphocytes structure, and their spreading in tissues (Walker, E. M., & Walker, S. M. 2000). An important consequence of iron excess in the organism is represented by the formation of free radicals, able to induce severe cellular damage. This event ispromoted by the natural ability of iron to stimulate one-electron reactions. For example,Fe^{2+} can easily reduce oxygen to superoxide radicals. Moreover, two superoxide molecules can dismutate, producing oxygen and hydrogen peroxide. Ferrous iron can induce the dissolution of peroxides giving birth to hydroxyl or alcoholic radicals. These chain reactions continue to increase the number of radicals in the organism. Animals can defend themselves against these insults through the action of antioxidants. Antioxidants are compounds able to prevent oxidation. Regarding iron, antioxidants such as vitamin E and C can act differently. For example, they can prevent peroxide formation from chelating the ferrous form or catch free radicals preventing them from inducing harmful effects (Fraga, C. G., & Oteiza, P. I. 2002). In the organism is not present an iron specific excretory pathway. Primarily Fe is eliminated with the bile because of the physiological disruption of hemoglobin and exfoliation of enteric epithelium. Thus, iron balance is mainly linked to digestive processes and diet intake (Dalzell, D. J. B., & Macfarlane, N. A. A. 1999). In fish, the ingestion route is not the only way of exposure; iron uptake can also occur through the respiratory system. In case of an excess of the metal, cases of acute iron toxicity can occur. Several ecotoxicology studies reported acute iron toxicity in fish, which led to mortal respiratory distress accompanied by locomotory dysfunctions, as seen in *Tilapia sparrmanii* and salmonids. In fish species, the main mechanism of iron intoxication is probably linked to gill surface exposure to the metal rather than its ingestion. Indeed, histological findings testify Fe deposits on gill epithelia and destruction of secondary

lamellae in specimens posed in contact with 0·8–1·7 mg l^{-1} of the trace element (Dalzell, D. J. B., & Macfarlane, N. A. A. 1999). Acute iron poisoning is rare in avian species, but chronic intoxications are more common. An exaggerated iron amount ingested with the diet can result in a great quantity of Fe stored in the spleen and liver. Iron can accumulate as haemosiderin inside hepatocytes and Kupffer cells in these organs. This condition is called hemosiderosis, and concurrently with hepatic diseases, it can aggravate general conditions of the host leading to hepatic failure. Moreover, hemosiderosis has been linked to the concurrent onset of infectious diseases (Cork S. C., 2000), demonstrating the implication of iron imbalance in the normal activity of the immune system. Problems in iron homeostasis can be due also to a deficiency of this important trace element in the animal organism. Although Fe deficiency in wildlife is extremely rare, it must be considered, especially when scientists witness major changes in marine wildlife habits, migration routes, and feeding habits due to recent massive climate change. Furthermore, iron depletion can lead to anemia, which has already been demonstrated in land mammals, including humans, weakening the body and lowering immune defenses, exposing the subject to the contraction of infectious and parasitic diseases. Iron is a crucial element for lymphocytes activation because of its role in the synthesis of DNA. Activated lymphocytes uptake Fe from transferrin. The mechanism is normally regulated following cellular needs. Unfortunately, iron deficiency can alter this regulation system, reducing the Fe saturation of transferrin andpreventing Fe's proper release (Brock, J. H., & Mulero, V. 2000). In marine mammalsand sea birds, iron is essential to guarantee primary life functions such as diving and swimming. Most of the oxygen in marine mammals is spent diving and derives from myoglobin (Polasek, L. K., & Davis, R. W., 2001). This implies that a dramatic reduction of iron's bioavailability can compromise hunting and gathering food. As forthe previous trace elements mentioned, Manganese (Mn) is included in the list of essential trace elements because of its role in numerous enzymatic activities. Manganese is a ubiquitous trace element highly exploited in industrial activities entering the constitution of pesticides, ceramic, and glass production. Therefore, it can

also be considered an emerging and persistent pollutant. For this reason, scientists are now paying more attention to the kinetics and potential toxicity of this metal. Manganese metalloenzymes detoxify reactive oxygen species, DNA synthesis, protein, and sugar catabolism. Mn stored in the organism and not exploited for enzymatic reactions is primarily used to remove free radicals such as superoxide (Costa, L. G., & Aschner, M., 2014). It is well known in humans the potential toxicity of manganese. Occupational diseases have been caused by excessive and constant aerogene exposure to manganese, leading to neurological alterations. This condition has been called "manganism". Its clinical signs are like Parkinson's disease because the metal accumulation occurs in the basal ganglia, the same cerebral structures involved in the pathogenicity of Parkinson's (Dobson et al., 2004). Manganese is an important micro-nutrient also in fish, but an internal concentration above the optimal threshold can lead to acute toxicity. In brook charr (*Salvelinus fontanilis*) exposed to a high dosage of manganese, post-mortem analysis revealed a destructive action on sodium homeostasis. Plasma sodium decrease was inversely associated with manganese binding on the gills' surface in these subjects. Moreover, manganese accumulation in the liver and filets caused modifications in sugar metabolism and electrolyte balance. Chronic exposure and relative effects in tilapia and goldfish were recorded, too, underlying a decrease in erythrocytes' number and hemorrhages (Vieira et al., 2012; Aliko et al., 2018). Mn exposure induced systemic oxidative stress in fish and demonstrated an organ-specific antioxidant defense involving different enzymatic classes (Vieira et al., 2012; Aliko et al., 2018). Recent studies have also been conducted on avian species to evaluate the toxic effects of Mn exposure. These studies demonstrated the role of manganese in inducing cytotoxicity in immune cells due to oxidative stress in cocks (*Gallus gallus*). After the exposure, birds showed increased ROS produced and lipid peroxidase activity. These conditions imply serious damage to internal organs, especially organs involved in the immune response. These damages can influence the onset of infectious and parasitic illnesses and the onset of cancers (Liu X. F. et al., 2013). In addition, birds analyzed in several studies focused on Mn

effects showed severe hepatic damages characterized by hepatocellular necrosis with a progressive and irreversible loss of parenchymal organization coupled with necrotic foci infiltrated by mononuclear cells. These histopathological findings translate clinically into severe hepatic dysfunction (Roy et al., 2015). Few bibliographic data are available regarding manganese investigation in marine mammals. Although some papers report the concentration of Mg detected in different cetaceans' species, the absence of knowledge about species-specific threshold values about Mn concentration tolerance prevents an in-depth analysis of the role of this element in the overall health status of the animals under investigation.

Zinc (Zn) is an essential trace element fundamental for the growth and correct functioning of the immune system. zinc is part of thousands of enzymes in animals. It can bind to metalloproteins and then be stored in the brain. In the central nervous system, zinc plays an important role in the proper functioning of synapses (Takeda, A.,2000). Moreover, Zn stimulates the production of metallothionines which affect detoxification mechanisms of non-essential trace elements (Richards, M. P., & Cousins, R. J.,1975). Nevertheless, deficiency or excess of Zinc can cause damage to animals. Zinc poisoning has been recorded in aquatic birds with pancreas and liver abnormalities (Beyer et al., 2004). For fish, zinc toxicity is strongly related to the water's physical and chemical variables, such as hardness, dissolved oxygen, and temperature. Just a few cases of Zn poisoning are reported in fish. Data testify liver is the target organ for accumulation and damage (Papagiannis et al., 2004). In marine mammals, zinc is investigated as an essential trace element, and in literature, it usually does not reach alarming concentrations. Although it is important to monitor zinc presence and levels to gain data on the general health status of the population. Selenium (Se) is an essential trace element involved in thyroid hormone metabolism, antioxidant defense, and immune activities. The biological function of Se is performed by selenium proteins, including selenocysteine residue in their core. Some of the most studied selenium proteins are glutathione peroxidase, which has a fundamental role as an antioxidant enzyme, and thioredoxin reductase, which regulates inflammatory

processes and chemotaxis. Thus, Selenium plays a fundamental role in the immune system's functioning, preventing mutations of viruses or inhibiting the growth of modified cells due to exposure to aflatoxins. Selenium also has a role in the physiological reproductive processes guarantying adequate mobility to sperm and enhancing the release of estrogens. The transport of selenium to tissues is deputed to selenoprotein P. Selenoprotein P helps the organism to react against non-essential trace elements, especially mercury, preventing damage due to the accumulation of these metals (Sobolev et al., 2018). The group of proteins constituted by selenium includes iodothyronine-deiodinase, the essential enzyme involved in the deiodination of thyroxine (T_4), and its transformation to the active form T_3 (triiodothyronine). For these reasons, indirectly, selenium regulates growth, metabolism, and thermogenesis through its action on the pituitary- gonadic axis (Arthur et al., 2003; Safonov V.A., 2008). In the context of ecotoxicology studies, it is essential to mention the role of selenium in the neutralization and detoxification of methylmercury. Indeed, Se is an important antagonist of Hg toxicity, although protective mechanisms of action are not clarified yet. Selenium may promote migration of MeHg from more sensitive organs such as kidneys, liver, and brain to fewer sensitive ones such as muscles. It has also been suggested that Se could act as a competitor with mayor chemical affinity than MeHg, for the same receptors. Furthermore, selenium could promote the conversion of MeHg into compounds of low toxicity, avoiding damage due to oxidative stress. Selenium can be present in the environment and food sources in different forms; the most investigated is selenomethionine because it has the highest absorption power in animalsand humans (Cabanero et al., 2005). Studies on fish species highlighted the protective role of selenium against the detrimental effects of methylmercury. It is demonstrated that selenomethionine increases the elimination of MeHg in zebrafish, shrimps, and goldfish (Gribble et al., 2016). Despite the great importance and protective role of selenium, an excess of this trace element can lead to toxicity. One of the most known damages inducted by Se poisoning is linked to the correct process of proteins synthesis. One of the fundamental elements of proteins' constitution is sulfur (S). Sulfur creates

linkages between two S atoms forming a bridge among amino-acids strains. This condition is fundamental to allowing the building of tertiary proteins in structure. If Selenium is present in the organism in an excessive quantity, it takes the place of sulfur in the formation of proteins, preventing the formation of disulfide bridges. This event leads to the synthesis of dysfunctional enzymes, which affect the normal reproductive process in fish, causing teratogenesis (Lemly A.D.,2002). Moreover, fish intoxicated by selenium showed gross and microscopic alteration of gills' tissue with edema of lamellae and secondary inefficient blood flow and inadequate gas exchange. These issues, in turn, stimulate the insurgence of oxidative stress. The onset of anemia also exacerbates the reduced gas exchange. Anemia is another adverse effect connected with selenium intoxication due to the ability of the metal to bind to hemoglobin, limiting the possibility to transport oxygen (Lemly A.D.,2002). Studies focused on avian species demonstrated the negative effects of an excess of selenium on eggs. Thiscondition has been investigated in multiple marine species, and data confirm it leads todeformities in chickens. Other field research on adult specimens showed liver damage, alopecia, emaciation, and necrosis of the beak related to constant exposure to selenium. The interesting result is that higher values have been recorded in all these studies for aquatic avian species, testifying how intrinsic variables such as a species-specific metabolism and diet can modify the answer of the organism to selenium exposure (Ohlendorf, H. M., & Heinz, G. H. 2011). In marine mammals, selenium has been studied primarily for its defensive action against toxic forms of mercury. Several studies focused on detecting and quantifying these two trace elements, mercuryspeciation, and calculation of the Se:Hg molar ratio to understand if there is a positiverelationship between the metals. Most of these articles related to different species worldwide confirm a strong connection between values of MeHg and Se in two internalorgans of different marine mammals species: the kidney and liver (Martinez-Lopez etal., 2019; Li et al., 2020; McCormack et al.,2020 Page-Karjian et al., 2020) with valuesof Se:Hg molar ratio higher than 1. This result is not surprising since these two anatomic compartments are responsible for metabolic and elimination processes for

xenobiotics. Moreover, studies on marine mammals confirm the chemical behavior of selenium and mercury, and the mechanisms of neutralization of Hg in the organism change with the age and life stages of the host (Li et al., 2020). Adult females analyzed during pregnancy or lactation showed a consistent reduction of Hg and Se concentrations in internal organs, corroborating the hypothesis of trace elements' maternal transfer to the fetus and calves. This hypothesis is also strengthened by analyzing biological samples from fetuses and calves that show high values of trace elements in the organism. While this mechanism of toxic transfer elimination may be beneficial to the adult female, it poses serious risks to the proper development of the puppy. Thus, these data are important because they help the scientific community build prediction models on the future health status of the population in the exam. A clear example of this condition is reported by Grajewska et al., 2019 in a study focused on the analysis of MeHg, Se and their molar ration in pregnant and lactating females of Baltic grey seals (*Halichoerus grypus grypus*), investigating placental and milk samples, and in the blood of their puppies.

Mercury is a non-essential trace element known for its toxicity in humans and wildlife. Mercury emissions can derive from fossil fuel combustion, mineral processing, or mining. Mercury can be transported on winds and water currents far from the source of discharge, amplifying the problem globally. It exists in different forms, distinguished in inorganic and organic mercury compounds. Inorganic Hg forms include metallic mercury and mercurous and mercuric salts. Organic Hg comprises chemical compounds in which the metal bounds to structures incorporating carbon atoms such as methyl or ethyl groups (Bernhof R.A., 2012). After being released into the environment, inorganic mercury can enter the aquatic ecosystem and undergo methylation. Methylmercury has a great affinity for proteins sulfhydryl groups and accumulates in organisms and biomagnify across the food chain. Because of the food chain biomagnification process, the highest trace element concentrations are reported in samples of apex predators such as tuna, swordfish, sharks, sea birds, and marine mammals. Mercury toxicity represents a global concern both for public health and

wildlife. Mercury is widely used in several human economic activities and quickly enters the natural environment. In humans, two main routes of exposure are reported: inhalation of mercury vapor. This condition is primarily linked to occupational diseases, and ingestion of methylmercury is mainly related to the consumption of fisheries products. Methylmercury is quickly absorbed by the organism and readily crosses the placenta and the blood-brain barrier creating deposits in the brain (EFSA, 2012). The first realization of the extreme toxicity of mercury came with discovering the Minamata disease. Minamata disease is a human syndrome due to mercury intoxication diagnosed for the first time in 1956 in Minamata bay in Japan. Despite this massive outbreak, just in 2013, an international convention, the "Minamata Convention", was finally instituted to prevent and limit mercury pollution. Minamata disease was related to an enormous discharge of methylmercury in the sea coming from industrial plants located in the bay. In this Japanese area, during the spring of 1956, 54 people died because of consuming fish contaminated with a high dosage of methylmercury (Eto, K.2000). Thousands of people were affected with irreversible damages. The syndrome included acute and chronic clinical signs. In adults, acute poisoning led to visual and hearing impairment, olfactory and gustatory alterations, and cerebellar ataxia. Minamata diseases also affect pregnant women with repercussions on the physiological development of the fetus. Severe motor and mental development alterations were recorded in children born from intoxicated mothers. Chronic cases of mercury intoxications have been diagnosed only some years later. Chronic poisoning patients displayed paraesthesia of distal extremities and sensory reduction (Ekino et al., 2007). Similar clinical signs have been observed in terrestrial mammals exposed to MeHg. The first signs of intoxication in rodents were anorexia and lethargy, followed by alterations in behavior, motricity, and convulsions. In small mammals, placental transfer was also demonstrated, with concentrations double in the brain's fetus than in the mother's brain. In addition, histopathological findings showed neuronal necrosis and phagocytosis of the cerebellum and cerebrum areas. Methyl mercury interferes with the immune system. Immunotoxicity of this trace element

includes a reduction of T cell proliferation and activity, helping the onset of infectious and parasitic diseases (Wolfe et al., 1998). Mercury can also accumulate in the endocrine system, especially in the hypothalamus, affecting the hypothalamus-hypophysis axis. In confirming this hypothesis, a study conducted on mineworkers chronically exposed to mercury through inhalation showed a metal concentration 3-4 times higher in the pituitary gland and thyroid than in the kidney. Mercury concentrations also vary between sex, probably because of a different mechanism of action of Hg on sexual hormones. In rats, it has been speculated that Hg may interfere with estrogens which have a fundamental role in thymic development. Thus, the interference of mercury on estrogens during pregnancy can affect the physiological development of the thymus in the fetus, causing a following impairment of the immune system. Other research on fish and mice demonstrated Hg influences the sex ratio of offspring. This event can affect the species' future survival, leading to an imbalance between female and male subjects (Tan et al., 2009).

Nickel (Ni) is a non-essential trace element. It is widely exploited in metallurgies activities. This event conduces to important environmental pollution posing at-risk humans and wildlife. Nickel is recognized as an immunotoxin, teratogenic, and carcinogen agent. Ni can induce contact dermatitis, alterations in the respiratory tract such as fibrosis or cancer, and cardiovascular diseases. Mitochondrial alterations due to impairment of membrane potential and DNA destruction can lead to severe forms of oxidative stress. Nickel also has adverse effects on reproduction, acting on Leydig cells and destroying them (Genchi et al., 2020). In fish, a study conducted on common carp showed nickel accumulates primarily in the gills, where it can induce edema, dyspnoea, and alteration of respiratory frequency (Elbeshti et al., 2018). Freshwater species, including gastropods, insects, and amphibians, are highly sensitive to nickel exposure. In marine environments, the most sensitive taxa include echinoderms, mollusks, and crustacea (Wang et al., 2020; Gosh et al.,2021). In those species, characterized by a filter-feeding behavior, short-term exposure at sublethal levels can seriously affect the organisms and increase the progressive accumulation of Ni in superior trophic levels

of the food chain (Nogueira et al.,2020). Nickel may induce detrimental effects in birds also. A recent study showed high levels of Ni accumulated in the liver and kidneys, which exceeded legal limits established for safe human consumption. At the same time, the brain and muscles displayed a lower concentration of the metal (Behrooz et al., 2021). Riaz et al., 2021 demonstrated that feathers could also be a reliable sample to investigate nickel contamination in birds, detecting high metal concentrations in different wild avian species. Results are concerning since levels seem high enough to alter immune and reproductive functions (Riaz et al., 2021). Similar potential adverse effects can be recorded in marine mammals even though most of the data collected in the bibliography show low contamination levels (Outridge, P. M., & Scheuhammer, A. M.,1993; Zeisler et al.,1993). Arsenic (As) is widely distributed in four different oxidative forms. The most common forms of Arsenic in the aquatic environment are arsenate and arsenide. Anthropogenic sources of As release in the environment are pesticides used in agriculture, pigments produced for paints, and coal combustion. Arsenate is a molecular analog of phosphate and exploits the specific phosphatase transport system to enter the organism. Moreover, arsenide form has the power to bind sulfhydryl groups, modifying the correct constitution of proteins (Oremland, R. S., & Stolz, J. F. 2003). Arsenic is useful for animals in small quantities, but an excess can induce severe damage. The most studied mechanism of arsenic toxicity is related to an imbalance between pro-oxidant and antioxidant substances that determines oxidative stress. In the aquatic environment, detrimental effects of Arsenic have been recorded for fish in the form of cytotoxicity in addition to reduced activity of antioxidant compounds and onset of oxidative stress. The first adverse effect in cells regards the reduction of functions of the mitochondrial membranes leading to the formation of superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals. In addition, arsenic can induce the formation of reactive nitrogen species. Arsenic exposure in small rodents and humans also demonstrated chromosomal aberrations testifying to the potential genotoxicity of the metal (Ventura-Lima et al., 2011). Fish are exposed to arsenic through gills, skin, and digestive systems. Although many aquatic species have

evolved with a defensive system of biotransformation to reduce the toxicity of this trace element, several studies showed accumulation of arsenic in gills and liver (Kumari et al., 2019) and pathological behavior linked to swimming alterations and loss of equilibrium. This condition is probably due to the neurotoxic effects of arsenic. Sublethal effects of arsenic include anemia, gallbladder inflammation, and liver modifications. One of the target organs for arsenic to explicit his toxic action is the kidney. This assumes an important value in fish because of the role of the cranial part of this organ in immunity. A recent study demonstrated fish exposed to arsenic show macrophages death, suppression of antibodies, reduction of phagocytic potential and cytokines (Ahmed et al., 2013; Kumari et al., 2019). Arsenic poisoning is also reported in avian species, leading to clinical signs such as depression, emaciation, and dyspnoea (Ghaffar et al., 2015). Other research established the adverse effect of arsenic in broilers with a consistent reduction of hepatic cytochromes activities and onset of oxidative stress and reactive oxygen species (Naraharissetti et al., 2009). In marine mammals, the primary source of contamination for arsenic is food. Different studies focused on different marine mammals species highlight a significant difference among concentration values detected in the liver of mammals feeding on cephalopods and crustaceans and marine mammals having a diverse diet. Indeed, the animals whose diet is closely linked to the consumption of cephalopods and crustaceans show higher levels of arsenic, which probably accumulate arsenic in greater amounts than other fish species. Arsenic can bioconcentrate, but it is not able to biomagnify. Correlation between age of the subjects and As concentrations detected has been reported positively for animals at a low position in the food chain, such as shrimps, but it has not been reported for predators at the apex of the food web (Kubota et al., 2001). The placental transfer has also been reported for arsenic in different species (Kubota et al., 2005; Simokon, M. V., & Trukhin, 2021).

Cadmium (Cd) is a non-essential metal widely used in anthropogenic activities. Cd is present in electronic devices such as televisions screen, lasers, and batteries. Cd is also detected in cosmetic products. In humans, two primary sources of Cd are identified:

inhalation and ingestion. After entering the organism, Cd is absorbed and bound to proteins such as metallothionines containing the sulfhydryl group. Most Cadmium absorbed deposits in the liver and kidney with a half-life of almost 25 years. Cadmium toxicity is linked primarily to induced oxidative stress, inhibition of heme production, modifications in DNA expression, and agonistic disturbance in Zn and Mn actions. Studies on humans highlighted the kidney as a target organ for Cd deposition. Cd affects mainly the proximal tubule resulting in inefficiency in the reabsorption of proteins, glucose, and bicarbonate. This condition is due to oxidative stress's insurgence that stimulates cellular apoptosis. Moreover, cadmium can alter vitamin D metabolism in the kidney, affecting the skeleton structure with osteomalacia and osteoporosis (Bernhof R.A., 2013; Genchi et al., 2013). In addition, the immune system is affected too by cadmium. Exposure during pregnancy can result in post-natal deficiency in lymphocyte T and natural killer cells production. Cadmium is considered an endocrine disruptor and can mimic the action of estrogens (Bernhof R.A., 2013; Genchi et al., 2013). Regarding birds, traces of cadmium have been detected in several avian species worldwide. Different biological samples have been analyzed, including internal organs such as kidneys, liver, and feathers. Data collected demonstrated accumulation of Cd primarily in the kidney, confirming the affinity of Cd for this organ. However, significant and concerning levels of cadmium were also detected in feathers. Verma et al. analyzed internal organs and feathers of different wild avian species in India to verify Cd contamination in these animals. Data obtained are concerning. Indeed, blue macaw feathers' showed Cd concentrations of 450 µg/g, and Eagle 225.6±0.80µg/g in its feathers (Verma et al., 2018). This research is interesting also because it confirms feathers can be exploited as reliable biological samples to investigate Cd contamination. This consideration is vital in wildlife ecotoxicology because it allows scientists to collect samples in a non-invasive way for the animals. Research has also been conducted on poultries; these studies are equally important for public health and the economy, as these are species bred worldwide and intended for human consumption. Data exhibit a species-specific sensitivity to Cd exposure;

nonetheless, above the tolerance threshold, all the species analyzed demonstrated a significant reduction in egg-laying and eggshell quality, dysorexia, and reduced vitality (Olgun et al.,2020). Cadmium uptake from the environment can occur through the skin, gills, and ingestion in fish. It has been suggested that Cd enters the organism by exploiting calcium channels, and inside the cells, it can interfere with enzymes and metallothionines. Despite cadmium stimulating the production of metallothioneins in gills, these proteins seem unable to carry out sequestration and detoxification processes of the metal, probably because of Cd's high affinity for calcium-binding sites in gills. Nephrotoxicity and immunotoxicity have been demonstrated in fish and mammals (Kumar, P., & Singh, A.,2010). Several studies focused on detecting and quantifying cadmium in different biological samples in marine mammals. Results show Cd widespread contamination in terms of both geography and species investigated. Nevertheless, cetaceans and pinnipeds seem to tolerate exposure to cadmium without manifesting severe adverse effects for most of the concentrations recorded in the bibliography. These species have probably evolved, building numerous detoxification systems focused mainly on the induction of metallothioneins which, stimulated by the entry of metal into the host, can sequester it and render it harmless to the organism. Despite that, it is crucial to continue monitoring marine mammals to gain information on species-specific threshold values beyond which adaptation and detoxification mechanisms may no longer be sufficient to protect the organism (Dorneles et al., 2018). Indeed, more recent field research on Mediterranean striped dolphins highlighted a higher level of Cadmium detected in kidneys than previously reported in the literature, emphasizing the importance of further ecotoxicological studies (Esposito et al., 2020).

Lead (Pb) is a trace element not involved in any biological process. The primary source of environmental contamination is the combustion of leaded gasoline, which is still used in non-industrialized countries (Gnassia-Barelli, M., & Romeo, M.,1993). Furthermore, due to lead's low cost and ductility, it has been used for different purposes, such as batteries, paints, shotshell ammunition, and fishing weights. In humans, lead toxicity has been well known since ancient times. The primary route of

humans and animals' exposure to lead is ingestion. Once entered the body, lead is rapidly absorbed. Its strong affinity for sulfhydryl groups in proteins can alter them, explicating its negative effect on several anatomic districts such as the central nervous system, kidney, cardiovascular, and immune system. It is essential to notice that, unlike other non-essential trace elements, no threshold value can be considered safe for lead. (Ab Latif Wani, A. A., & Usmani, J. A.,2015; Gidlow D.A.,2015). Clinical signs of acute and intense intoxication are usually represented by anomalies in the behavior linked to damage to the brain and neuromuscular apparatus. Chronic exposure results in depression, gastro-intestinal alterations, memory, and coordination loss, tingling of extremities, and anemia (Ab Latif Wani, A. A., & Usmani, J. A.,2015; Gidlow D.A.,2015). Lead environmental pollution is a severe problem for wildlife. Numerous studies focused on aquatic species, especially on aquatic birds that can mistake lead spent shotgun for food entering in contact with the contaminant through accidental ingestion. A secondary problem is related to raptorial species, which prey on animals contaminated with lead (Scheuhammer, A. M., and S. L. Norris, 1996). Acute intoxication in birds is caused by the progressive absorption of lead ingested and accumulated in the gizzard. Then can enter the bloodstream through reabsorption by the gut and distributes to organs and tissues. Signs of intoxication include abnormal head position, incapacity to fly and feed due to a paralytic condition that affects the digestive system, and green diarrhea. The paralysis is progressive and extends to wings and legs, preventing the animal from moving (De Francisco et al., 2003; Pain et al.,2019). Chronic conditions include the onset of oxidative stress, reduction of the normal function of the immune system, inhibition of reproductive ability, and difficulties in hatching (Williams et al., 2017; Manning et al.,2019). Furthermore, data recorded on bred avian species demonstrated the active role of Pb in drastically reducing immune defenses also in specimens vaccinated for infectious illnesses such as Newcastle disease (Yussef et al.,1996) also if also administered at a low dosage. This evidence confirms the ability of lead to impair birds' immune system (Yussef et al., 1996). Fish are exposed to lead through the digestive and respiratory systems. Once

entered the organism, lead is processed in the liver, the elective organ for detoxification. In fish, Pb is bound to steroids in the bile. These compounds are then reabsorbed in the enteric tract or excreted with feces. It is interesting to underline that lead is one of the most cumulative metals in fish, and its excretion percentage is very low. Lead contamination in fish causes a vital alteration of electrolytic channels. Sodium, calcium, and potassium homeostasis are perturbated. Ca ions have a competitive behavior toward Pb ions, and the protective action of calcium against lead toxicity is documented in different fish species. These conditions determine the onset of oxidative stress, alteration in the immune system with a reduced white cell count, and reduced synthesis of cytokines (Lee et al., 2019). Lead exposure has also been investigated in marine mammals. Numerous studies report the detection of lead in the internal organs of different marine mammals. Like in other mammals, the toxic effects of lead in cetaceans and pinnipeds regard mainly the renal compartment, potentially affecting its function. Marine mammals have developed a highly sophisticated urinary apparatus characterized by peculiar processes due to the marine environment. Therefore, an impairment of the physiological functioning of the kidneys poses a severe threat to the host's survival.

AIM OF THE STUDY

This study aimed to collect data on different species belonging to the aquatic environment in European geographic areas to deepen the toxicokinetic knowledge of numerous xenobiotics and verify the detrimental effects of ubiquitous pollutants that are potentially dangerous to aquatic ecosystems and public health. The research has deliberately ranged between species at different food chain trophic levels, geographic areas, and aquatic ecosystems. This allowed us to obtain a broader and more complete picture of the state of pollution and contamination of the marine environments taken into consideration, as well as a deeper and more exhaustive knowledge of the state of exposure of wildlife and the defense and pathology mechanisms found in the individual animal species under study. The choice to range in research between different species in different aquatic environments has also allowed us to collaborate with numerous research groups and evaluate multiple aspects of scientific research regarding the approach to sampling, laboratory investigations and technologies, and the final teamwork of writing publications. It is essential to notice that for our study, we exploited many different laboratory methodologies depending on the nature of the organic matrices under investigation, the xenobiotics to be detected, and the most authoritative recent bibliography available.

MATERIALS AND METHODS

Paper n°1

Samples have been collected during necropsies of animals found dead or had died after being referred to the Wildlife Recovery Centers in the study areas of Galicia and Asturias. After necropsy, 57 specimens were frozen and stored at −20 °C until samples were prepared for analysis. From each subject, 3 g of subcutaneous adipose tissue was taken, placed individually in plastic bags, and stored at −20 °C. Eleven OCPs (including metabolites) were assayed: isomer mixture of hexachlorocyclohexane (HCH) consisting of β and γ-HCH; DDT and its metabolites (namely 4,4′-DDD and 4,4′-DDE); hexachlorobenzene (HCB); and the cyclodiene insecticides heptachlor epoxide, dieldrin, endrin, endosulfan, and endosulfan sulfate. Similarly, 7 indicator PCBs (CBs 28, 52, 101, 118, 138, 153, and 180) were targeted, as they are predominantly present in biotic and abiotic matrices and have been recognized as compounds representative of the whole group of PCBs by the Agency for Toxic Substances and Disease Registry (ATSDR, 2000). Reference materials supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) with a purity of 97–99.7% were used for OCPs standard preparation, with concentrations ranging from 10 ppb to 10 ppm. Similarly, a commercial mix of 7 PCBs from SpexCertiPrep (Stanmore, UK) (10 µg/ml in isooctane) was used to quantify PCB congeners IUPAC 28, 52, 101, 118, 138, 153, and 180. Stock solutions (500 µg/ml) were˜prepared by dissolving reference standards in acetone (Panreac) and stored at −20 °C. Working solutions for sample fortification and injection in the GC systems were prepared by diluting stock solutions in n-hexane (Panreac). The protocol followed to perform the PCB and OCP extraction was adapted from a procedure used by Mateo et al. (2012). Samples were thawed at room temperature, and 0.7 g of the tissue was chopped and mixed with 7 ml of n-hexane. The mixture was homogenized and frozen overnight, allowing the fat to precipitate. Five ml of the supernatant were added with 2 ml of H2SO4. Subsequently, the tubes

were shaken in an orbital shaker for 10 min, sonicated for 5 min, and centrifuged at 1000×g for 5 min; the acid-containing phase was discarded. The above procedure was repeated until the acidic phase was completely clear. The resulting extract was evaporated, re-suspended in 200 µl n-hexane, and then used for OCPs and PCB concentration measurements. A Bruker Scion 456 triple quadrupole gas chromatograph-mass spectrometer was used to analyze the samples. Analyte separation was achieved on a Rxi-5 Sil MS column (30 m x 0.25 mm, i.d. x 0.25 film thickness). The results were analyzed using specific GCMS software. The multiple- ramp temperature program used involved a first step of 3.5 min at 70 °C, then the temperature was raised to 180 °C at a rate of 25 °C/min. This was followed by an increase to 300 °C at a rate of 15 °C/ min and a final increase to 325 °C at a rate of 50
°C/min and maintained for 5 min. The vaporized samples were injected in spitless mode at a 1.20 ml/min column flow rate. The injection port, detector, and interface temperatures were 280 °C, 280 °C, and 300 °C, respectively. PCB and OCP residues were quantitatively evaluated using the internal standard method (with 25 µg/l of PCB180 added at the beginning of the extraction process). The calibration curves were obtained by determining the relationship between the peak area and the concentration of the different standards. Solvent blanks (consisting of 500 µl n-hexane instead of tissue) were processed in parallel to the samples to ensure the quality of the analyses. accuracy was estimated recovery percentage, analyzing blank adipose tissue samples (n = 10) spiked at five concentrations levels of PCB and OCP mixtures. Previously, the blank sample was analyzed to determine the content of analytes in triplicate. Recoveries were obtained as the ratio (in %) between the calculated concentration of spiked samples and the theoretical concentration added. The recovery percentages for PCB spiked samples were found between 89% and 109% (CV < 20%), while the recovery percentages for OCPs were between 80% and 128% (CV < 20%). The limit of quantification (LOQ) was established as the lowest concentration level validated with satisfactory values of recovery (70–110%) and precision (RSD < 20%). The limit of detection (LOD) was estimated as the analyte concentration that produced a peak

signal of three times the background noise in the chromatogram at the lowest fortification level studied for each compound. The LODs of those analytes present in the blank tissue sample were estimated from the chromatograms corresponding to the analyzed blank sample (Hernández et al., 2005). The LODs for PCBs and OCPs ranged between 0.006 and 0.079 μg/kg and 0.070–1.124 μg/kg lipid weight (l.w.), respectively. Data were analyzed using statistical software Prism 5 version 5.03 for Windows (GraphPad software, Inc., CA). Normality and homoscedasticity of data were assessed. Since data did not show a normal distribution and the variances were not homogeneous, the statistical analyses were performed using a non-parametric Mann Whitney U-test to evaluate the differences related to both gender and age. Finally, a Spearman test was performed to determine the correlations among chemical levels. Results were expressed as mean ± SEM and range, and the level for statistical significance was defined as $P < 0.05$. Statistical assessments were limited to those chemicals that could be detected in > 50% of the samples. A value of 50% of the LOD was assigned to samples with an undetectable contaminant concentration. These values were included in the dataset for statistical testing, a technique that minimizes nominal type I error rates (Clarke, 1998).

Paper n°2 PBDEs

Tissue samples were collected on-site during necropsy, placed in the aluminum paper, identified by the ID of each whale and by the tissue, and immediately stored at −20 °C at the University of Bologna facilities until analysis. Before shipping to the laboratories of the CSIC (Barcelona, Spain), samples were freeze-dried, and water content was assessed. Sample preparation to detect PBDE, HBCD, DEC, and MeO-PBDE analyses were carried out using a previously optimized sample extraction method (de la Cal et al., 2003; Labandeira et al., 2007). 0.5–1 g dry weight (dw) was spiked with 13C PBDEs and 13C-syn-DP. Samples were kept overnight to equilibrate prior to the pressurized liquid extraction (PLE) with a mixture of hexane:dichloromethane (1:1). Extraction consisted of 2 static cycles of 10 min at 100 °C and working at 1500 psi.

Lipid content was determined gravimetrically after the extraction. Afterward, organic content was redissolved in hexane and treated with H_2SO_4, followed by a solid-phase extraction (SPE) using alumina cartridges (Al-N, 5 g). SPE cartridges were conditioned with hexane and eluted with hexane:dichloromethane (1:2). Extracts were evaporated to incipient dryness under a gentle nitrogen stream at 30 °C and reconstituted to a final volume of 40 µL prior to the instrumental analysis. PBDEs and MeO-PBDEs were analyzed with an Agilent 7890A gas chromatograph coupled to an Agilent 7000B triple quadrupole mass spectrometer. Chromatographic separation was carried out with a DB5 ms column (15 m × 0.25 mm × 0.1 mm of film thickness). The instrumental conditions and elution program were based on our previous works (Eljarrat et al., 2007; Eljarrat et al., 2002). For the spectrometric determination (Barón et al., 2014), electronic ionization at 300 °C was used, with helium as carrier gas. For the analysis of DECs (Barón et al., 2015a), the chromatographic separation was carried out with another DB-5 ms column. Negative chemical ionization at 175 °C was used, with methane as ionization gas and helium as carrier gas. On the other hand, HBCD analyses were carried out by liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) using electrospray ionization (ESI), following the protocol optimized by Guerra et al. (2008). Selective reaction monitoring (SRM) mode was used for all compounds, with two transitions monitored for each analyte. The most intense transition was used for quantification, while the second provided confirmation. Instrumental parameters such as recoveries, reproducibility, method limits of detection (mLODs), and method limits of quantification (mLOQ) are summarised in Supplementary information. Recoveries ranged between 61 and 105%, always within the acceptability range (40–120%) for analytical methods based on quantification by isotopic dilution. mLODs and mLOQs ranged from 0.01 to 1.59 and from 0.03 to 5.30 ng/g lipid weight (lw).

PYRETHROIDS

Paper submitted

Samples were collected during the sperm whales' mass stranding in Vasto in September 2014. Brain, liver, muscles, blubber, heart, feces, and umbilical cord were properly stored and frozen until further investigations. Organic solvents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Solid-phase extraction C18 (2g/15ml) and basic alumina (5g/25 ml) cartridges were obtained from Isolute Biotage and Interchim. Bifenthrin, λ-cyhalothrin, fluvalinate, resmethrin, and a mixture of pyrethroids containing cyfluthrin, cypermethrin, deltamethrin, fenvalerate, permethrin, and tetramethrin were used as analytical standards. D6-transpermethirn and d6-trans-cypermethrin represented internal standards. Pyrethoids' detection was performed by gas chromatography and tandem mass spectrometry (GC-MS.MS) with negative chemical ionization. For Sample extraction, blubber, brain, heart, liver, and muscle of sperm whales (0.1 g dry weight (dw)) were pierced with deuterated internal standards (2 ng of d6-trans- permethrin and 1 ng of d6-trans-cypermethrin). The sample was stirred and extracted by sonication with 20 ml of hexane:dichloromethane (2:1) for 15 min and centrifuged at 3500 rpm for 7 min twice. The two organic phases were mixed in a vial, and the solvent was changed to 10 ml of acetonitrile. Solid-phase extraction (SPE) C18 (2 g/15 ml) and basic alumina (5 g/25 ml) cartridges were taken from Isolute Biotage and Interchim, respectively. The cartridges were conditioned with 25 ml of acetonitrile. The extract was filtered through the cartridges and restored. The extract vials were washed with 20 ml of acetonitrile. The eluate was evaporated and re-dissolved with 100 µl of ethyl acetate. To compute the lipid amount in the samples, 1 g of the liver was also extracted with 40 ml of hexane:dichloromethane (2:1) and evaporated. Finally, the lipid content was measured gravimetrically. The pyrethroid test was carried out with an Agilent 7890A gas chromatograph coupled with an Agilent 7000B triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) as set out by Feo et al. (2011). Compounds were separated with a DB5ms capillary column (15 m × 0.25 mm, 0.1 µm film thickness) with the following thermal

program 100 °C for the first minute, then raised from 100 to 230 °C for 8 min, then from 230 to 310 °C for 8 min and, hold at 310 °C for 2 min. Injector temperature was equal to 270 °C; injection volume was 3 μl; carrier gas was He at1 ml min^{-1}. The mass spectrometer worked in the negative chemical ionization (NCI) mode, 34, using ammonia at 2 × 10−4 torr as reagent gas. The ion source temperature was 250 °C. Selective reaction monitoring (SRM) mode was used, with two transitions monitored for each compound. The most intense transition was used for quantification, and the second transition provided a confirmation comparing the SRM1/SRM2 ratio calculated for the samples with the ratio found in the standards. Details of the MS/MS conditions and the selected transitions in the SRM mode are found in Feo et al. (2010). The analytical method was selected to monitor 18 different pyrethroids: aletrina, bifenthrin, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, fenvalerate, flumethrin, fluvalinate, imiprothrin, kadenthrin, permethrin, phenothrin, pralethrin, resmethrin, tetramethrin, tralomethrin, trasfluthrin. Method recoveries ranged from 53 to 116 %, with relative standard deviations (RSD) consistently below 20%. Method detection limits (MDLs) and method quantification limits (MQLs) ranged from 0.02 to 0.46 ng g−1 lipid weight (lw) in the former and from 0.08 to 1.54 ng g^{-1} lw in the latter.

ATRAZINE

Paper on press

25 serum samples from *Caretta caretta* have been collected from the animal's jugular vein on the same day it was stranded and admitted to the Cetacea Foundation. The samples were individually labeled and stored in the freezer until laboratory analysis. Freshwater samples were taken at two different points of the Savio river course (Fig.13,14) and of the surface waters of the Adriatic Sea in front of the Cesenatico coast; samples were individually labeled and kept until further analysis. Water and blood samples were thawed and centrifuged for 15 minutes at 1000 RMP. Then samples were tested for Atrazine using an antigen-antibody chromatographic method: the ELISA (Enzyme-linked immunosorbent assay) method (ABNOVA, www.abnova.com). The kit was tested for serum and validated by the manufacturers specifically for this study. 50 µL of certified standard blood and water were placed in each well. We carefully added 50 µL of standard (Positive Calibrator containing 10 µg/mL of atrazine) and water and serum samples to the bottom of each well. We slightly tapped the side of the strip holder to distribute the sample evenly and added 100 µL of ATZ-ALP conjugate to each well. Slightly tapped the side of the strip holder to mix the sample and enzyme conjugate properly. We incubated at room temperature for 40 minutes. After incubation, we disposed of the solution in the wells by inverting and shaking. We washed microtiter wells 3 times with wash buffer to remove the non-bound conjugate. After that, we added 100 µL of pNPP substrate to each well and incubated at room temperature for 20 min. Then we added 50 µL of Stop Solution to each well and tapped the strip holder for proper mixing. In the end, we read absorbance at 405 nm using an ELISA reader. Results have been discussed evaluating positive correlation among intrinsic variables of subjects included in the study, such as the total length of carapace and, therefore, the approximative estimated age of the animal and the sex of the specimen.

Heavy metals

Paper n° 3

After octopus captures, the samples' preparation included the record of octopus weight, sex, and total length. Samples were immediately sealed in individual polyethylene bags, frozen at −20 ◦C, and kept at the same temperature until dissection. In the laboratory, the digestive gland of each organism was removed under partially defrost conditions without rupture of the outer membrane. Subsequently, the digestive gland was treated separately from the remaining tissues, and an interior portion was sampled for metal analysis. Arms and mantle were dissected, including the skin. Subsequently, each tissue was homogenized using a laboratory mixer and stored at −20 ◦C until further analyses. Glassware and laboratory equipment were decontaminated before use with diluted ultrapure 65% HNO3 (Romil UpA, Cambridge, UK) and rinsed with Milli Q water (Millipore, Bedford, MA, USA). Aliquots of each sample (0.50 ± 0.02 g) were digested in 5 mL of ultrapure 65% HNO3 and 2 mL of 30% H2O2 (Romil UpA, Cambridge, UK) in a microwave digestion system (Milestone, Bergamo, Italy). The final volume was obtained by adding Milli-Q water. Metal concentrations in the digested samples were determined with an atomic absorption spectrometer (Analyst 600, Perkin-Elmer, Madrid, Spain) equipped with a graphite furnace and a L'vov platform for Pb and Cd. A flow injection analysis hydride system (FIAS 100, Perkin-Elmer) was used to determine the total Hg. The equipment was calibrated with standard solutions (Perkin-Elmer), resulting in a calibration curve with three concentrations for Pb and Hg and four concentrations for Cd. Recovery of the metals was determined by adding known amounts to metal-free samples, which were then subjected to the same digestion procedure. The resulting solutions were analyzed for metal concentrations. Recovery of metals from spiked samples ranged from 85 to 120%. Concentrations of each heavy metal were expressed as milligrams per kilogram wet weight. Quality was monitored by analyzing procedural blanks, duplicate samples, and standard solutions. Standard solutions of analytes were prepared from certified stock solutions of Cd, Pb, and Hg with a relative matrix modifier (atomic spectroscopy standard, Perkin Elmer).

Concentrations for each set of samples were determined in the medium range of the calibration curve. The method's performance was assessed through participation in interlaboratory studies organized by FAPAS (Food Analysis Performance Assessment Scheme, Sand Hutton, UK). The FAPAS studies were conducted with fish tissue. The limit of detection (LOD) and the limit of quantification (LOQ) was calculated by determining the standard deviation of 10 independent blanks spiked at 1, 2, 4, and 8 μg g^{-1} for Cd and 25, 50, and 100 μg g^{-1} for both Pb and Hg, with an external standardization curve. All metal concentrations were expressed in wet weight as mean ± SEM (standard error). Factorial analysis of variance was used to test the statistical significance of the influence of the sampling site (Napoli versus Castellammare di Stabia), the target tissue for metal accumulation (muscle Molecules 2019, 24, 2401 6 of 7 versus digestive gland) on the concentration of heavy metals. Moreover, ANOVA and Mann-Whitney test was used to detect differences between metal concentration in muscle and digestive gland and sampling area. Statistical significance between the concentration of metals and the variables (total weight and gender) were analyzed using multiple regression. The One-Sample Kolmogorov-Smirnov Test confirmed the normal distribution of data. Statistical analyses were performed using MedCalc for Windows, version 18.11.3 (MedCalc Software, Ostend, Belgium). The result, of $p < 0.05$, was considered significant.

Paper 4

Sixty samples of red swamp crayfish were collected during the summer of 2017. Crayfishes were captured using baited traps placed at Villa Literno (ViL), near the Volturno River, and at Sessa Aurunca (SeA), near the Garigliano River in the Campania region. Specimens were then transferred alive in refrigerated boxes (4–8 ◦C) to the laboratory. In our facility, crayfish were weighed and sexed. Furthermore, we measured each carapace length using a caliper from the tip of the rostrum to the edge of the carapace. Crayfishes were euthanized by thermal shock (−80 ◦C for 30 min). Subsequently, the abdominal muscle and the hepatopancreas were removed under

partially defrosting conditions and stored in Falcon tubes at −20 ◦C until further analyses. Each sample was homogenized, and 0.5 ± 0.2 g of tissue was added to 5 mL of 65% HNO3 and 2.0 mL of 30% H2O2. Microwave-assisted digestion was performed with a specific mineralization program for 25 min at 190 °C. Samples were cooled at 32 °C, the digested mixture was transferred into a 50.0 mL flask, and the final volume was obtained by adding Milli-Q water. Trace elements detection and quantification were determined by the ICP-OES technique using a Perkin Elmer Optima 2100 DV instrument coupled with a CETAC U5000AT. Subsequently, both metals quantification and quality assurance procedure were performed as described by Zaccaroni et al. LODs values (limit of detection values) as wet weight were: 0.024 µg g−1 for As; 0.0002 µg g−1 for Cu; 0.006 µg g−1 for Zn; 0.001 µg g−1 for Cr; 0.0018 µg g−1 for Cd; 0.011 µg g−1 for Pb; 0.001 µg g−1 for Hg. The method's performance has been defined by interlaboratory studies organized by FAPAS (Food Analysis Performance Assessment Scheme, Sand Hutton, York, UK). Results are reported in wet weight as mean ± SEM (standard error). Statistical significance of the influence of sampling sites (ViL Vs. SeA) and statistical significance in concentrations of trace elements in target organs (muscle vs. hepatopancreas) was tested using factorial analysis of variance. Furthermore, we apply the ANOVA test to highlight differences between trace element accumulation in the hepatopancreas and the muscle and between the sampling areas. Multiple regression was used to discover statistical significance between trace element concentration and intrinsic variables (total weight and gender of specimens). One-Sample Kolmogorov–Smirnov Test confirmed the normal distribution of our data. Our statistical analyses have been performed using MedCalc for Windows, version 18.11.3 (MedCalc Software, Ostend, Belgium). Significant value has been established at $p < 0.05$.

Paper 5

Cuprous chloride (CuCl, purity 99.9%, code 651,745) and zinc sulfate (ZnSO4, purity 99.9%, code 307,491) were purchased from Sigma-Aldrich (St. Louis, MO, USA). We

set the higher concentration of CuCl and ZnSO4 to below the median 96 h LC50 for invertebrates (240 and 1200 µg/L, respectively) (http:// gestis-en.itrust.de). Prior to the experiment, the concentration of 50 µg/L CuCl and 1000 µg/L ZnSO4 were tested for lethality. Once the higher concentrations were determined, we set up the experiment in which specimens were exposed via a bath-mediated exposure to two concentrations of both compounds: 50 and 5 µg/L CuCl and 1000 and 100 µg/L $ZnSO_4$. Primary stock solutions of CuCl and $ZnSO_4$ were prepared at 0.005 g/L and 0.1 g/L, respectively. A single lot of 245 specimens of *H. bialatus* was purchased from a local dealer for aquarists. The pearl mussels were acclimated for 15 days in 8-L tanks filled with oxygenated filtered artesian well water (20 C±1, pH 7.5±0.3, average dissolved oxygen 8.7 mg/l) at the Experimental Station of the Department of Agricultural, Forest, and Food Sciences (DISAFA), University of Turin (Italy). After acclimation and prior to the experiment, the lethality of 50 µg/L CuCl and 1000 µg/L ZnSO4 was checked. Twenty mussels were placed in two tanks (10 specimens each), one for each compound, under the same conditions described above. Assessment ran for 16 days. Water was changed every day, and compounds were renewed daily. Mortality was checked daily. Since no mortality was recorded, the final experiment was conducted using 50 and 5 µg/L CuCl or 1000 and 100 µg/L ZnSO4. The lower concentration of both compounds was set at tenfold lower than the higher concentration. The remaining 225 mussels were placed in 15 tanks (15 specimens per tank) under the same acclimation conditions. For each group, three tanks were used: control, CuCl (50 and 5 µg/L), and ZnSO4 (1000 and 100 µg/L). The experiment was carried out for a total of 16 days. Every 4 days, 9 mussels from each group (3 from each tank) were sampled. We carried out a trial of 2 weeks and 5 endpoints spaced 24 h and then 96 h apart to monitor both the early effect and the effects over time of biocides on *H. bialatu*s. The digestive gland and the gills were dissected, placed in labeled test tubes, and stored at−80 °C until chemical and biochemical analysis. Metal accumulation Determination of Cu and Zn was performed on the digestive gland and the gills by inductively coupled plasma-optic emission spectrometry (ICP-OES, Perkin Elmer Optima 2100 DV,

PerkinElmer, Inc., Shelton, CT, USA). Samples were homogenized and microwave-digested in a Milestone ETHOS ONE oven (Milestone, Sorisole, Italy) using 4 mL HNO3 and 1 mL H2O2. All reagents were from Merck (Darmstadt, Germany); acids were of Suprapur grade. Results are presented as mg/kg wet weight (w.w.). Instrument performance was checked with blank reagents for each session by processing certified reference material (HISS-1). The limit of detection (LOD) was 0.0003 mg/kg and 0.0007 mg/ kg for Cu and Zn, respectively. Percentages of recovery ranged from 103 to 113% for Cu and Zn, respectively. Oxidative stress biomarkers Oxidative stress biomarker levels were measured in triplicate by spectrophotometry (Varian Cary 50, Santa Clara, CA, USA) at 25 °C on the cytosolic fraction of the digestive gland and the gills. Metallothionein levels were measured in three pooled samples (three specimens each) of the digestive gland or the gills. Tissues were homogenized (1:4) in a 0.02 M TRIS/HCl buffer, added with 0.5 M sucrose, 0.1 mg/ml bacitracin, 0.008 TIU/ml aprotinin, 87 μg/ ml phenylmethylsulfonylfluoride (PMSF), and 0.1 μl/ ml α-mercaptoethanol. Homogenates were centrifuged at 14.500×g to obtain the cytosolic fraction. The supernatants were purified in a chloroform/ethanol solution and then in HCl/ethanol to obtain the metallothionein fraction. Pellets were washed in ethanol:chloroform:TRIS/HCl (87:1:12) solution and resuspended in 0.25 M NaCl. Destabilizing solution (1 N HCl+4 mM EDTA) and Ellman's reagent (DTNB: 5,5 dithiobis-2-nitrobenzoic acid) were added to each sample. Sulfhydryl residue contents (−SH) were quantified at 412 nm. The absorbance of each sample was related to a reduced glutathione calibration curve (1 mg/ml GSH) to obtain the metallothionein concentration. Total glutathione (GSH+2GSSG) was assessed according to a method previously used in invertebrates (Dörr et al., 2020). Thiol concentration was measured in 100 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 unit of GR, 4 mg ml−1 NADPH, and 1.5-mg ml−1 5,5′-dithiobis(2- nitrobenzoic acid) (DTNB), both dissolved in 0.5% NaCO3. The digestive gland and the gill tissues (0.1–0.2 g) were homogenized in 5% sulfosalicylic acid, 4 mM EDTA and centrifuged at 30,000×g for 30 min (4°C). Absorbance was recorded at 412 nm, and glutathione disulfide was used

as a reference. Analysis of MDA was conducted according to the method previously standardized in mussels (Magara et al., 2021). The digestive glands or the gills of three pooled samples of three specimens each were homogenized (1:5) in TRIS/HCl 20 mM buffer pH 7.4 with 0.5 M butylated hydroxytoluene (BHT) and centrifuged. The supernatant was derivatized in 1-methyl-2-phenylindole, HCl, and TRIS/HCl pH 7.4, sample or MDA standard. After incubation at 45°C for 60 min, the samples were centrifuged at 15,000×g and read spectrophotometrically at 586 nm. Enzymatic assays were conducted on the digestive gland and the gills. Tissues were homogenized in 100 mM KP buffer pH 7.5 with 0.1 mg/ml bacitracin, 0.008 TIU/ ml aprotinin, and 2.5% sodium chloride (NaCl) and centrifuged at 30,000 × g. The supernatant was divided into aliquots for each enzymatic assay. The analytical methods for SOD, CAT,GPx, GST, and GR in mussels are described elsewhere (Magara et al. 2021). Briefly, SOD activity was evaluated in 50 mM Na2CO3 buffer pH 10 with 0.1 mM EDTA, 500 mM cytochrome C, 1 mM hypoxanthine, and xanthine oxidase. CAT was determined following the consumption of H2O2. GPx was assessed following NADPHoxidation at 340 nm using H2O2 as substrate. GST levels were measured following theformation of thioether with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. GR activity was determined following the oxidation of NADPH at 340 nm. According to Lowry et al. (1951), the cytosolic total protein concentration was determined tonormalize enzyme activity. Integrated biomarker responses version 2 The integrated biomarker responses version 2 index was calculated following the original method of Beliaef and Burgeot (2002), later modified by Sanchez et al. (2013). In this method, avalue is obtained that summarizes the response of a representative panel of biomarkers.The ratio between the data of each biomarker (Xi) and the mean reference data (X0) was calculated and then log-transformed to reduce variance: The Yi values are relatedto the general mean (μ) and standard deviation(s) of each biomarker and standardized:The mean standardized biomarker response (Zi) and the mean of the reference biomarker data (Z0) were used to determine the biomarker deviation index (A). This allowed us to assume the mean of the control group as the baseline value and then

present in a star plot the biomarker variations we observed in the groups accordingly: Finally, A values for each biomarker in each group were summed. Statistical analysis Normality of biochemical data was tested with the Shapiro–Wilk test. Statistical analysis was performed using two-way ANOVA with concentration, time, and concentration × time interaction as independent variables, followed by Bonferroni's post hoc test.

RESULTS AND DISCUSSION

Results and discussion of the data obtained from our research are summarised here to provide a general picture of the situation that emerged from our studies, thus allowing us to assess the possible contamination and its degree in different wild speciesfollowing exposure to various contaminants of primarily anthropogenic origin. Comparing the different studies we conducted allows us to examine the strengths and weaknesses of ecotoxicology studies applied to wild species. Identifying these conditions will enable us to focus on finding innovative solutions to bypass these gaps and make the studies carried out more efficient and effective.

Data obtained from the analysis of lipidic tissue of Spanish gulls for detecting 18 different POPs in the period going from 2014 to 2016 are concerning. The concern arises mainly because all the pollutants have been detected in the fatty tissue of the 57 specimens included in the study, even though the use of several compounds has been forbidden since the 80s, such as DDT and its metabolites. Moreover, more recent restrictive and prohibitive measures established during the Stockholm Convention in 2001 also comprehended newer compounds we included in the research, such as the cyclodiene insecticides. Our data reflect the persistent pollution from which the Mediterranean area suffers despite the protection measures officially adopted over the past decades. Our findings on the avian species in question, and our subsequent considerations, are corroborated and strengthened by our study on sperm whales stranded on the Italian Adriatic coast. Sperm whales stranded in Italy in 2014 were analyzed to detect halogenated flame retardants in different biological samples. As far we know, this was the first study to investigate this class of compounds in sperm whales. Having no bibliographic data to compare with and given the scarcity of previous references on cetaceans, we wanted to investigate all possible tissue types, including maternal and fetal adnexa and the fetus's tissues. This research helped the international scientific community answer important questions on the vertical transfer of xenobiotics in cetaceans. Our data confirmed the previous hypothesis of this mechanism, which leads mothers to eliminate an important quantity of accumulated toxins but can influence the correct development of the fetus before birth. Even if the only male that was possible to investigate in our study was the fetus, our report suggests an important correlation between values of pollutants accumulated and gender in marine mammals due to pregnancy and following long-term lactation. Correlation among gender and age of subjects analyzed have also been performed for our study on yellow legs gulls, reinforcing just partially the thesis of an existing statistical link between gender and grade of contamination detected in the host. Indeed, a correlation

in 4’4 DDE compound’s concentration has been recorded in birds analyzed, which displayed a higher level of 4’4 DDE in female specimens than in males. As described above for marine mammals, in birds also, these divergences in results obtained can be attributed to differences in metabolism, hormones, and reproduction process. These considerations allow us to draw attention to a fundamental and highly limiting aspect of ecotoxicology studies. Although scientific attempts are made to precisely define any correlations between the data obtained and the intrinsic variables of the subject, such as sex and age, it must be remembered that the study of wild species includes a series of issues difficult to overcome. In this case, the limitation placed on the study is primarily related to the fact that animals are normally simultaneously exposed to a diversity of pollutants. This condition makes it almost impossible to discern which individual pollutant induces each harmful effect with absolute precision. Moreover, it is often difficult to establish which type of synergy or antagonism between the various xenobiotics exists within the host and what these interactions ultimately entail. It is important to underline this since this condition is part of the major problem that makes it almost impossible to establish with certainty a cause-effect link between contaminants and pathological effects in wild species. Another problem is that scientific knowledge of physiology and biology is often poor for most aquatic fauna. The lack of knowledge about the physiological functioning of organs, apparatuses, and evolutionary adaptations to the surrounding environment, which is, moreover, constantly, and rapidly changing, often makes it impossible to establish clearly what the real pathological events are and how serious their consequences are in the short and long term. For instance, in literature, most of the ecotoxicology articles on marine mammals’ wildlife tend to focus primarily on numerical comparisons of the values obtained from laboratory analysis. However, although this is important and sometimes necessary, as often the data available is scarce or non-existent, often this comparison loses scientific significance since there are no previous cases in the literature that can define with scientific certainty threshold values, sub-lethal or lethal values for a specific xenobiotic against a specific animal species. In this sense, *in vitro* studies acquire great importance because this type of research is repeatable under standard conditions and can provide unequivocal data used as reliable parameters applied in field studies. Unfortunately, *in vitro* studies cannot give exhaustive information on multiple xenobiotics' contemporary actions and effects. Moreover, it is impossible to perform them in many wild animal species. For example, only a few sperm whales ’cellular lines are available to be tested in the laboratory. We wanted to perform the

most complete investigation on the 4 subjects stranded on the Adriatic coasts. We decided to investigate all the biological tissues that could be sampled to compensate for the lack of bibliographic information on the species regarding PBDEs' exposure, accumulation, and toxicokinetic in sperm whales. Our data clearly show how these pollutants' distribution reaches high levels in anatomic compartments other than lipidic tissues, such as the liver and muscles, including the cardiac tissue. This evidence should make us reflect on the need to expand studies and extend the investigation to a wider typology of biological matrices to be included in the studies and raise many scientific questions that mainly concern the pathogenesis and kinetics of these toxicants in the species under consideration. In this study, we had the opportunity to analyze three subjects belonging to the same family, which resulted in a sisterhood relationship. This is important information because it allows us to make some considerations that can help us understand the pathological processes these animals have gone through. First, considering the species' biology, we know females sperm whales remain together for all their lives under the guidance of the oldest one. Therefore, it is reasonable to think that the subjects have frequented the same places while feeding on the same sources. These considerations are fundamental since they erase multiple variables that we would normally think could affect the outcome of the data obtained. Results of analyses of PBDEs in the heart and muscles of the youngest subject, a nulliparous one, were higher than the ones recorded for the other sexually mature female and for the oldest one, which was pregnant. Thanks to the genetic and biological knowledge available, we can assume that these divergences in data must be related to the life stage of the animals and their reproductive status. In yellow legs seagulls, the diffuse POPs contamination we highlighted and differences related to gender pose questions on the hormonal response of the subjects to pollutants' exposure, which could be different in males and females. The organism's need to face the repeated insult of persistent pollutants could induce the specimens to change male ethological behavior, prioritizing their survival rather than reproduction processes. This event is linked to the interference of endocrine disruptors with corticosterone, which is involved in response to stress, enhanced by exposure to POPs. Furthermore, OCs mime steroids and bind to their receptors, inducing an altered response in reproductive behavior. Reproductive problems are aggravated by a significant statistical relationship between xenobiotic accumulation and age. According to previous literature, our data show a strong correlation between increasing concentration levels and age. Sexually mature adults have the highest contaminants values, corroborating the hypothesis that endocrine disruptors can alter

the breeding success of the species, posing a serious risk to the species future survival. Even though the data we have collected and the toxicokinetic studies of the chemicals support these hypotheses, further investigations are indispensable for clearly defining the harmful role of these contaminants in the reproductive processes of the species. Subsequent analyses should consider a larger number of subjects and be performed on a representative sampling of all of the population, including specimens of all ages and genders in numbers comparable to each other. This consideration highlights another important limitation that often characterizes ecotoxicological studies on wildlife. Indeed, collecting samples that optimally meet the needs of the study is often impossible. Firstly, because usually wildlife is highly protected. Therefore, the samples obtained for research are generally occasional. The fact that the samples available are occasional makes it difficult to obtain an overall representative sample of the population. It is also important to consider the state of preservation of the samples themselves, as they generally come from animals that have died of unknown causes at an unknown time. In addition, it is not always possible to carry out exhaustive studies investigating all possible biological samples. Especially when working with live animals, wildlife protection requires that samples be obtained minimally invasive as a matter of ethics. This implies that only feathers, fur, skin, feces, and urines are often available for investigations. This is important to understand because it often prevents a thorough study of the behavior of the chemical species within the host. However, detecting old compounds, some of which are banned in Europe or have been restricted in their use, in two different wild species of the Mediterranean basin occupying the apex of the food chain is alarming. Even if these data underline how old and well-known compounds can perpetrate even very long-term damage to aquatic wildlife, we did not want to underestimate the toxic potential of newer chemical compounds. Therefore, we wanted to extend the study on sperm whales, also analyzing newer chemical compounds such as pyrethroids. Pyrethroids in the aquatic environment are persistent due to their hydrophobicity and reduced water solubility. They can enter the food chain primarily through the gills and the gastroenteric system of fish and top predators. Potential toxicity attributed to these insecticides include neurotoxic, carcinogenic, reproductive, and immune suppression adverse effects. Few data are available in the literature on pyrethroids. This is probably due to the ability of these insecticides to be quickly converted into non-toxic metabolites both in mammals and fish. These pollutants are relatively new in use. The data we collected have not been published yet. As far as we know, this is the first study conducted on sperm whales and

focused on pyrethroids detection. We decided to test these compounds because ecotoxicology must keep up with the development of new chemical compounds and their introduction in the market to monitor and detect any adverse environmental effects at the earliest possible stage. Moreover, there was a complete absence of bibliographic data about these chemicals and their behavior in marine mammals. We selected 17 different pyrethroids to be tested: bifenthrin, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, fenvalerate, flumethrin, fluvalinate, imiprothrin, kadenthrin, permethrin, phenothrin, pralethrin, resmethrin, tetramethrin, tralomethrin,and trasfluthrin. Samples available for this analysis were blubber, heart, liver, and muscle investigated in all the three females stranded. Moreover, we had the chance toanalyze the brain of the two older specimens and the umbilical cord of the oldest, pregnant one. Of 17 pyrethroids investigated, only three were detected in the samples available. Tetramethrin was the chemical compound reaching the highest valuesdetected, and surprisingly its highest concentrations were recorded in the muscles of the three subjects. This find could be linked to the mobilization of pollutants in the organisms of the three females since they were found emaciated and affected by infectious diseases confirmed by viral analysis in all the subjects, including the fetus and kidney failure of the oldest specimen. Myoglobin in the muscles of marine mammals is an essential protein for oxygen transportation and storage. Most of the oxygen marine mammals exploit to carry out deep dives derives from muscle mass andmyoglobin. Alterations in these structures and their functions could affect diving and hunting. Nevertheless, in this specific case, the results obtained by the investigations conducted on heart tissues, which are considerably high in the three cetaceans, rather suggest a higher chemical affinity of pyrethroids for muscle tissues and their components than for other anatomical districts. This is an interesting finding because our data shows that tetramethrin concentrations detected are higher than the blubber concentrations, which are traditionally considered the target organ for detecting these chemicals. Once more, this underlines the importance of investigating all the possiblebiological samples available. In addition, since this is one of the first studies focused on pyrethroids in cetaceans, including the opportunity to analyze also maternal-fetus adnexa, it is impressive to observe that even if tetramethrin reaches the highest concentrations in our samples, it has not been identified in the umbilical cord asking questions about whether the contaminant is transferred to the fetus through a vertical route. On the contrary, permethrin, the other pyrethroid detected in most samples, reaches lower concentrations in all the anatomical compartments investigated in all

three sperm whales. It has also been identified in the umbilical cord. The presence of pyrethroids in the maternal fetus adnexa could have a double meaning and, therefore, double interpretation. Indeed, the detection of permethrin in the umbilical cord may be linked to sequestration and accumulation of the contaminant in the umbilical cord, thus preventing or reducing its transfer to the fetus. Nevertheless, it is also possible that the detection of permethrin in the umbilical cord demonstrates the ability of the compound to permeate the maternal-fetal adnexa and accumulate in the pup itself. To obtain more information on the kinetics of permethrin related to its transfer to the fetus, it would have been essential to analyze fetal tissues. Still unfortunately, due to the scarcity of samples available to the various research groups, this opportunity could not be exploited. However, it is possible to compare these results with those of the PBDEs tested. This comparison suggests that it is possible that, as with PBDEs, the detection of pyrethrin in the umbilical cord corresponds to the presence of the compound in fetal tissues. One last meaningful consideration that emerges from our study is that although few samples were analyzed, there is no clear correlation between the concentrations of pyrethroids found in the three subjects and their age. This results in the need for further studies to investigate the behavior of these chemicals in the sperm whale. Eliminating the possibility of interference from external variables that acted on the three specimens, i.e., mainly their different geographical distribution in the Mediterranean and a difference in diet, this finding suggests that the discrepancies found may be conditioned by intrinsic subjective variables, such as metabolic rate, endocrine balance, synergies and antagonism among pollutants accumulated, immune system functions and concomitant diseases. Ecotoxicology is a science that needs to face on a daily basis with advances in technology and the use of increasingly modern compounds, without neglecting the pollutants that have been regulated and banned in the past decades but continue to leave a negative impact on wildlife. Therefore in the light of the results of these initial studies, which show that there is significant residual contamination of large marine vertebrates with chemical compounds that have not been in use for decades, we decided to perform research focused on atrazine and its detection in surface fresh and marine water of Cesenatico area and in serum of loggerhead turtles (*Caretta caretta*) stranded alive in the same geographic area. Even though atrazine is a well-known pesticide that has been widely used for decades in Europe and has chemical characteristics that ensure its persistence in aquatic environments, few studies on its detection and possible native effects on aquatic apex predators are available in the literature. Few studies have previously been conducted on reptile eggs, particularly

exposing eggs during the hatching period to an atrazine solution. These trials showed a reduction in the number of eggs able to hatch, particularly when exposed to concentrations between 2 and 200 μg/L. In addition, a study of *Nerodia sipedon* aquatic snakes, in which laying females were exposed to the same atrazine levels as above, showed that very few of these snakes survived (Van Der Kraak et al.; 2014). The literature available on research into the harmful effects of acute and chronic atrazine exposure on turtles is extremely limited. Nonetheless, research carried out on freshwater species (*Trachemys scripta*) has shown that contamination of these animals with atrazine is correlated with drastic changes in the correct functioning of the immune system, with an appreciable reduction in the physiological phagocytic and macrophagic response, thus making the subjects easily attacked by infections of various kinds which, following the acquired immunodepression, can overwhelm the organism. A significant reduction in lysozyme release was found in red-eared turtles exposed to atrazine. In reptiles, lysozyme plays a key role in counteracting pathogens that have entered the animal body. Lysozyme is an important marker of the pro- inflammatory response in reptiles, has antibacterial functions, and measures the innate immune response. Specifically, this condition was also found by Walsh et al.; in a 2010study carried out on *Caretta caretta*, in which they were able to demonstrate the correlation between herbicide poisoning and exposure to algal toxins, with a significantreduction in the production and release of lysozyme into the circulatory stream(Soltanian, S.; 2016). Second, research on the analysis of atrazine present in *Caretta caretta* eggs demonstrated high contamination, raising the question regarding the contextual contamination of several different chemical compounds and their possible synergy (Allan et al.; 2017). Another research instead sought to verify the potential endocrine disruptor action exerted by atrazine on the reproductive efficiency of turtles by searching for a possible correlation between atrazine action, exposure temperature of eggs in the nest, and sex ratio of the unborn children to find out whether atrazine had activity on sex hormones. This study demonstrates the correlation between atrazine, and turtle nest temperature, the influence of these synergies on the sex ratiosof unborn hatchlings, and how higher nest temperatures increase the endocrine-disrupting activity of atrazine on animal organisms (Willingam E.; 2005). Further investigations into atrazine exposure in turtle breeding systems have revealed that even at low concentrations, this herbicide can act by de-masculinizing individuals and thus preventing normal reproduction in the species under investigation (Hayes et al.; 2011). Our results are very alarming, considering that all serum and water samples tested

positive for atrazine even though this herbicide was officially banned in Italy in 1990. The concentrations of atrazine found in the blood of common turtles analyzed in our study show levels very similar to the average concentrations found in 2016 in L. kempii turtles from Mexico. The highest value found in our study is 38.9 ng/ml, which is not an insignificant level of contamination considering that in our country, the use of substances containing atrazine has been banned since 1992, while in Mexico, its use is still permitted. It should be stressed that the use of blood samples as a research matrix always and only allows us to analyze a 'snapshot' condition, i.e., to photograph a precise and fleeting moment of the contaminant's pharmacokinetics within the body, as the blood simply acts as a means of transport for the pesticide. Although atrazine concentrations are high compared with those found in sea turtle serum from countries where atrazine is still legal, we cannot predict the future of the substance in our study subjects. Because we were unable to analyze other tissues that typically act as storage sites for the chemicals, such as the liver, kidney, or muscle, we cannot determine whether and how much of the atrazine found in the animals' circulatory streams was eliminated or accumulated and thus posed a potential risk to their survival. In addition, it should also be noted that, unlike the research carried out on the Mexican specimens, the animals we assessed were not in an optimal state of health but were all defecated, stranded in a severe condition, underweight, parasitized, or suffering from trauma and infection. Therefore, it is reasonable to assume that some of the atrazine found in the circulatory stream may have been released from storage organs in response to the body's mobilization of lipid energy reserves in distress. This evaluation is very important because, considering this hypothesis, the presence of atrazine in the serum we analyzed may not necessarily be recent but simply represent the movement of a previous accumulation of contaminants in the body that has not yet been disposed of. No positive correlation has been found between atrazine serum concentration and the total length or sex of the animals included in the study. However, it must be noted that the samples we collected are unfortunately small in number and that further important information on the kinetic dynamics of the toxicant could be obtained by also analyzing the components of the eggs laid by the females to discriminate whether part of the contaminant is eliminated from the individuals through the process of egg formation and laying. a condition that is impossible for us to achieve now as the study region is not a usual nesting site for this species. This could be true, at least partly, considering that the presence of atrazine also found in the water samples analyzed raises the suspicion of current exposure to the compound. these preliminary results certainly

represent an important starting point for continuing the monitoring of the region's coastal waters and marine fauna. indeed, to date, constant and prolonged monitoring is the best method for acquiring significant data to assess the true state of atrazine contamination of the aquatic ecosystem. Our research wanted to include also other animal species occupying different and lower trophic levels than the apex predators, which may be prey for the latter and traditional and regular food consumed by humans. There are two main reasons for this choice: firstly, it allows us to obtain an overview of the pollution situation in aquatic ecosystems by analyzing biological indicators belonging to different levels of the trophic chain; secondly, it allows us to assess the risk to public health and the final human consumer. The three research we conducted focused on common octopus (*Octopus vulgaris*), red swamp crayfish (*Procambarus clarkii*), and shark fin mussel (*Hyriopsis bialatus*), which represent three aquatic species belonging to different Phyla, different aquatic environments, different ecology, and biology, and which are exploited by humans for different purposes. These species have in common that they are considered excellent bio-indicators of the health of the ecosystem of which they are part and that humans use them for economic or food purposes. The studies we carried out fit into this perspective and in this context. We decided to conduct studies focused on the quali-quantitative detection of trace elements considering both essential and non-essential ones. We analyzed the presence of Cadmium, Lead, and Mercury in muscles and digestive glands of common octopuses collected in two different coastal sites of the Italian south Tyrrhenian Sea. Cephalopods are optimal bioindicators for the marine environment and represent an important food source regularly consumed by local people. Therefore, monitoring non-essential trace elements in this species acquires an important double meaning. Italian coastal areas have suffered numerous changes in the last years, including an important anthropogenic pressure. The common octopus is a benthonic, resident species feeding mainly on gastropods, crustaceans, and bivalve mollusks, living in direct contact with the seabed, representing a potential source for uptake of pollutants. Non-essential trace elements can accumulate in common octopus. This condition may threaten the host’s survival and indirectly represent a risk for humans. Our data demonstrated the presence of all three metals in both muscle and digestive glands of octopus sampled in the coastal area of Napoli and Castellamare di Stabia. Although these two geographic areas are well known for their severe degree of pollution, the levels recorded in our study do not comply with the parameters of the law regarding food safety for the consumer. This is crucial information because lead concentrations recorded in Castellamare exclude the

product from human consumption. Our data confirm the importance of constant monitoring to maintain public health and the importance of continuing to monitor the geographical area and its pollution status. Moreover, it should be noted that all samples were positive in the investigations carried out, a condition that should not be considered normal. This research points out also the importance of carrying out constant and lasting monitoring over time linked to the fishing economy. Indeed, the possibility of periodically checking the product's safety also makes it possible to guarantee the opportunity for many categories of workers to carry out their profession adequately. Furthermore, our data show a substantial difference between the concentrations found in the Naples and Castellamare samples, highlighting the different pollution profiles in these two geographic areas. In Naples samples, higher levels of Cd and Hg were recorded from samples of Castellamare, while Pb concentrations were higher in Castellamare samples. This evidence shows no correlations with gender and size of animals according to what was previously reported in the literature for cephalopods. Results obtained in our study display an important difference in the order of magnitude for all the three trace elements analyzed in the digestive gland compared with the muscles. This confirms the fundamental role of the digestive gland as the primary site for accumulation and detoxification mechanisms to defend the organism. To assess the state of pollution of some Italian aquatic environments and at the same time to evaluate the healthiness of fishery products regularly consumed by the local population and therefore linked to the economic activities of the country, we carried out a study focused on the species *Procambarus clarkii.* This freshwater crustacean is one of the most common species in Italy, and there has been a strong increase in its consumption in recent years as a traditional dish in some Italian regions. To maintain a strong link between our research, we have carried out a quali-quantitative analysis for trace elements in edible tissue samples of Red Swamp Crayfish collected in the Campania region. The decision to focus the study on this species was motivated by several factors. firstly, we sought to make our overall work homogeneous, so just as with the study on the common octopus, we decided to focus on a widely consumed species of economic and health interest that was a good bioindicator comparable in terms of the trophic level occupied in the food chain and that was sampled in the same Italia region in two well-known polluted areas.

Data gained from this study are interesting because they show appreciable differences in the concentrations of trace elements from the two sampling areas. Overall, the values

recorded for the samples collected in Villa Literno are significantly higher than those found in the samples from Sessa Aurunca. This condition is particularly evident for zinc and arsenic values. The results obtained for arsenic and zinc are of great scientific importance. Although they are counted among the essential trace elements, an excess of them in the food can affect the development of diseases in the final consumer. Although this is well known, no European or Italian regulation about maximum legal values established for edible portions of crustaceans exists concerning As, Cr, Cu, and Zn. Nevertheless, American and European institutions have indicated some tolerable upper intake values for public health risk assessment. Regarding this specific concept, our data cause concern as the zinc and copper values detected in the Villa Literno area to exceed the established safe threshold value, being also considerably higher than the available bibliographic data. Furthermore, our data on the detection and accumulation of arsenic in Red Swamp Crayfish's muscle are also very interesting. Indeed, even if no upper intake level has been established for arsenic by any governmental institution, a maximum concentration of 50 $\mu g\ L^{-1}$ has been identified. This threshold value is well below the mean arsenic concentrations we detected in our muscles' samples, especially for the Villa Literno site. Although it is reported in the literature that generally, most of the arsenic accumulated in crustaceans is present in its organic form, which is defined as less toxic than the inorganic one, our data again underline the importance of continuing to monitor the species in this area and to investigate further, including the speciation of the contaminant, to carry out a proper risk analysis with scientific certainty. Concerning non-essential trace elements, our results Our data identified low levels of contamination of the species, even recording values below the detection limit for mercury. Surprisingly, therefore, the results obtained from our study show that the consumption of Red Swamp Crayfish could potentially be unsafe due to the accumulation of trace elements considered essential rather than due to non-essential trace elements reiterating the urgency of always performing comprehensive ecotoxicological studies, including a broad spectrum of trace elements to be investigated and analyzing not only the target organs for the considered xenobiotics.

As found previously in our studies, although high concentrations of pollutants were found in the target organs, such as in this case, the hepatopancreas, the data obtained from other tissues were equally important and meaningful. To conclude our survey, we decided to carry out research focused on the potential toxicity of trace elements to the pearl mussel *Hyriopsis bialatus*. We decided to focus the study on the kinetics and effects of copper and zinc on the mollusk. This decision is partly linked to the unexpected data obtained from the Red Swamp Crayfish study and partly to the characteristics of this shellfish. Continuing to follow our thread in the research, we have chosen *Hyriopsis bialatus* because it is a multi-utility aquatic organism. It is an optimal bioindicator of the freshwater environment mainly because it siphons nutrients from all the water columns; it has an important economic value because of its quick reproductive cycle, which is continuous throughout the year. Moreover, it has important defense systems located primarily in the digestive gland constituted by antioxidant and biotransformation enzymes, which have the role of detoxifying the mussel. Nevertheless, the scientific community has begun to devote attention to these mussels because of their recent dramatic decline linked to environmental deterioration and poor water quality. One of the worldwide recognized causes of poor water quality is the wide use of anti-fouling paint. Biofouling is a global problem that the shipping industry has always led. Thus, metal-based anti-fouling paints were widely diffused to preserve submerged portions of ships. In recent decades, after the ban in 2008 of anti-fouling paints composed of tributyltin, the naval industry has tried to replace these environmentally harmful compounds with more eco-sustainable paints. These eco-friendly mixtures are rich in copper and zinc and result in the release of large quantities of zinc and copper into the aquatic environment contributing to the increasing pollution of the ecosystem. As far as we know, our study is the first to investigate the effects of copper and zinc forms on a pearl mussel. Under standardized laboratory factors, we exposed the mussel for 16 days to cuprous chloride (CuCl 50 and 5 µg/L) and zinc sulfate (ZnSO4 1000 and 100 µg/L). Then, on days 1,4,8,12, and 16, we measured the oxidative stress by evaluating selected oxidative stress biomarkers. To ensure

standardization of research, we processed the gills and digestive gland samples using the same preparation and digestion protocols of our studies mentioned above. Then glutathione, thiol, MDA (Malondialdehyde), SOD (superoxide dismutase), CAT (catalase), GPx (Glutathione peroxidase), GST (glutathione S transferase), GR (glutathione reductase) were measured. Our results displayed a time-related accumulation of copper both in the gills and digestive gland. While zinc demonstrated just a transient and reduced accumulation in the gills of the mussels. Nevertheless, our study showed that copper and zinc compounds could induce oxidative stress inmussels. Thanks to using an integrated biomarkers response index, we calculated the response of enzymes to exposure to trace elements. Both digestive gland and gill samples reacted with increased MT and GPx activity. A reduction of total glutathionewas also recorded and linked to the defense mechanism against metals driven by thiol.It is interesting to notice that although these data confirm the activation of powerful mechanisms to inactivate or reduce the toxic reactions on the mussels regarding copperexposure, we recorded a scarce stimulation in MT synthesis after zinc contamination even if bibliographic data witness metalloproteins are largely induced by zinc exposure in several species of mussels. This finding contrasts with our study's substantial absence of zinc accumulation. This leads to the assumption that other defense mechanisms are put in place by the species in question to inactivate the action of zinc.The lack of Zn in gills and the digestive gland may depend on the ability of the metal to bind with glutathione, forming complexes that can easily be excreted. Another interesting result we recorded is linked to the activity of SOD and CAT enzymes whichappear severely compromised by trace elements exposure, especially by zinc. So, in contrast with previous data available, our study suggests that zinc exposure can interfere with the physiological protective enzymatic mechanisms in *Hyriopsis bialatus*, weakening the antioxidant power of the host. Therefore, this event may reduce the effectiveness of protective mechanisms against xenobiotics or even promotethe ultimate production of free radicals. We collected important data not only becausethey are the first to be recorded on this species but also because they investigate in-

depth the kinetics and toxicity of two trace elements that are considered essential. Just as in the Red Swamp Crayfish study, our research demonstrates the importance of focusing on contaminants that are considered strictly harmful and those chemicals that are traditionally counted as less dangerous because they are also involved in physiological homeostatic mechanisms. Moreover, our research highlights the complexity of the potential interactions between xenobiotics and innate immune protective mechanisms. Our study also demonstrates how the use of animal species adaptable to laboratory procedures and considered good bio-indicators makes it possible, through experimentation carried out under controllable and standardized conditions and the application of suitable mathematical models, both to discern the activity carried out on the host by each contaminant and to assess the synergies or antagonisms between the substances that interfere with the animal organism. Therefore, study models such as ours can open the door to a new way of approaching ecotoxicology and investigating highly complex processes such as wildlife exposure to xenobiotic mixtures. From an economic and public health point of view, our study demonstrates how pollutants in the aquatic environment can interfere with the survival of the species, thus affecting the economy and representing a potential risk to consumers' health. This is especially because the mollusks are consumed whole, which includes the ingestion of the digestive gland, the preferential site of accumulation of large quantities of xenobiotics.

Overall, the collected data are not encouraging and require careful evaluation to implement future ecotoxicological studies.

PART A: POPS

Paper n.1: **Concentrations of chlorinated pollutants in adipose tissue of yellow-legged gulls (*Larus michahellis*) from Spain: Role of gender and age**

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Concentrations of chlorinated pollutants in adipose tissue of yellow-legged gulls (*Larus michahellis*) from Spain: Role of gender and age

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ABSTRACT

Concentrations of 7 different polychlorinated biphenyl (PCB) congeners, and eleven organochlorine pesticides (OCPs) and metabolites, including DDTs (dichlorodiphenyltrichloroethane), HCHs (hexachlorocyclohexane isomers), Endosulfan, Endosulfan sulfate, Endrin, Dieldrin and HCB (hexachlorobenzene), were determined in adipose tissue of 57 yellow-legged gulls collected from NW and N Spain. Furthermore, the possible differences due to two endogenous factors, age and gender, were determined. All the analyzed PCBs were detected in over 66% of the samples, with levels of 291.9 (PCB 180), 34.5 (PCB 118), 0.7 (PCB 28), 432.6 (PCB 153), 225.5 (PCB 138), 1.3 (PCB 101) and 0.4 (PCB 52) μg/kg of adipose tissue. With respect to the OCPs and metabolites, only 4,4′-DDE and HCB were detected in more than 50% of the samples, with means of 360.6 and 2.5 μg/kg of adipose tissue, respectively. From all the considered contaminants, only 4,4′-DDE levels presented significant differences depending on the gender, with females showing higher values than males ($p < 0.01$). Significant differences ($p < 0.001$) were also found related to age for the levels of PCBs 180, 138, 101, 28 and 153, as well as 4,4′-DDE, with adult levels being higher than those in young birds. The results of the present study constitute a baseline to better assess the environmental impacts of PCB and OCP contamination at other coastal sites for future bio-monitoring studies, with particular emphasis on gender- and age-related differences.

1. Introduction

Polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) are included within the group of persistent organic pollutants (POPs). POPs are widely used chemical compounds of environmental concern, and feature long-range transport, resistance to metabolism and potential toxicity (Ashraf, 2017). The presence of these compounds are generally a result of industrial, commercial and agricultural activities. Moreover, they have become ubiquitous in the environment where, in recent decades, they have been found to cause adverse effects on humans and wildlife. Several of these compounds have been implicated, for example, in decreased reproductive success in fish-eating water-bird populations in contaminated areas (Choi et al., 2001a) and it has been shown that they can affect oxidative stress levels (Fenstad et al., 2016). These contaminants are fat soluble and not readily degradable in the environment. Furthermore, they have the potential to biomagnify and to accumulate in high concentrations in animals at the top of the food chain, considered at risk. Aquatic organisms, and those exploiting aquatic resources, are particularly exposed to increasing levels of pollutants since aquatic systems are usually the ultimate pollutant sink, either due to diffuse sources, or direct discharges from the environment (Ramos et al., 2013). Both OCPs and PCBs are a cause for concern for nearshore marine ecosystems already threatened by a variety of human activities and pressures (Good et al., 2014).

Coastal and estuarine areas from Galicia (29,575 km^2) and Asturias (10,603.57 km^2), two regions located along the northern

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Spanish coastline, are characterized by touristic, industrial, fishing, shipping, dredging and aquaculture activities, and/or contamination events. Even if there are well-preserved natural areas in both regions, there are also several important cities and industrial plants (for example, close to the cities of Vigo, A Coruña, Ferrol and Gijón). The NW/N Atlantic coast of Iberia is known to host over 80,000 yellow-legged gull (*Larus michahellis*) breeding pairs, as well as a wintering population comprising several gull species, most of which are yellow-legged gulls travelling from the Mediterranean (Arizaga et al., 2013). These seabirds, considered an integral part of aquatic ecosystems, have become sensitive biomonitors of the changes occurring due to both natural and anthropogenic factors in similar areas of Europe (Morales et al., 2016). Indeed, since the condition and reproductive success of seabirds are influenced both by the conditions of breeding areas and in remote places where they live outside the breeding season, they can be used as proxies to assess the impact of many variables affecting their environment in temporal and spatial terms (Falkowska et al., 2016). Seabirds are generally high consumers and subject to accumulation of marine pollution, and are commonly used as sentinel species for exposure to persistent contaminants. In addition, *Larus michahellis* is markedly adaptable when choosing habitats, often in the vicinity of coastal population centers. In those habitats, they can breed successfully in buildings, feeding on residues from both dumps and discards from local fishing activities. Fledglings, which have not yet been subjected to pollutant bioaccumulation, are useful for monitoring temporal and spatial changes in pollution levels around the breeding area (Abdennadher et al., 2010). Thus evaluation and measure of the effects of contaminants in living organisms and their environment are also influenced by endogenous factors such as gender and age (Burger, 2007). However, many endogenous factors have received considerable attention in wildlife, being gender one of them. The sex of a bird can affect exposure and accumulation of pollutants. One conventional explanation for differences in chemical burden suggests their transfer from breeding females to the eggs. However, results from studies on the effect of gender on toxic burden in birds are not consistent nor established for every chemical.

The aim of the present study was to evaluate the levels of different POPs in adipose tissue of gulls from different regions of the Atlantic coast of Spain, in order to determine whether organic contaminant exposure poses a threat to the environment under study. In addition, with the interest of determining the suitability of this seabird species as a bioindicator, the possible differences related to two endogenous factors, age and gender, on POPs levels was also investigated.

2. Material and methods

2.1. Study areas and sampling

Gulls were collected during the period of 2014–2016 in the regions of Galicia and Asturias (respectively situated in the NW and N of Spain) (Fig. 1). Collected animals were found dead or had died after being injured and referred to the Wildlife Recovery Centers situated in the study areas. Recovered birds suffered mainly from physical injuries, including electrocution, fall from the nest due to inexperience in flying, and others of unknown origin. Injured birds included in the study were those that had not been held at the Recovery Centre for more than 5 days before dying (the average stay was approximately 2 days). Diet during recovery was likely free of environmental contaminants. Different species of fresh fish were bought in the fish market and was for human consumption. The species were chosen depending on the size (preferable small), the protein/fat ratio, and the price.

During necropsy, several parameters such as mass measurements (g), organ weights (g), bill development and physical condition were registered. Age was determined based on the color plumage, as there is a significant color range to pure white adults, and 1-year-old juvenile gulls can easily be discerned from adult conspecifics using plumage characteristics (Grant, 1986). Gender was determined through observation of the gonads during necropsy. Twelve female juveniles, 16 female adults, 13 male juveniles and 16 male adults were identified. After sampling, the remains were destroyed hygienically by incineration, under current European legislation.

After necropsy, all specimens ($n = 57$) were immediately frozen and stored at −20 °C until samples were prepared for analysis. From each corpse, a portion of approximately 3 g of subcutaneous adipose tissue was taken, placed individually in plastic bags, and stored at −20 °C. The complete data set included 25 juveniles and 32 adults. In terms of gender, there were 29 males and 28 females.

2.2. Reagents and quantification of chlorinated compounds by GC/ MS analysis

POPs were analyzed in adipose tissue. Eleven OCPs (including metabolites) were assayed: isomer mixture of hexachlorocyclohexane (HCH) consisting of β and γ-HCH; DDT and its metabolites (namely 4,4′-DDD and 4,4′-DDE); hexachlorobenzene (HCB); and the cyclodiene insecticides heptachlor epoxide, dieldrin, endrin, endosulfan, and endosulfan sulfate. Similarly, 7 indicator PCBs (CBs 28, 52, 101, 118, 138, 153, and 180) were targeted, as they are predominantly present in biotic and abiotic matrices and have been recognized as compounds representative of the whole group of PCBs by the Agency for Toxic Substances and Disease Registry (ATSDR, 2000). Reference materials supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) with a purity of 97–99.7% were used for OCPs standard preparation, with concentrations ranging from 10 ppb to 10 ppm. Similarly, a commercial mix of 7 PCBs from SpexCertiPrep (Stanmore, UK) (10 μg/ml in isooctane) was used for single quantification of PCB congeners IUPAC 28, 52, 101, 118, 138, 153, and 180. Stock solutions (500 μg/ml) were prepared by dissolving reference standards in acetone (Panreac) and stored at −20 °C. Working solutions for sample fortification and for injection in the GC systems were prepared by diluting stock solutions in n-hexane (Panreac®).

The protocol followed to perform the PCB and OCP extraction was adapted from a procedure used by Mateo et al. (2012). Briefly, samples were thawed at room temperature and 0.7 g of the tissue was chopped and mixed with 7 ml of n-hexane. The mixture was homogenized and frozen overnight, allowing the fat to precipitate. Five ml of the supernatant were added with 2 ml of H_2SO_4, the tubes were subsequently shaken in an orbital shaker for 10 min, sonicated for 5 min and centrifuged at 1000 × *g* for 5 min, and the acid-containing phase discarded. The above procedure was repeated until the acidic phase was completely clear. The resulted extract was evaporated, re-suspended in 200 μl n-hexane and then used for OCPs and PCBs concentration measurements.

A Bruker Scion 456 triple quadrupole gas chromatograph mass spectrometer was used to analyze the samples. Analyte separation was achieved on an Rxi-5 Sil MS column (30 m x 0.25 mm, i.d. x 0.25 film thickness). The results were analyzed using specific GCMS software. The multiple-ramp temperature program used involved a first step of 3.5 min at 70 °C, then the temperature was raised to 180 °C at a rate of 25 °C/min. This was followed by an increase to 300 °C at a rate of 15 °C/min and a final increase to 325 °C at a rate of 50 °C/min, and maintained for 5 min. The vaporized samples were injected in splitless mode at a column flow rate of 1.20 ml/min. The temperatures of the injection port, detector and interface were 280 °C, 280 °C and 300 °C, respectively. PCB and OCP residues were quantitatively evaluated carrying out the internal standard method (with 25 μg/l of PCB180 added at the beginning of the extraction process). The calibration curves were obtained by determining the relationship between the peak area and the concentration of the different standards. Solvent blanks (consisting of 500 μl n-hexane instead of tissue) were processed in parallel to the samples to assure the quality of the analyses.

To verify the suitability and performance of the procedure, the

Fig. 1. Sampling area, located in the coastal regions of Galicia and Asturias (from West to East), in NW-N Spain. The red points indicate the Wildlife Rehabilitation Centers where animals/samples were submitted.

Table 1
Concentration of PCBs and OCPs (expressed in μg/kg l.w.) in adipose tissue samples of *Larus michahellis* (n = 57).

Concentration	%	Mean	SEM	Median	Minimum	Maximum
PCBs						
PCB 28	100	0.7	0.2	0.2	0.1	7.2
PCB 52	66.7	0.4	0.1	0.2	< LOD	2.7
PCB 101	98.3	1.3	0.3	0.6	< LOD	11.5
PCB 118	100	34.5	7.7	8.4	0.9	241.6
PCB 138	100	225.3	48.7	50.1	8.5	1280
PCB 153	100	432.6	94.0	107.7	13.6	2856
PCB 180	100	291.9	65.8	68.3	7.2	2009
OCPs and metabolites						
4,4'-DDE	100	360.6	121.3	74.7	5.7	6970
HCB	70.2	2.5	1.1	1.1	< LOD	9.2
Heptachlor epoxide	40.4	1.0	0.3	0.3	< LOD	16.4
4,4'-DDD	35.1	124.3	37.7	36.4	< LOD	1233
Endrin	28.1	0.8	0.3	0.1	< LOD	3.5
Endosulfan	15.8	0.6	0.1	0.4	< LOD	1.2
γ-HCH	12.3	0.8	0.2	0.4	< LOD	11.3
Endosulfan sulfate	5.3	0.7	0.2	0.2	< LOD	2.9
Dieldrin	3.6	0.5	0.2	0.4	< LOD	1.1

% – represents the % of the total amount of samples that contained the specific chemical (samples where the chemical was detected). SEM: standard error mean.
LOD (limit of detection) – PCB 101: 0.028, PCB52: 0.073, HCB: 0.214, heptachlor epoxide: 0.258, 4,4'-DDD: 0.572, endrin: 0.080, endosulfan: 0.324, γ-HCH: 0.379, endosulfan sulfate: 0.167, and dieldrin: 0.231.

accuracy was estimated by means of recovery experiments, analyzing blank adipose tissue samples (n = 10) spiked at five concentrations levels of PCB and OCP mixtures. Previously, the blank sample was analyzed to determine the content of analytes in triplicate. Recoveries were obtained as the ratio (in %) between the calculated concentration of spiked samples and the theoretical concentration added. The recovery percentages for PCB spiked samples were found between 89% and 109% (CV < 20%), while the recovery percentages for OCPs were between 80% and 128% (CV < 20%). The limit of quantification (LOQ) was established as the lowest concentration level validated with satisfactory values of recovery (70–110%) and precision (RSD < 20%). The limit of detection (LOD) was estimated as the analyte concentration that produced a peak signal of three times the background noise in the chromatogram at the lowest fortification level studied for each compound. The LODs of those analytes present in the blank tissue sample were estimated from the chromatograms corresponding to the analyzed blank sample (Hernández et al., 2005). The LODs for PCBs and OCPs ranged between 0.006 and 0.079 μg/kg and 0.070–1.124 μg/kg lipid weight (l.w.), respectively.

2.3. Statistical analysis

Data were analyzed using statistical software Prism 5 version 5.03 for Windows (GraphPad software, Inc., CA). Normality and homoscedasticity of data were assessed. Since data did not show a normal distribution and the variances were not homogeneous, the statistical analyses were performed using a non-parametric Mann Whitney *U*-test, to evaluate the differences related to both gender and age. Finally, a Spearman test was performed to determine the correlations among chemical levels. Results were expressed as mean ± SEM and range, and the level for statistical significance was defined as $P < 0.05$. Statistical assessments were limited to those chemicals that could be detected in > 50% of the samples. A value of 50% of the LOD was assigned to samples with an undetectable contaminant concentration. These values were included in the data-set for statistical testing, a technique that minimizes nominal type I error rates (Clarke, 1998).

3. Results and discussion

3.1. Levels of chlorinated contaminants

Levels of PCBs and OCPs, including metabolites, were determined in adipose tissues from 57 *Larus michahellis* from different locations along the coast line of NW and N Spain. The concentrations (given as mean and standard error) of individual PCBs and OCPs are presented in Table 1, and reported on a lipid weight basis (μg/kg l.w.). Concentrations of both groups of contaminants are known to be higher in adipose tissue and provide a more representative evaluation of the cumulative internal exposure than those found in different tissues. This is due to the fact that measurement in other tissues (for example blood) will fluctuate during lipid mobilization (i.e., body weight loss, or breeding), rendering adipose tissue samples as preferable bioindicators of body burden, when available (Achour et al., 2017).

PCBs were the dominant compounds among various organochlorine compounds (OC) analyzed in terms of percentage of positive samples, although the concentration of both congeners 101 and 52 were below the limit of detection in 1 and 19 samples, respectively. On the other hand, the five remaining congeners were detected in 100% of the analyzed samples, with highest mean and maximum concentrations corresponding to PCBs 153 and 180.

Regarding OCPs and their metabolites, and in agreement with

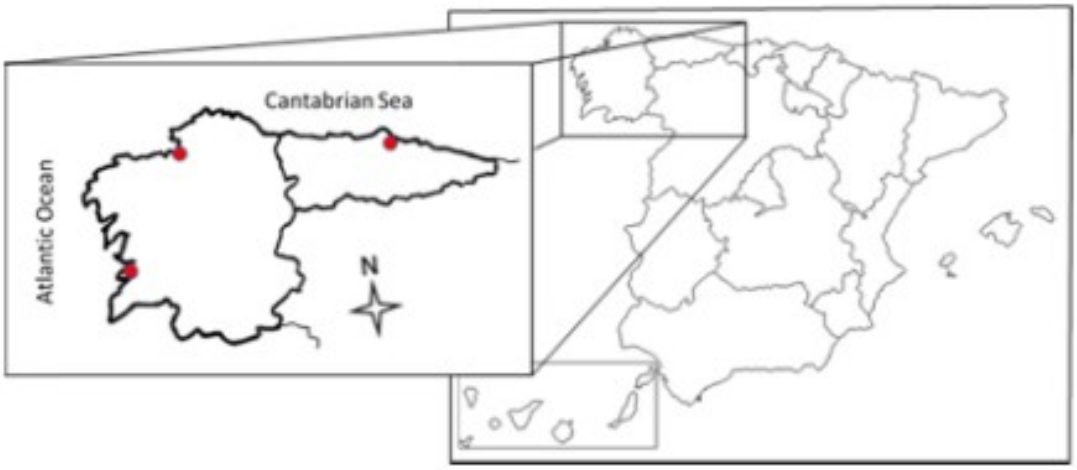

Fig. 1. Sampling area, located in the coastal regions of Galicia and Asturias (from West to East), in NW-N Spain. The red points indicate the Wildlife Rehabilitation Centers where animals/samples were submitted.

Table 1
Concentration of PCBs and OCPs (expressed in μg/kg l.w.) in adipose tissue samples of *Larus michahellis* (n = 57).

Concentration	%	Mean	SEM	Median	Minimum	Maximum
PCBs						
PCB 28	100	0.7	0.2	0.2	0.1	7.2
PCB 52	66.7	0.4	0.1	0.2	< LOD	2.7
PCB 101	98.3	1.3	0.3	0.6	< LOD	11.5
PCB 118	100	34.5	7.7	8.4	0.9	241.6
PCB 138	100	225.3	48.7	50.1	8.5	1280
PCB 153	100	432.6	94.0	107.7	13.6	2856
PCB 180	100	291.9	65.8	68.3	7.2	2009
OCPs and metabolites						
4,4'-DDE	100	360.6	121.3	74.7	5.7	6970
HCB	70.2	2.5	1.1	1.1	< LOD	9.2
Heptachlor epoxide	40.4	1.0	0.3	0.3	< LOD	16.4
4,4'-DDD	35.1	124.3	37.7	36.4	< LOD	1233
Endrin	28.1	0.8	0.3	0.1	< LOD	3.5
Endosulfan	15.8	0.6	0.1	0.4	< LOD	1.2
γ-HCH	12.3	0.8	0.2	0.4	< LOD	11.3
Endosulfan sulfate	5.3	0.7	0.2	0.2	< LOD	2.9
Dieldrin	3.6	0.5	0.2	0.4	< LOD	1.1

% – represents the % of the total amount of samples that contained the specific chemical (samples where the chemical was detected). SEM: standard error mean.
LOD (limit of detection) – PCB 101: 0.028, PCB52: 0.073, HCB: 0.214, heptachlor epoxide: 0.258, 4,4'-DDD: 0.572, endrin: 0.080, endosulfan: 0.324, γ-HCH: 0.379, endosulfan sulfate: 0.167, and dieldrin: 0.231.

accuracy was estimated by means of recovery experiments, analyzing blank adipose tissue samples (n = 10) spiked at five concentrations levels of PCB and OCP mixtures. Previously, the blank sample was analyzed to determine the content of analytes in triplicate. Recoveries were obtained as the ratio (in %) between the calculated concentration of spiked samples and the theoretical concentration added. The recovery percentages for PCB spiked samples were found between 89% and 109% (CV < 20%), while the recovery percentages for OCPs were between 80% and 128% (CV < 20%). The limit of quantification (LOQ) was established as the lowest concentration level validated with satisfactory values of recovery (70–110%) and precision (RSD < 20%). The limit of detection (LOD) was estimated as the analyte concentration that produced a peak signal of three times the background noise in the chromatogram at the lowest fortification level studied for each compound. The LODs of those analytes present in the blank tissue sample were estimated from the chromatograms corresponding to the analyzed blank sample (Hernández et al., 2005). The LODs for PCBs and OCPs ranged between 0.006 and 0.079 μg/kg and 0.070–1.124 μg/kg lipid weight (l.w.), respectively.

2.3. Statistical analysis

Data were analyzed using statistical software Prism 5 version 5.03 for Windows (GraphPad software, Inc., CA). Normality and homoscedasticity of data were assessed. Since data did not show a normal distribution and the variances were not homogeneous, the statistical analyses were performed using a non-parametric Mann Whitney *U*-test, to evaluate the differences related to both gender and age. Finally, a Spearman test was performed to determine the correlations among chemical levels. Results were expressed as mean ± SEM and range, and the level for statistical significance was defined as $P < 0.05$. Statistical assessments were limited to those chemicals that could be detected in > 50% of the samples. A value of 50% of the LOD was assigned to samples with an undetectable contaminant concentration. These values were included in the data-set for statistical testing, a technique that minimizes nominal type I error rates (Clarke, 1998).

3. Results and discussion

3.1. Levels of chlorinated contaminants

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gull adipose tissue, in agreement with the few previous investigations in seabirds (Espín et al., 2010; Fromant et al., 2016). Correlations between POPs of different chemical families have previously been documented in seabird plasma, and strongly suggest that contaminant exposure happens by feeding on prey containing similar relative amounts of both PCBs and DDTs (Finkelstein et al., 2006). In the present study, both contaminant groups (PCBs and OCPs) were positively correlated in males (correlation coefficient: 0.55, and p-value = 0.002) and females (correlation coefficient: 0.6, and p-value = 0.001). It is important to note that correlation studies are important with respect to exposure assessment and determination of how well a measure of one specific contaminant can reflect that from another xenobiotic.

3.2. Gender and age-related differences

Comparisons among sub-groups of samples were performed (juvenile males, juvenile females, adult males and adult females). Results for male-female and juvenile-adult comparisons are shown here. It would appear that the uptake, biokinetics and response to contaminants differs significantly between male and female individuals. This may be due to differences in metabolic rates, hormonal or reproductive states and size (Burger, 2007). As observed in Fig. 2, only the levels of 4,4′-DDE statistically differed according to gender, with females showing a higher mean level than males (606.2 and 115.6 μg/kg adipose tissue, respectively). Conversely, levels of PCBs 180, 138, 101, 28 and 153 were slightly higher in males than in females; however, those differences were not statistically significant.

As shown in Fig. 3, PCBs 180, 138, 101 28 and 153, as well as the pesticide 4,4′-DDE presented significant differences associated with age ($p < 0.001$with higher levels in adult animals compared to young ones. The mean highest concentrations for adult animals corresponded to PCB 153, 4,4′-DDE, PCB 180 and PCB 138 (871.2, 770.6, 588.3 and 457.1 μg/kg adipose tissue, respectively) whereas these same mean values corresponding to young animals were markedly lower (89.9, 40.2, 60.4 and 44.2 μg/kg adipose tissue, respectively).

Significant gender differences in lipid-corrected concentrations have been observed only for DDT in liver of different Arctic seabird species. The present study showed similar results for adipose tissue, suggesting that reproduction (and more specifically egg laying) did not have a lasting effect on OC concentrations (Buckman et al., 2004). In the same way, gender was not found to have significant relevance on the concentrations of different POPs in liver of glaucous gulls sampled in Greenland (Cleemann et al., 2000), even if the concentrations found in adult males were in general higher than those quantified in adult females, and a similar result was obtained with razorbill samples from SW Spain (Espín et al., 2010). However, other studies have confirmed a gender-related influence, associated with the fact that breeding females transfer some of their tissue contaminant burden to their eggs, also suggesting that maternal transfer favors the less persistent congeners

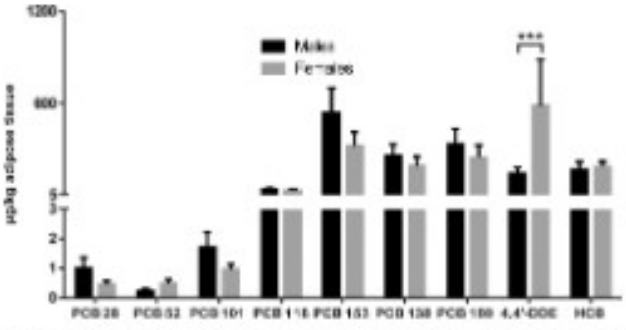
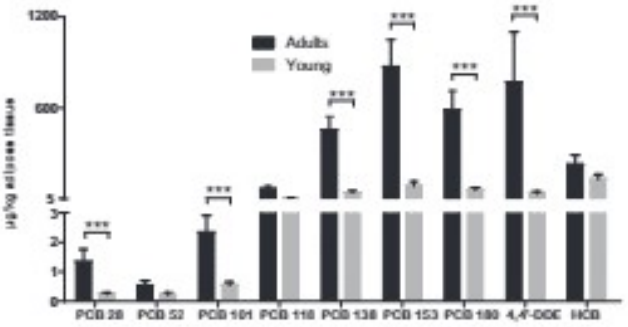


Fig. 2. Levels of chlorinated contaminants (mean ± SEM) according to gender (male vs. female). ***differences were statistically significant ($p < 0.001$) between males (n = 29) and females (n = 28).



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(Bustnes et al., 2008). This statement could not be confirmed with the results found in the present study. Although gender can clearly affect animal exposure, toxicokinetics and levels of contaminants, the effects of gender on POP bioaccumulation do seem to differ among species, populations, organs, and specific contaminant, rendering the study of gender-related differences of extreme interest. More specifically, one of the most challenging aspects of the study of gender-related effects will be in the understanding of mixtures, since living organisms in nature are not exposed to only one, two, or even three contaminants. Information from field studies, like the present study, could offer information about the similar patterns followed by different contaminant groups, which can show correlation in their accumulation. Furthermore, the time course of exposure varies, and the order of exposure may differentially affect outcomes (Burger, 2007). Erikstad et al. (2013) suggested that pollution stress might produce different hormonal responses in males and females, leading to sex-dependent differences in breeding effort. In this sense, males could redirect their behaviour towards their own survival by reducing their breeding investment, due to the higher levels of corticosterone in males than in females provoked by pollution stress. OCs may act as endocrine disrupters by mimicking steroids and binding to hormone receptors, and different hormonal responses related to breeding effort among male and female in gulls seem possible. These data suggest that further study on gender-related evaluation would be of great relevance for future biomonitoring.

When determining the effect of age, it must be considered that bioaccumulation is a key feature in any study on persistent pollutants and is directly correlated with the age of the bird. Based on bioaccumulation, it is reasonable to suppose that older birds exhibit higher concentrations of contaminants in similar diets and geographical ranges (Cipro et al., 2013). This is in accordance to the findings of the present study, where statistical differences ($p < 0.001$) were found between adults (ΣPCBs = 1990 ± 403 μg/kg, ΣOCPs = 1002 ± 340 μg/kg) and young (ΣPCBs = 139 ± 17 μg/kg, ΣOCPs = 171 ± 33 μg/kg). In fact, the majority of studies show a positive association between age and concentrations of both DDT/DDE and PCBs. The few studies that do not show a relationship generally had small sample sizes, a limited age range to allow detection of an age difference, or a low exposure population. Age may also be a marker for cohort-related changes in exposure levels, with older individuals being exposed to higher levels in the past, as well as a marker for age-related shifts in weight and metabolism. Significant differences between adult and young glaucous gulls were found in a study carried out in Greenland (Cleemann et al., 2000); all compounds analyzed, especially PCBs and DDTs, were accumulated with age, and the PCB burden in adults comprised a higher proportion of the higher chlorinated PCBs than that found in young gulls. Similarly, a significant positive relationship was found between age and OC levels in adipose tissues of razorbills from Spain, with the highest levels in adults (Espín et al., 2010). In Gyrfalcons (*Falco*

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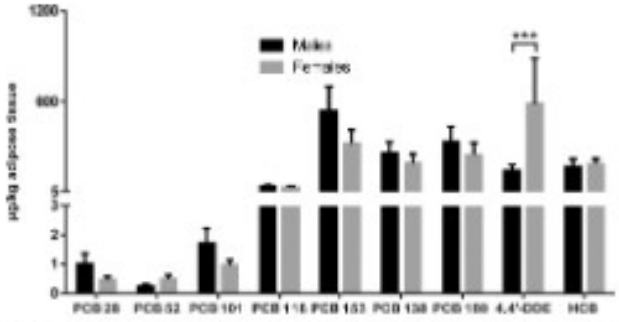

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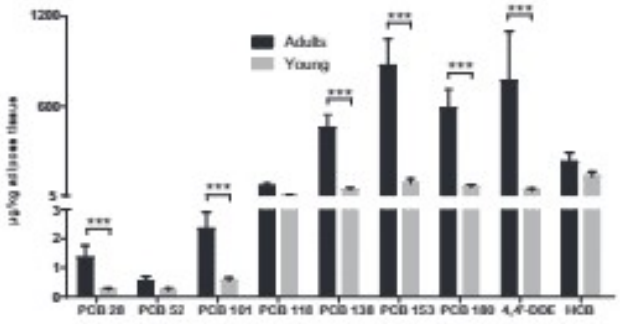

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rusticolus) (Ólafsdóttir et al., 1995), the OC levels were markedly related to age, so that the PCB levels had increased about 100-fold from newly hatched chicks to an 18-months old bird. Phillips et al. (2003) showed that the total PCB concentrations and total PCB body burdens in the adult albatross were higher than those in the chicks, and similarly, there was a general increase in PCB concentrations with increasing age observed in common cormorants from Japan (Guruge et al., 2000). However, contrasting results have been reported in two different seabird species, black guillemots (*Cepphus grille*) in Iceland (Ólafsdóttir et al., 2005) and thickbilled murres *(Uria lomvia)* in Canada (Donaldson et al., 1997), with no evidence of accumulation of PCBs and DDT with age (1–9 years). Moreover, some studies have suggested that steady-state levels are reached at different ages for different chemical compounds. For OC, it was suggested that intake equals the elimination at a yearly basis and that the steady-state equilibrium is reached around the age of reproduction in birds (Erikstad et al., 2013). This is the case for PCBs, which can reach an equilibrium more rapidly than DDE. This may be related to the individual diet in seabirds, which can overshadow the effect of age, perhaps rendering the trophic level at which those animals feed of great relevance (Bustnes et al., 2003).

4. Conclusion

Results from the present study constitute a baseline to better know the environmental levels of PCB and OCP contamination, filling a gap of knowledge related to the specific accumulation of OC in adipose tissue from seabirds. Results will be useful for further environmental biomonitoring studies developed at other coastal sites. Due to the variability in results depending on the species, feeding, pollutant exposure and other conditioners, differences related to gender and age, as well as other directly related factors, must undoubtedly be considered for further biomonitoring studies.

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Paper n.2: **Halogenated flame retardants in stranded sperm whales (*Physeter macrocephalus*) from the Mediterranean Sea**

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This paper is presented here, although it was published just before the beginning of the PhD, as it should be considered prodromic for the following paper on pyrethroids in sperm whales, submitted for publication.

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Halogenated flame retardants in stranded sperm whales (*Physeter macrocephalus*) from the Mediterranean Sea

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HIGHLIGHTS

- Samples from freshly dead sperm whales were collected in 2014.
- Different halogenated compounds were detected in many tissues, including foetus' ones.
- Mother-embryo transfer of halogenated contaminants was proved.
- Detected concentrations could have contributed to patho-physiological alterations observed.

GRAPHICAL ABSTRACT

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ABSTRACT

In recent years, decline of marine mammals' populations and increased frequency of strandings have arised the interest on the role that pollution may have in these events. The present work aimed at quantifying levels of brominated flame retardants (BFRs) and dechloranes (DECs) in tissues of 3 adult females and one foetus of sperm whales stranded in the Southern Adriatic Sea coasts (Italy). Results proved the presence of different flame retardants (FRs) in tissues of sperm whales, including various polybrominated diphenyl ethers (PBDE) congeners (47, 99, 100, 154, entering the composition of PentaBDE mixture), hexabromocyclodecanes (HBCDs), Dec 602 and methoxylated polibrominated diphenyl ethers (MeO-BDEs). In blubber, a target tissue for contaminant accumulation, ΣPBDEs reached values of 160, 158 and 183 ng/g lw, α-HBCD of 5.75 ng/g lw, Dec 602 of 1632 ng/g lw and MeO-BDEs of 563 ng/g lw. The availability of foetal tissues allowed evaluating the potential maternal transfer on many of these compounds, and to discuss the potential adverse effects on foetal health. To the best of our knowledge, obtained data are the first reporting placental transfer of FRs in sperm whales. PBDE levels detected in foetus suggested a potentially long-term exposure to BFRs, which could cause severe damages to the developing organism, likely at the cerebral, endocrine and immunologic levels. Dec 602, which was detected at the highest concentrations among all FRs considered, could potentially cause dysfunctional effects on the immune system of adult females.

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1. Introduction

In recent years, decline of marine mammals' populations and increases in strandings have arised the interest on the role that pollution may have in these events. Consequently, investigations on contaminant exposure and effects in marine mammals have dramatically increased (Vos et al., 2003). Most studies assessing levels of inorganic and organic contaminants in marine mammals are affected by the availability of stranded, wild animals. Thus, for many of these pollutants it is difficult to define ranges of normality and safety thresholds. Anyway, we can compare data on a timeline basis, taking into account also intrinsic and extrinsic factors (geographical distribution, feeding areas, diet, gender, age) which can modulate accumulation and effects of pollutants (Vos et al., 2003). Analytical data can also be biased by the conservation status of tissues, as in many cases carcasses are found days after death.

In toxicological studies, great attention is paid to persistent organic pollutants (POPs), among which brominated flame retardants (BFRs) are considered as "chemical for priority action" for which routine monitoring is established (OSPAR, 2000; Simmonds et al., 2001). BFRs comprise compounds of anthropogenic origin such as polybrominated diphenyl ethers (PBDEs), and hexabromocyclododecane (HBCD), which are banned due to their environmental impact. However similar natural compounds as methoxylated polibrominated diphenyl ethers (MeO-PBDEs) have also been subject of interest in studies on marine pollution.

PBDEs are lipophilic, persistent and toxic to wildlife and humans (Alaee, 2003; de Wit et al., 2010). In mammals, they have been related to disruption of thyroid functions, of neurobiological development and to foetal toxicity/teratogenicity (Alonso et al., 2014). Hall et al. (2003) established the threshold level for PBDEs in blubber (1500 ng/g lipid weight, Lw.) at which thyroid endocrine disruption in juvenile grey seals was observed.

Records on PBDE detections in marine mammals are reported since the end of the 20th century, at levels high enough to induce adverse effects (de Boer et al., 1998). Odontocetes seems to accumulate higher PBDE levels than Mysticetes, due to their different trophic level (Alonso et al., 2014; Moon et al., 2010).

The most common target tissue for monitoring concentrations of PBDEs is blubber, where these compounds accumulate due to their lipophilicity. Furthermore, a mother-calf transfer with lactation can be hypothesised for these compounds, as showed in adult female pilot whales that displayed lower PBDE concentrations than their offspring (Lindström et al., 1999a). In sperm whales (*Physeter macrocephalus*) PBDEs concentrations up to 130 ng/g wet weight (ww) and 4 ng/g ww were found in blubber and liver, respectively (de Boer et al., 1998). No apparent differences in patterns of PBDE congeners accumulation are reported amongst cetacean species. Indeed, hepatic congener profiles were similar amongst *Tursiops truncatus*, *Stenella coeruleoalba*, *Globicephalas melas*, *Grampus griseus* sampled in the Mediterranean Sea, with BDE-47 having the highest values, followed by BDE-99 and BDE-100 (Pettersson et al., 2004). PBDEs have also been detected in blood, kidney and muscle of cetaceans from different areas of the World (Alonso et al., 2014; Isobe et al., 2009; Kannan et al., 2005; Nomiyama et al., 2011; Ramu et al., 2005).

Cetaceans also present well detectable levels of HBCD, which are supposed to be bioaccumulated through the food chain (Morris et al., 2004; Shaw et al., 2009; Sørmo et al., 2006). HBCDs occur in three different isomers (α, β and γ), and the most frequently detected form is α-HBCD, which has a higher lipophilicity and is less metabolized by cetaceans' hepatic microsomes with respect to the β and the γ isomers (Becher, 2005; Covaci et al., 2006; Esslinger et al., 2011).

Other compounds used as FRs are dechloranes (DEC), including Dechlorane Plus (DP), Dechlorane 602 (Dec 602), Dechlorane 603 (Dec 603) and Dechlorane 604 (Dec 604), which have a high chemical stability and a reduced photodegradation. Only recently DECs have been considered in ecotoxicology studies, while proved to bioaccumulate and biomagnify along food chains (Feo et al., 2012). They are likely to interfere with immune system, reducing lymphocytes counts and impairing the balance between Th2 and Th1 cytokines (Feng et al., 2016).

MeO-PBDEs are compounds of natural origin, produced by sponges, mollusks, cyanobacteria and algae. Compounds presenting the MeO- in meta or para position seems to be PBDEs derivatives (Feo et al., 2012; Sun et al., 2013; Yu et al., 2013).

The present work is aimed at quantifying levels of BFRs and DECs in tissues of 3 adult females and one foetus of stranded sperm whales from Southern Adriatic Sea coasts (Italy). Besides the specific focus of the present study, reported investigations contributed to a wider collaborative research effort encompassing several (multidisciplinary) approaches (Mazzariol et al., Submitted) through which we attempted evaluating whether pollutants accumulation assessed in stranded animals could relate to patho-physiological and biochemical alterations.

2. Materials and methods

2.1. The event and the sampling

On September 12th, 2014 seven sperm whale females stranded along the coasts of Punta Penna (Vasto), 42° 07′00″ North, 14° 42′ 00″ East, Southern Adriatic Sea, Italy (Fig. 1). Four of the animals live stranded and were successfully returned to the sea, while three died upon stranding. This event has to be considered as exceptional, since this species is rarely sighted in the Adriatic Sea due to low bathymetry and to the scarce prey availability. The animals have been necropsied just after death, and during necropsy the pregnancy status of the oldest female was assessed. Given its advanced development, sampling was possible also from the foetus. Following necropsy and sample collection, several multidisciplinary analyses (including parasitology and pathology) were also performed (Mazzariol et al., Submitted). Stranded animals were all females (SW1, SW2, SW3), while the foetus (SW1B) was a male.

Tissue samples were collected on site during necropsy, placed in aluminium paper, identified by the ID of each whale and by the tissue, and immediately stored at −20 °C at the University of Bologna facilities until analysis. Sampling of liver and brain was performed in the foetus, given the reduced dimension of the organs and the difficulties in accessing to the brain in such a small whale. Before shipping to the laboratories of the CSIC (Barcelona, Spain) samples were freeze dried and water content was assessed.

2.2. Chemical analyses

2.2.1. Chemicals

Syn- and *anti-* isomers of DP and ^{13}C-*syn*-DP were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Both native and mass-labelled PBDE mixtures, containing 8 PBDE congeners (BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209, and the ^{13}C-labelled, respectively), as well as the standard mixture of MeO-PBDEs (5-MeO-BDE-47, 6-MeO-BDE-47, 4′-MeO-BDE-49, 2′-MeO-BDE-68, 5′-MeO-BDE-99, 5′-MeO-BDE-100, 4′-MeO-BDE-101 and 4′-MeO-BDE-103), and α-, β- and γ-HBCD and d_{18}-labelled-HBCDs were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada). Dec 602 (95%), Dec 603 (98%) and Dec 604 (98%) were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada).

2.2.2. Sample preparation

PBDE, HBCD, DEC and MeO-PBDE analyses were carried out using a previously optimized sample extraction method (de la Cal et al., 2003; Labandeira et al., 2007). 0.5–1 g dry weight (dw) was spiked with ^{13}C-PBDEs and ^{13}C-*syn*-DP. Samples were kept overnight to equilibrate prior to the pressurized liquid extraction (PLE) with a mixture of hexane:dichloromethane (1:1). Extraction consisted in 2 static cycles of

Fig. 1. Map of the sampling site on the coast of Punta Penna (Vasto), 42° 07' 00" North, 14° 42' 00" East, Southern Adriatic Sea, Italy.

10 min at 100 °C and working at 1500 psi. Lipid content was determined gravimetrically after the extraction. Afterwards, organic content was re-dissolved in hexane and treated with H_2SO_4 (conc.) followed by a solid phase extraction (SPE) using alumina cartridges (Al-N, 5 g). SPE cartridges were conditioned with hexane and eluted with hexane:dichloromethane (1:2). Extracts were evaporated to incipient dryness under a gentle nitrogen stream at 30 °C and reconstituted to a final volume of 40 μl prior to the instrumental analysis.

2.2.3. Instrumental analysis

PBDEs and MeO-PBDEs were analysed with an Agilent 7890A gas chromatograph coupled to an Agilent 7000B triple quadrupole mass spectrometer. Chromatographic separation was carried out with a DB-5 ms column (15 m × 0.25 mm × 0.1 mm of film thickness). The instrumental conditions and elution program were based on our previous works (Eljarrat et al., 2007; Eljarrat et al., 2002). For the spectrometric determination (Barón et al., 2014), electronic ionization at 300 °C was used, with helium as carrier gas. For the analysis of DECs (Barón et al., 2015a), the chromatographic separation was carried out with another DB-5 ms column. Negative chemical ionization at 175 °C was used, with methane as ionization gas and helium as carrier gas.

On the other hand, HBCD analyses were carried out by liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) using electrospray ionization (ESI), following the protocol optimized by Guerra et al. (2008).

Selective reaction monitoring (SRM) mode was used for all compounds with two transitions monitored for each analyte. The most intense transition was used for quantification, while the second provided confirmation.

2.2.4. Quality parameters

Instrumental parameters such as recoveries, reproducibility, method limits of detection (mLODs) and method limits of quantification (mLOQ) are summarised in Supplementary information (Table S1). Recoveries ranged between 61 and 105%, always being within the range of acceptability (40–120%) for analytical methods based on quantification by isotopic dilution. mLODs and mLOQs ranged from 0.01 to 1.59 and from 0.03 to 5.30 ng/g lipid weight (lw), respectively.

2.3. Statistical analysis

Statistical analyses were performed by permutation multivariate analysis of variance (PERMANOVA) using the PERMANOVA+ add-on in PRIMER v6 (PRIMER-E Ltd., UK). First, ∑PBDE, ∑HBCD, ∑DEC, ∑MeO-PBDE values for each adult specimen and tissue were used to calculate similarity matrices based on the Euclidean distance (999 permutations). "Specimen", "tissue type" and the measured biometric parameters ("age", "body weight", and "body length") were selected as fixed factors to determine differences contaminant concentrations due to individual variability or tissue type. Pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance (Anderson et al., 2008). The same statistical inference was employed basing on a similarity matrix (based on the Euclidean distance, 999 permutations) obtained from the dataset of single congener concentrations. Both similarity matrices were also submitted to ordination analysis (performed by non-metric multidimentional scaling, NMDS, analyses) to explore data clustering.

3. Results and discussion

3.1. Animals

Biometrics of stranded animals is reported in Table 1. Basing on length and estimated age, SW1 and SW2 are to be considered as adult females, while SW3 should be considered as a sub-adult (although very close to sexual maturation). Indeed it is considered that female sperm whales reach adulthood at the age of 7, while sexual maturity

Table 1
Biometric parameters of stranded sperm whales.

I.D.	Length (m)	Age (years)	Weight (t)
SW1	8.95	35	8.84
SW2	8.38	14	7.35
SW3	7.33	7	5.11
SWEI	0.98	/	/

is reached at the age of 9 (Mesnik, 2014). Genetic analyses revealed the degree of kinship among the females: SW2 and SW3 were true sisters (same father and mother), while SW1 shared with the others only the mother (Mazzariol, et al., Submitted). Taking into account social organization of sperm whale groups, SW1 was considered as the matriarch (Mesnik, 2014).

All animals resulted severely starved before death; given the degree of emptiness of gastrointestinal tract, it was estimated that starvation lasted 3 to 7 days before stranding, if not longer. Some plastic bags were found in the stomach of SW1.

All animals presented a severe chronic reactive lymphoadenopaty with lymphocyte depletion. Furthermore, SW1 showed a severe renal degeneration with renal stones, which probably lead to a general compromission of kidney activity. SW2 presented a luteinic cyst. Analysis of the cyst's fluid for estrogens levels, performed with Radio Immuno Assay at the Veterinary Physiology Laboratories of the University of Bologna, revealed the following concentrations: estradiol 76.3 pg/mL and testosterone 609 pg/mL. It was not possible to quantify progesterone (Mazzariol, et al., Submitted). SW3 showed an extramedullary hematopoiesis phenomenon, indicative of alterations in hematopoietic system, presumably due to anemia or myeloid aplasia (Mazzariol, et al., Submitted).

3.2. Levels of halogenated compounds

Halogenated flame retardants (HFRs), including PBDEs, HBCD and DECs, as well as naturally occurring MeO-PBDEs were analysed in the different tissue samples of stranded sperm whales. All the results are presented in Table 2.

3.2.1. PBDEs

Several PBDEs were detected in all the analysed samples (except for feces), reaching concentrations up to 183 ng/g lw. Blubber was the tissue presenting the highest ΣPBDE concentrations in all adult females, with values of 160, 158 and 183 ng/g lw in SW1, SW2 and SW3, respectively. Congeners detected were BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154 and BDE-209. BDE-47 is the most frequently detected congener in all the tissues except for liver samples. According to published data (Dorneles et al., 2010; Ikonomou and Addison, 2008), besides BDE-47, the most frequently detected congeners are BDE-99, −100 and −154. Obtained data are in agreement with those reported in different Arctic food chain, all of them involving marine mammals (Corsolini et al., 2006; Kelly et al., 2008; Wolkers et al., 2004).

Lipid content of tissues and animal trophic level can drastically influence the concentrations of brominated compounds. Bachman et al. (2014) evaluated PBDEs in blubber of different cetaceans from Pacific Ocean, showing that species highest in their food chains (*i.e.*, orcas, *Orcinus orca*, and sperm whales) also had comparatively higher levels of these compounds. Since other cetacean species feeding on cephalopods and in deep-see environments also showed relatively higher levels of these pollutants, we can hypothesise that concentrations assessed in sperm whale blubber may depend, at least partially, on types of prey and depth of hunting (Bachman et al., 2014; Lindström et al., 1999a; Lindström et al., 1999b; Pettersson et al., 2004; Van Bavel et al., 1999). Although little information is available on content of PBDEs in deep-sea cephalopods, which are the most common preys of sperm whales, data reported by Romero-Romero et al. (2017) highlighted the potential for these compounds to biomagnify along typical trophic food webs in which sperm whales are reported as top predators. Therefore, deep-see preying may represent a relevant source of PBDEs for these mammals. Furthermore, it is interesting to note that PBDEs levels reported by Romero-Romero et al. (2017) for sperm whale blubber (149 ng/g l. w.) are remarkably different from those found in dolphin (about 82 ng/g l.w.) sampled in the same area. This finding may suggest that trophic transfer could not be ruled out also in the present work.

Scarce information is available on PBDE levels in sperm whales. de Boer et al. (1998) reported PBDE levels in the range of 188–347 ng/g lw. These concentrations are higher than those observed in this study, likely owing to differences in metabolic and physiological status of the two pods, in the stranding periods and in the geographical areas. Sperm whales individuals analysed by de Boer et al. (1998) were indeed severely starved and were found along Dutch coasts. No information is available concerning age and gender of the animals, therefore comparison for gender or age cannot be performed. Finally, it should be considered that in the '90s PBDEs were largely used as flame retardants, so their presence in the environment was higher. The inclusion of PBDEs in the Stockholm Convention in 2009 (Stockholm Convention, 2009a; Stockholm Convention, 2009b; Stockholm Convention, 2012), implying the total ban of the production of these compounds, lead to a decrease in their release in the environment, which is not followed by a similar drastic reduction in air, marine mammals and humans tissues, if not in the first years after the ban (Bjurlid et al., 2018; Parry et al., 2018; Shunthirasingham et al., 2018; Simond et al., 2017). More recently, Pinzone et al. (2015) evaluated PBDE concentrations in blubber biopsies of sperm whales from the Mediterranean Sea. Again, reported levels (≤781 ng/g lw) were higher than those observed in the present study.

Table 2
Concentration levels (expressed in ng/g lw) of HFRs (PBDEs, HBCDs and DECs) and naturally occurring MeO-PBDEs in different tissue samples from sperm whales.

	Blubber				Muscle				Heart				Liver			Brain		Feces	Umb. cord
	SW1	SW2	SW3	SWB1	SW1	SW2	SW3	SWB1	SW1	SW2	SW3	SWB1	SW1	SW2	SW3	SW1	SW2	SW1	SW1
BDE-28	2.40	2.05	nq	2.16	nd	nd	nq	nq	nq	nq	nq	nq	nq	nq	nq	nq	nd	nd	nd
BDE-47	78.8	65.1	99.8	38.5	15.5	59.9	71.6	57.2	40.5	46.1	85.3	32.1	nq	nq	nq	15.9	7.26	nd	22.8
BDE-100	27.7	26.8	32.0	15.7	nd	nq	nq	nq	nq	nd	28.5	9.14	nd	nq	17.8	nd	nq	nd	8.85
BDE-99	20.3	24.1	29.7	16.2	nd	nq	nq	34.3	nq	22.0	23.7	nq	23.8	nq	14.8	nd	nq	nd	10.4
BDE-154	31.2	40.3	21.5	25.5	nq	nd	nq	nq	17.5	17.3	nd	nq	27.5	40.9	33.9	nq	3.27	nd	nq
BDE-153	nd	nd	nd	4.55	nq	nq	nq	nd	nq	nq	nd	nq	nq	89.6	4.65	nq	nq	nd	nq
BDE-209	nd	nd	nq	nq	nd	12.8	8.11	nd	nq	nd	nd	4.10	nd	6.12	22.40	nq	nd	nd	nd
ΣPBDEs	**160**	**158**	**183**	**103**	**15.5**	**72.7**	**79.7**	**91.5**	**58.0**	**85.4**	**138**	**45.3**	**51.3**	**137**	**93.6**	**15.9**	**10.5**	**nd**	**42.1**
α-HBCD	5.75	2.59	3.48	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	11.3	nd
β-HBCD	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	12.4	nd
γ-HBCD	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	16.1	nd
ΣHBCDs	**5.75**	**2.59**	**3.48**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**39.8**	**nd**
Dec 602	**1632**	**519**	**713**	**nd**	**513**	**71.8**	**169**	**nd**	**nd**	**298**	**138**	**nd**	**402**	**426**	**206**	**nd**	**nd**	**nd**	**16.0**
ΣHFRs	**1798**	**680**	**900**	**103**	**529**	**145**	**249**	**91.5**	**58.0**	**383**	**276**	**45.3**	**453**	**563**	**300**	**15.9**	**10.5**	**39.8**	**58.1**
6-MeO-BDE-47	258	209	75.7	115	nd	188	nd	nd	117	108	nq	61.1	163	257	63.7	nd	nd	nd	79.6
2'-MeO-BDE-68	305	232	145	194	454	215	184	162	nq	157	168	96.8	239	nq	113	nd	nd	nd	102
ΣMeO-PBDEs	**563**	**442**	**221**	**309**	**454**	**403**	**184**	**162**	**117**	**265**	**168**	**158**	**402**	**257**	**177**	**nd**	**nd**	**nd**	**182**

The bold numbers are the total amounts of each analysed family.

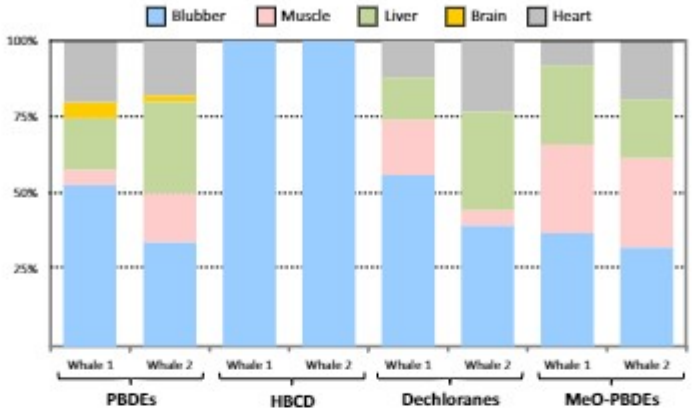

Fig. 3. Distribution of contaminants in each analysed tissue for SW1 and SW2.

3.2.3. DECs

Dechlorane 602 was the only decloran detected in sperm whale samples analysed in the present study (Table 2). High levels of Dec 602 were detected in tissues of adult sperm whales (in particular in the blubber, with levels up to 1632 ng/g lw), but not in those of the foetus. Anyway, it should be noted that this compound was found in umbilical cord which may suggest umbilical cord as a protective tissue for the developing organisms, acting as a barrier for placental transfer of these contaminants.

On the whole, ΣHFR levels (ΣPBDEs + ΣHBCDs + ΣDECs) measured in blubber of adult sperm whales ranged between 680 and 1798 ng/g lw. Therefore, DECs contributed for about the 76% to the 91% total HFR contamination. This predominance of DECs was also observed in other analysed tissues (between 50% and 97%, and 69% and 89% in muscle and liver, respectively).

Little is known about Dec 602 levels in ceteaceans. De La Torre et al. (2012) reported Dec 602 concentrations in *Pontoporia blainville* being about 0.38 ng/g lw, levels that lower than those observed in the present study. Barón et al. (2015a) determined Dec 602 in blubber samples of 3 cetacean species (*Delphinus delphis*, *Globicephala melas* and *Tursiops truncatus*) from the Strait of Gibraltar and the Gulf of Cadiz (Spain) during 2012. Measured concentrations were up to 29.6 ng/g lw, that are much lower than those reported in the present study. No additional information on toxicokinetics and potential adverse effects of Dec 602 on cetaceans are currently available, hence, no tentative conclusions can be argued about the potential effects of the relatively high concentrations reported in sperm whale in the present study. In mice subjected to a 7-day treatment at relatively low concentrations (1 and 10 μg/kg body weight per day), Dec 602 impaired immune system activity by modifying CD4+ and CD8+ cell populations and Th2:Th1 cytokines ratio (Feng et al., 2016). A potential immunodepression triggered by exposure to relatively high levels of Dec 602 could have contributed to increase the sensitivity of sperm whales towards the effects of *Herpesvirus* and *Morbillivirus*, which were detected in adult females and foetus.

3.2.4. MeO-PBDEs

On the whole, MeO-PBDEs were detected in all analysed tissues, with the exception of brain and feces, at concentrations up to 563 ng/g lw. Only two congeners were detected, 2′-MeO-BDE-68 and 6-MeO-BDE-47, both tetra-brominated congeners. These two compounds are the two main MeO-PBDEs normally found in marine mammals (Alonso et al., 2014). In blubber samples from adult sperm whales, 2′-MeO-BDE-68 and 6-MeO-BDE-47 contributed between 53 and 66% and 34–47% to the ΣMeO-PBDEs, respectively.

Since MeO-PBDE are compounds of natural origin, their observed accumulation in tissues of sperm whale leads to hypothesise the presence in the Mediterranean Sea of organisms producing these molecules, as it occurs in other areas (Dahlberg et al., 2016; Ochiai et al., 2017; Yin et al., 2017). Nevertheless, the concentrations observed in the present study are lower than those reported by Dorneles et al., (2010) for cetaceans from the Brazilian coasts. It should also considered that concentrations reported by Dorneles et al. (2010) are the highest ever reported, even higher than those previously detected in Australian *Kogia breviceps* (3760 ng/g ww) by Vetter and Jun (2003). High concentrations of 6-MeO-BDE-47 and 2′-MeO-BDE-68 in aquatic environment are generally related to the presence of sponges and algae (El Megdiche et al., 2017; Po et al., 2017). It has been supposed that these organisms use these compounds as a chemical defensive mechanism, or for a hormonal-like activity (Dam, 2011). Furthermore, cephalopods can represent the route of MeO-BDEs transfer from benthos towards cetaceans, also because they represent the main prey items for many Odontocetes (Dorneles et al., 2010).

If we compare ΣPBDEs and ΣMeO-PBDEs, the observed levels of the synthetic compounds were always lower than those of the naturally occurring compounds, with the exception of brain samples in which only synthetic PBDEs were detected. However, Barón et al. (2015b) reported the presence of 6-MeO-BDE-47 and 2′-MeO-BDE-68 in brain samples of 3 cetacean species (*Delphinus delphis*, *Globicephala melas* and *Tursiops truncatus*) from the Alboran Sea (Spain). A different behaviour of the blood-brain barrier (BBB) between dolphins and sperm whales could be one potential explanation of this difference, as observed in other species (Alonso et al., 2017; Barón et al., 2015b; Nomiyama et al., 2017; Yang, 2016).

Table 3
PERMANOVA results based on a Euclidean matrix obtained from ∑PBDEs, ∑MeO-PBDEs, ∑DECs, ∑HBCDs in adult sperm whales. Values with $P < 0.05$ are highlighted in bold. df: degree of freedom. Pseudo-F: in adult sperm whales. F value by permutation (Anderson et al., 2008); P (perm): probability of pseudo-F.

Source	df	Pseudo-F	P(perm)
Specimen	2	**3.887**	**0.019**
Tissue type	3	**7.721**	**0.005**
Age	2	1.997	0.305
Weight	2	1.998	0.347
Length	2	1.997	0.337

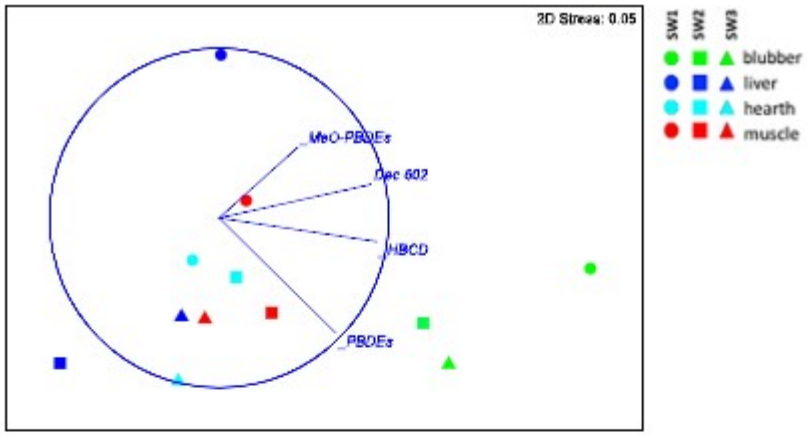

Fig. 4. Non-metric multidimensional scaling (NMDS) of ∑PBDE, ∑HBCD, ∑DEC, ∑MeO-PBDE concentrations in different tissues of adult female sperm whales stranded in the Adriatic Sea (Euclidean matrix; 999 permutations).

3.2.5. Tissue distribution

A comparison of the compound distributions in various sperm whale tissues was carried out using data obtained from SW1 and SW2, for which samples from all the 5 different tissues were available. Fig. 3 showed the percentage tissue distribution of each contaminant. Distribution of the chemicals amongst the different tissues was similar between the two investigated specimens studied. Nevertheless, it is worth noting that only PBDEs were detected in brain samples, indicating that these pollutants can pass through the BBB and reach the brain, whereas BBB seems to be efficient in protecting the rbin from HBCDs, DECs and MeO–PBDEs accumulation. It is also important to remark that blubber was the only tissue in which HBCDs were detected.

On the whole, PERMANOVA analyses carried out on data from all adult specimen ($N = 3$; Table 3) showed that the factor "specimen" and "tissue type" significantly affected total brominated compound levels in analysed samples, giving a further support to the differential tissue distribution displayed by the compounds.

A more in-depth analysis of data distribution was performed by means of non-metric multidimensional scaling (NMDS) ordination. This ordination inference allows exploring sample clustering by means of the multivariate dataset employed, here represented by the tissue levels of ∑PBDE, ∑MeO, Dec, ∑HCB assessed in adult sperm whale. This statistical approach helps to visualize similarity (or distances) amongst samples and proved suitable to disclose those tissues showing a peculiar contaminant profile (Fig. 4). Indeed, the statistic outcome reported in Fig. 4 showed that blubber samples from different specimens clustered together independently from the analysed specimen, while the other tissues have a more disperse distribution. SW1 was quite different from the other specimens in the values measured in hearth and muscle, and to a lesser extent in the blubber. All the compounds showed a preferential accumulation in the blubber, confirming this tissue as a target organ for organic compound accumulations in sperm whale, although a certain degree of individual variability should be also considered.

When levels of single congeners are considered (Fig. 5; Table 2), it can be observed that most part of PBDEs and Dec 602 showed a preferential accumulation in the blubber, and the remaining PBDE congeners and MeO-PBDEs in liver and heart.

4. Conclusions

The present study proved the accumulation of different FRs in tissues of sperm whales, including various PBDEs congeners (47, 99, 100, 154,

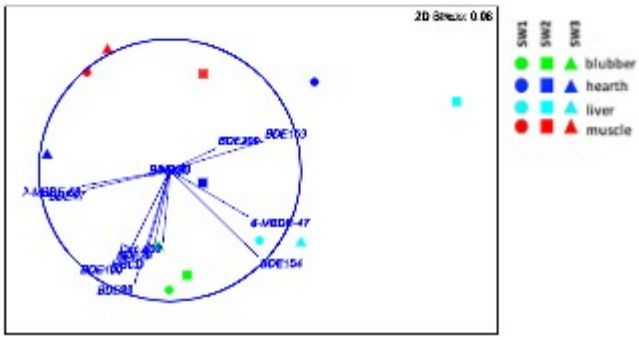

Fig. 5. Non-metric multidimensional scaling (NMDS) of single BDE congener concentrations in different tissues of adult female sperm whales stranded in the Adriatic Sea (Euclidean matrix; 999 permutations).

Although concentrations obtained in blubber samples from the three sperm whales were very similar (158, 160 and 183 ng/g lw), levels measured in muscle or heart were higher in SW3 specimen (a sub-adult female) compared to SW1 and SW2 (both adult specimen), suggesting that nulliparous females may have higher contaminant burdens with respect to actively reproducing females.

Comparing concentrations and distributions of single PBDE congeners showed that the adult females had different residual profiles, which might be linked to different metabolic efficiencies likely related to specimen ages. All females are indeed siblings, and given the social organization of female sperm whales (Cantor and Whitehead, 2015; Whitehead and Mann, 2000), variability in the routes of contaminant exposures can be excluded. For SW1, an additional factor likely affecting congener profile is pregnancy, which alters mother's metabolism.

All foetal tissues showed noticeable PBDE amounts, hence suggesting the placental transfer as an additional route of excretion for these compounds in adult females, as already observed in *Delphinapterus leucas* (Desforges et al., 2012; Reiner et al., 2012). SWB1 showed higher muscle ΣPBDEs concentrations compared to all specimen considered. When comparing PBDE residues between mother and foetus, the same congeners are present in all tissues considered. This observation strengthens the hypothesis of placental transfer and provides evidence that reproduction is one of main excretion route for females. Congener profiles in foetus reflect those of the mother in blubber and heart, but not in muscle, where BDE-47 levels were higher in SWB1 than in SW1. Additionally, detectable levels of BDE-99 were observed only in foetus tissues (Fig. 2).

As previously reported in humans (Covaci et al., 2008) and mice (Staskal et al., 2006), different congener profiles between foetus and mother could reflect different metabolic activities (reduced in foetus). Staskal et al. (2006) reported that after administration of BDE-47, −99, −100 and −153 to mice, the congeners are preferentially stored in adipose tissue, and that rank of liver metabolic activity towards the congeners was as follows: BDE-47 > BDE-99 > BDE-100 > BDE-153. It is worth noting that BDE-47 was not detected in liver of all females analysed in this study, BDE-100 was detected in one out of three animals, BDE-99 in two and BDE-154 in all three and at levels comparatively higher than the formerly listed congeners. Although comparisons between mouse and sperm whale metabolic capacities are not straightforward, we can speculate that sperm whales are likely to show a similar ranking of metabolic potency towards the different BDE congeners as that reported in mice (Staskal et al., 2006). Furthermore, Boon et al. (2000) reported sperm whale cytochromes to display differential metabolic efficiencies towards different PBDE congeners, leading to congener-specific metabolic dynamics clearance processes. The same was observed *in vitro* by McKinney et al. (2006) in beluga whale hepatocytes, which proved to be very effective in metabolizing BDE-15 and -28, but not BDE-47, −99, −100, −153 and −154.

PBDE levels observed in foetus, which are comparable to those found in females, raised concern on the possible health effects of exposure to high levels of FRs during developmental stages, potentially leading to severe neurologic, immunologic and endocrine impairments.

3.2.2. HBCDs

The only HBCD isomer detected in present study was α-HBCD, which was found only in blubber of females, and not in foetus tissues. The β- and γ-isomers were detected only in feces of SW1, suggesting that metabolic processes are more efficient toward β-HBCD and γ-HBCD elimination with respect to α-HBCD, as reported previously (Becher, 2005; Zegers et al., 2005), or that these compounds are not accumulated by sperm whales.

The preferential accumulation of the α-HBCD isomer is in agreement with previous studies (Frederiksen et al., 2007; Johnson-Restrepo et al., 2008; Shaw et al., 2009; Shaw et al., 2012) reporting the specific accumulation of this isomer in biota, despite it accounts only for the 10% of the commercial pesticide mixture. The α-HBCD isomer is the less efficiently metabolized by marine mammals (Becher, 2005). Similarly, Hakk (2015) reported that only the α-HBCD isomer accumulated in adipose tissue of male rats, while 42.42%, 59.36% and 53.03% of administered dose of α-HBCD, β-HBCD and γ-HBCD respectively was recovered in the feces. Since to the best of our knowledge no further data concerning HBCD isomers distribution in sperm whale are currently available, a comparison can be performed with other cetacean species. For example, Reiner et al. (2012) showed that α-HBCD isomers only could be detected in tissues of beluga whales (5 ng/g ww). Isobe et al. (2007) reported evaluated α-HBCD blubber concentrations in *Neophocaena phocaenoides* and *Sousa chinensis* in the range of 4.7–55 ng/g lw and 31–380 ng/g lw respectively. Interestingly, the highest total concentrations (380 ng/g lw) were detected in *S. chinensis* living closer to the coastal areas hosting industrial activities. Thus, it can be hypothesised that, similarly to *Neophocaena phocaenoides*, sperm whales living in deeper, oceanic waters could experience a relatively lower α-HBCD exposure with respect to species living in coastal environments.

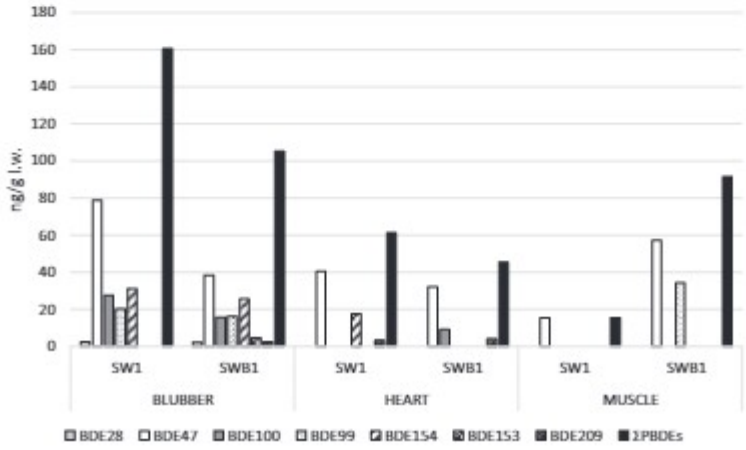

Fig. 2. BDE-28, −47, −99, −100 concentrations in maternal (SW1) and foetal (SWB1) tissues.

matching the composition of commercial PentaBDE mixture), HBCDs, Dec 602 and MeO-BDEs.

Many studies focused on the evaluation of FR residues in blubber, as it is considered the target tissues for their quantification. By addressing various "non-target" or non-conventionally analysed tissues, this study clearly showed the importance of widening the range of tissues to be analysed during monitoring investigations in order to obtain more realistic information on toxicokinetics of these compounds in cetaceans. Relatively high contaminant levels on such diverse body compartments could lead to unexpected adverse outcomes (for examples, immunodepression or endocrine disruption) which can severely impair sperm whale health.

The unique possibility of sampling foetal tissues allowed to evaluate the potential maternal transfer of some of these compounds, and to draft some considerations on potential adverse effects on foetal health. To the best of our knowledge, obtained data are the first reporting a potential placental transfer of FRs in sperm whales. PBDE levels detected in foetal tissues suggested that these animals may be potentially exposed to BFRs throughout their whole life cycle, which may lead to unpredictable health outcomes in the adult stage. Dec 602, which was detected at the highest concentrations among all FRs considered, have the potential to cause dysfunctional effects on the immune system of adult females.

By focusing on FR residues in an apex species regularly present in the Mediterranean Sea, the present study provided a further hint on the relevant contamination burden by FRs and (likely) other POPs in the area and the potentially relevant negative impacts on wildlife physiology and conservation. To this concern, data reported in this study on differential tissue distribution of the selected contaminant highlights the importance of performing more complete toxicological studies in cetaceans, and in sperm whale in particular, to better understand the impacts of these compounds on the health of the species. These studies will also aid in developing more effective strategies for the conservation of the species.

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Paper n. A. **A preliminary analysis of pyrethroids in stranded sperm whales (*Physeter macrocephalus*) on the Italian Adriatic Coast**

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Paper on submission

A preliminary analysis of pirethroids in stranded Sperm whales (*Physeter macrocephalus*) on the Italian Adriatic coast.

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Introduction

Pyrethroids are a class of synthetic chemicals used as insecticides and derived from pyrethrins, which are natural compounds of *chrysanthemum* flowers. Pyrethroids have been used since 1980, progressively replacing organophosphorus and carbamate compounds because of their equal efficacy but lower toxicity. Chemically, pyrethrins are esters of cyclopropane carboxylic acid and a cyclopentenolone alcohol modified to enhance insecticidal power and lengthen their life in the presence of atmospheric agents such as sunlight and water (Burns, C. J., & Pastoor, 2018). The chemistry of pyrethroids is similar among the entire class; indeed, all the compounds conserve the acid-alcoholic composition of pyrethrins. Pyrethroids are organic pollutants with extremely reduced water solubility (about few μg/L) and high hydrophobicity (log Kow ranging between 5.7 and 7.6) (Laskowski, 2002). Due to their high lipophilic nature, removing them from the organism is difficult. The pyrethroids class includes around 18 compounds and comprises some of the most widely used pesticides, such as cypermethrin and deltamethrin (Burns, C. J., & Pastoor, 2018). The mechanism of action of pyrethroids consists in a disruption of sodium channels in nervous cells, without interfering with acetylcholinesterase release. Since pyrethroids can be easily converted in inert metabolites, through hydrolysis processes in mammals and through

oxidative metabolism in fish, traditionally the scientific community has always considered these compounds not able to bioaccumulate in the food chain (Chambers, 1980; Demoute, 1989; Godin et al., 2007). Despite this, more recent studies demonstrated the presence of pyrethroids in human breast milk, with levels up to 1200 ng/g lipid weight (lw) (median value) (Bouwman, 2009; Feo et al., 2012) condition that poses new questions and requires further scientific investigation to fully understand the behaviour of this class of contaminants in the environment and in the food web. Even though pyrethroids overall low toxicity for the surrounding environment, these pesticides are highly toxic to aquatic wildlife. Especially long-term, low-dose exposure is dangerous for aquatic fauna, potentially leading to harmful effects on animals' genetic, immune, vascular, nervous, and endocrine systems (Tang et al.,2018). These pollutants enter the water cycle, transported from agricultural and urban areas through surface runoff and drainage channels arriving at the sea. Once reached the aquatic environment, pyrethroids are metabolized by bacteria, fish, and other organisms. In fish, these pollutants may enter through the gills, and then they can be transported into the circulatory stream, and from blood, they can reach all anatomical compartments (Tang et al.,2018). In fish, pyrethroids may interfere with dopamine release, which regulates the final synthesis of steroids. Alterations of brain enzymes such as aromatase have been reported following pyrethroids exposure, leading to variations in the circulating level of oestradiol and testosterone. Decreased expression of genes responsible for steroidogenesis has been recorded both in female and male fish, resulting in difficulties in reproduction (Brander et al., 2016). Although the harmful effects inducted by

pyrethroids on fish are well known, there are still few studies available focused on the possible detrimental effects of this class of pesticides on marine mammals. Alonso et al., in 2012 detected high levels of permethrin and cypermethrin in franciscana dolphins (*Plontoporia blainvillei*). they also identified a positive correlation between the levels of pyrethroids and the age of the subjects analysed, with the highest concentrations detected in the youngest animals and a progressive decrease as the age of the subjects increased. This trend was recorded until the animals reached sexual maturity. After that, the levels of pyrethroids began to rise again. Another study conducted on striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea showed pyrethroids concentrations in tissues of the animals in the same order of magnitude as those reported by Alonso et al. in franciscana dolphins (Aznar-Alemany et al., 2017). The aim of this study is to perform a qualy-quantitative analysis of different pyrethroid compounds in different sperm whales' tissues and to verify the possible transplacental transfer of these pollutants from mother to foetus. As far as we know this is the first study focused on the detection of pyrethroids in sperm whales.

MATERIALS AND METHODS

Biological samples have been collected during on site necropsies of three female sperm whales stranded alive and the died in Punta Penna, Vasto on the central east Italian Adriatic Coast in September 2014.

Figure 1 necropsy and sample collection of stranded sperm whales

The oldest female was pregnant, so we were able to collect also sample of maternal fetal adnexa. Samples have been immediately labelled and adequately stored at – 4 °C until further investigations. We collected samples of brain, heart, blubber, muscle, liver, feces, and umbilical cord (Table 1). We decided to test 18 compounds belonging to the class of pyrethroids: aletrina, bifenthrin, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, fenvalerate, flumethrin, fluvalinate, imiprothrin, kadenthrin, permethrin, phenothrin, pralethrin, resmethrin, tetramethrin, tralomethrin, trasfluthrin.

Sample	subject			
Blubber	Sw1	Sw2	Sw3	fetus
Heart	Sw1	Sw2	Sw3	fetus
Muscle	Sw1	Sw2	Sw3	fetus
Feces	Sw1			
Umbilical cord	Sw1			
Brain	Sw1	Sw2		
Liver	Sw1	Sw2	Sw3	

Table 1 samples collected

Samples preparation and laboratory analysis

Organic solvents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Solid phase extraction (SPE) C18 (2 g/15 ml) and basic alumina (5 g/25 ml) cartridges were obtained from Isolute Biotage and Interchim, respectively. Standard solutions were prepared in ethyl acetate. Bifenthrin, λ-cyhalothrin, fluvalinate, resmethrin and a mixture of pyrethroids containing cyfluthrin, cypermethrin, deltamethrin, fenvalerate, permethrin and teramethrin were used as analytical standards. Internal standards were d6-trans-permethrin and d6-trans-cypermethrin. All of them were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Sample extraction was in agreement with Feo et al., (2012). Blubber, brain, heart, liver, and muscle of sperm whales (0.1 g dry weight (dw)) were pierced with deuterated internal standards (2 ng of d6-trans- permethrin and 1 ng of d6-trans-cypermethrin). The sample was stirred and extracted by sonication

with 20 ml of hexane:dichloromethane (2:1) for 15 min and centrifuged at 3500 rpm for 7 min twice. The two organic phases were mixed in a vial and the solvent was changed to 10 ml of acetonitrile. Solid phase extraction (SPE) C18 (2 g/15 ml) and basic alumina (5 g/25 ml) cartridges were taken from Isolute Biotage and Interchim, respectively. The cartridges were conditioned with 25 ml of acetonitrile. The extract was filtered through the cartridges and restored. The extract vials were washed with 20 ml of acetonitrile that was mixed to the extract. The eluate was evaporated and re-dissolved with 100 µl of ethyl acetate. To compute the lipid amount in the samples, 1 g of liver was also extracted with 40 ml of hexane:dichloromethane (2:1) and evaporated. Finally, the lipid content was measured gravimetrically. The pyrethroid detection was carried out with an Agilent 7890A gas chromatograph coupled with an Agilent 7000B triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) as set out by Feo et al., (2010). Compounds were separated with a DB 5ms capillary column (15 m × 0.25 mm, 0.1 µm film thickness) with the following thermal program 100 °C for the first minute, then raised from 100 to 230 °C for 8 min, then from 230 to 310 °C for 8 min and, hold at 310 °C for 2 min. Injector temperature was equal to 270 °C; injection volume was 3 µl; carrier gas was equal to He at1 ml min−1. The mass spectrometer worked in the negative chemical ionization (NCI) mode, 34 using ammonia at 2 × 10−4 torr as reagent gas. The ion source temperature was 250 °C. Selective reaction monitoring (SRM) mode was used with two transitions monitored for each compound. For quantification, the most intense transition was used, and the second transition provided a confirmation comparing the SRM1/SRM2 ratio

calculated for the samples with the ratio found in the standards. Details of the MS/MS conditions and of the selected transitions in the SRM mode are found in Feo et al., (2011). Method recoveries ranged from 53 to 116 %, with relative standard deviations (RSD) always below 20%. Method detection limits (MDLs) and method quantification limits (MQLs) ranged from 0.02 to 0.46 ng g−1 lipid weight (lw) the former and from 0.08 to 1.54 ng g−1 lw the latter.

RESULTS AND DISCUSSION

Results of our study are reported in table 2 (Tab.2).

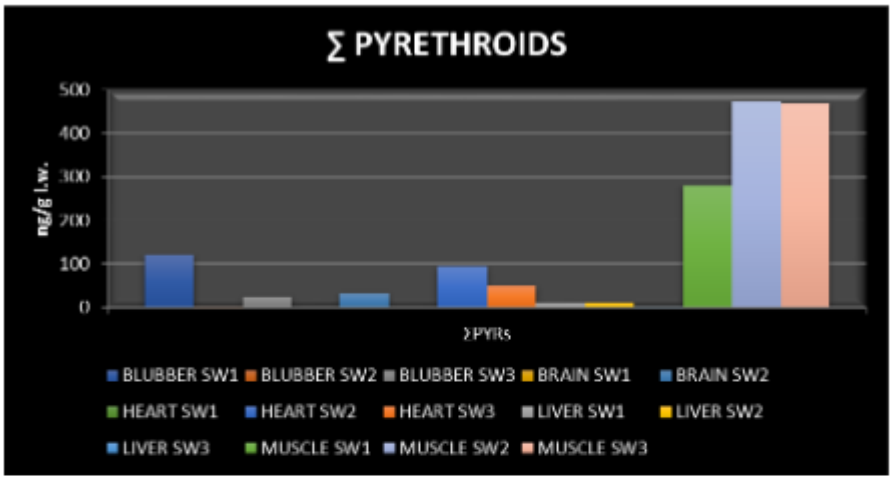
SAMPLE	INDIVIDUAL	PYRETHROID			
		TRANSFLUTHRIN	TETRAMETHRIN	PERMETHRIN	∑PYRs
BLUBBER	SW1	n.d	n.d	120	120
	SW2	2.70	n.d	n.d	2.70
	SW3	n.d	23.9	n.d	23.9
BRAIN	SW1	n.d	n.d	n.d	n.a
	SW2	n.d	26.7	4.67	31.3
HEART	SW1	n.d	n.d	n.q	n.a
	SW2	n.d	92.9	1.19	94.1
	SW3	n.d	43.3	5.02	48.4
LIVER	SW1	n.d	n.d	9.84	9.84
	SW2	n.d	n.d	11.7	11.7
	SW3	n.d	n.d	1.20	1.20
MUSCLE	SW1	n.d	278	3.26	281
	SW2	n.d	458	14.9	473
	SW3	n.d	464	4.94	469
UMBILICAL CORD	SW1	n.d	n.d	6.51	6.51

n.d = below mLOD. n.q = below mLOQ. n.a = not applicable.

Fig.2 results Values of pyrethroids (ng/g l.w.) in tissues of Sperm whale. Only compounds with values above BDL in at least each condition are reported.

We investigated the presence of 17 pyrethroids in different biological samples of sperm whales. We identified only three pyrethroid compounds in the biological samples available. Among them, Tetramethrin was the chemical compound reaching

the highest values detected, and surprisingly its highest concentrations were recorded in the muscles of the three subjects (Graphic 1).



graph.1 Concentration of pyrethroids in sperm whales' tissues

This find could be linked to the mobilization of pollutants in the organisms of the three females since they were found emaciated and affected by infectious diseases confirmed by viral analysis in all the subjects, including the foetus and kidney failure of the oldest specimen. Myoglobin in the muscles of marine mammals is an essential protein for oxygen transportation and storage. Most of the oxygen marine mammals exploit to carry out deep dives derives from muscle mass and myoglobin. Alterations in these structures and their functions could affect diving and hunting. Nevertheless, in this specific case, the results obtained by the investigations conducted on heart tissues, which are considerably high in the three cetaceans, rather suggest a higher chemical affinity of pyrethroids for muscle tissues and their components than for other

anatomical districts. This is an interesting finding because our data shows that tetramethrin concentrations detected are an order of magnitude higher than the concentrations found in the blubber, which is traditionally considered the target organ for detecting these chemicals. Once more, this underlines the importance of investigating all the possible biological samples available. In addition, since this is one of the first studies focused on pyrethroids in cetaceans, including the opportunity to analyse also maternal-foetus adnexa, it is impressive to observe that even if tetramethrin reaches the highest concentrations in our samples, it has not been identified in the umbilical cord asking questions about whether the contaminant is transferred to the foetus through a vertical route. On the contrary, permethrin, the other pyrethroid detected in most samples, reaches lower concentrations in all the anatomical compartments investigated in all three sperm whales. It has also been identified in the umbilical cord. The presence of pyrethroids in the maternal foetus adnexa could have a double meaning and, therefore, double interpretation. Indeed, the detection of permethrin in the umbilical cord may be linked to sequestration and accumulation of the contaminant in the umbilical cord, thus preventing or reducing its transfer to the foetus. Nevertheless, it is also possible that the detection of permethrin in the umbilical cord demonstrates the ability of the compound to permeate the maternal-foetal adnexa and accumulate in the pup itself. To obtain more information on the kinetics of permethrin related to its transfer to the foetus, it would have been essential to analyse foetal tissues. Still, unfortunately, due to the scarcity of samples available to the various research groups, this opportunity could not be exploited. However, it is possible to

compare these results with those of the PBDEs tested on the same subjects (Zaccaroni et al., 2019). This comparison suggests that it is possible that, as with PBDEs, the detection of pyrethrin in the umbilical cord corresponds to the presence of the compound in foetal tissues. One last meaningful consideration that emerges from our study is that although few samples were analysed, there is no clear correlation between the concentrations of pyrethroids found in the three subjects and their age. This results in the need for further studies to investigate the behaviour of these chemicals in the sperm whale. Eliminating the possibility of interference from external variables that acted on the three specimens, i.e., mainly their different geographical distribution in the Mediterranean and a difference in diet, this finding suggests that the discrepancies found may be conditioned by intrinsic subjective variables, such as metabolic rate, endocrine balance, synergies, and antagonism among pollutants accumulated, immune system functions and concomitant diseases.

Conclusions

Further investigations need to be conducted to elucidate the effective role of pyrethroids of marine mammals' health. our study certainly supports the hypothesis of a consistent transfer of contaminants from mother to foetus, a condition that could affect the health of the new-born, threatening its survival and ability to reproduce, and thus affecting the survival of future generations. although only three of the chemical compounds we researched were found, their detection must be a wake-up call to continue monitoring the species whenever the opportunity arises. In addition, the finding of high concentrations of pyrethroids in tissues that are not strictly considered

as targets, such as the muscles, should make the scientific community reflect on the urgency of extending the field of research to biological matrices other than those traditionally considered as targets for the accumulation of these contaminants. In fact, this new research approach can provide interesting new data to understand the kinetics of the pollutant within the animal organism and broaden the knowledge of the possible short- and long-term effects on the health of the individual subject examined. the collection of such data represents an initial key point to allow the construction of predictive models to define and implement the protection of these marine species.

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Paper n.B: **Atrazine monitoring in plasma of rescued loggerhead turtles from Northern Adriatic Sea**

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Paper on submission

Atrazine monitoring in plasma of rescued loggerhead turtles from Northern Adriatic Sea

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INTRODUCTION

Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) is a compound chemically classified as chloro-triazine (Stoker et al.; 2002). It was introduced in the 1950s and is still widely used in many countries worldwide as a low-cost and efficient herbicide, acting affecting the normal process of chlorophyll photosynthesis. However, the scientific community has recently raised concerns about its chemical characteristics. It is highly persistent in icy and dry environments, stable in all pH environments, and readily found in the subsurface and aquatic ecosystems. Atrazine is most effective when applied to wet soil; therefore, it is usually used after heavy rainfall, when the ground is most receptive. For this reason, atrazine can more easily enter the soil substrate and water systems, eventually flowing into rivers and streams and thus into the marine environment. One of the earliest significant scientific achievements in detecting Atrazine was in 1992 by Christopher and Bird. They linked the die-off of wild fish and avian species in the Chesapeake Bay to high levels of Atrazine in its waters and the subsequent disappearance of aquatic plants in the Bay. Despite these persistent and environmentally resistant characteristics and its widespread use worldwide, it is surprising that only a few ecotoxicological studies have been carried out to detect its presence and estimate its amount in natural ecosystems and wild species. Atrazine may be found in aquatic ecosystems as a toxic compound, or it may undergo a series of degradation processes leading to the formation of new metabolites, which show varying degrees of toxicity and persistence. The degradation of atrazine can be attributed to five different mechanisms: hydrolysis, adsorption, photodegradation, microbiological degradation, and volatilization. However, eco-toxicological research shows that the base compound is the most found in water. It is the most toxic, together with only a few of its metabolites (DEA, DIA, and DDA). Few studies have previously been conducted on reptile eggs, particularly exposing eggs during the hatching period to an atrazine solution. These trials showed a reduction in the number of eggs able to hatch, particularly when exposed to concentrations between 2 and 200 µg/L. In addition, a study of *Nerodia sipedon* aquatic snakes, in which laying females were

exposed to the same atrazine levels as above, showed that very few of these snakes survived (Van Der Kraak et al.; 2014). The literature available on research into the harmful effects of acute and chronic atrazine exposure on turtles is extremely limited. Nonetheless, research carried out on freshwater species (*Trachemys scripta*) has shown that contamination of these animals with atrazine is correlated with drastic changes in the correct functioning of the immune system, with an appreciable reduction in the physiological phagocytic and macrophagic response, thus making the subjects easily attacked by infections of various kinds which, following the acquired immunodepression, can overwhelm the organism. A significant reduction in lysozyme release was found in red-eared turtles exposed to atrazine. In reptiles, lysozyme plays a key role in counteracting pathogens that have entered the animal body. Lysozyme is an important marker of the pro-inflammatory response in reptiles, has antibacterial functions, and measures the innate immune response. Specifically, this condition was also found by Walsh et al.; in a 2010 study carried out on *Caretta caretta,* in which they were able to demonstrate the correlation between herbicide poisoning and exposure to algal toxins, with a significant reduction in the production and release of lysozyme into the circulatory stream (Soltanian, S.; 2016). Second research on the analysis of atrazine present in *Caretta caretta* eggs demonstrated high contamination, raising the question regarding the contextual contamination of several different chemical compounds and their possible synergy (Allan et al.; 2017). Another research instead sought to verify the potential endocrine disruptor action exerted by atrazine on the reproductive efficiency of turtles by searching for a possible correlation between atrazine action, exposure temperature of eggs in the nest, and sex ratio of the unborn children to find out whether atrazine had activity on sex hormones. This study demonstrates the correlation between atrazine, turtle nest temperature, and the influence of these synergies on the sex ratios of unborn hatchlings and how higher nest temperatures increase the endocrine-disrupting activity of atrazine on animal organisms (Willingam E.; 2005). In Italy, the use of atrazine has been indiscriminate for many years. Its use was initially restricted in 1986 due to the discovery of groundwater contamination, mainly in northern regions. Then in 1990, the compound was legally and officially banned from the whole country. As far as the international situation is concerned, since 2004, Europe has decided to revoke the authorizations of plant protection products containing the chemical compound. In Italy today, therefore, surface, and non-surface water contamination is due to past use and the highly persistent environmental characteristics of the herbicide (Ispra 2005). The three-yearly ISPRA reports in the geographical area of the Po Valley, including in the

monitoring programs the sampling sites of the Romagna coastline, have proved the still high and persistent contamination of atrazine and its metabolites. This latest official document shows that triazine herbicides are among the most frequently detected in surface and groundwater in the Po River basin at concentrations exceeding environmental quality standards. The choice of atrazine as a reference substance is based precisely on the fact that, since it has not been used for over two decades, its presence in water depends solely on its chemical and physical characteristics and environmental dynamics. To make the situation even more worrying, a 2017 study found high concentrations of atrazine still present in the freshwater network in northern Italy and the northern Adriatic, testifying to the spread and persistence of the substance in the marine environment. In this study, atrazine was found at all the points selected for sampling, which were carried out in 2011. Furthermore, the other sampling sites examined in the Mediterranean basin also reported significant positives for this contaminant. We mention the high concentrations found in the Greek maritime waters of the Aegean Sea, as our study uses as sample species common turtles that insist in the Mediterranean basin and that are not sedentary in the Adriatic but make annual journeys from different origins, including the Greek basin (Nodler et al.; 2013). The present study aims to search for the presence of atrazine in the circulatory stream of common sea turtles (Caretta caretta), stranded on the northern, eastern Adriatic coast. This research will evaluate the possible positivity of coastal seawater samples from the stranding sites of the same animals' understudy and some river water samples from the geographical area under investigation. One of the main reasons that led us to investigate the possible presence of this compound in the blood of *Caretta caretta* is linked to the chemical nature of this substance, which is characterized by a high persistence in water and sediments, still detected by recent reports in our study area. Moreover, concerning the danger and health damage that this compound could cause to exposed turtles, it should be remembered that atrazine is classified as an endocrine disruptor capable of inducing feminization of the male gonads in reptiles. Nonetheless, it remains a relatively new chemical compound to wildlife toxicology studies, and therefore, worthy of further investigation; also, because it is still legally usable in many non-European countries. Therefore, the evaluation and possible finding of traces of atrazine represents potentially an interesting fact to reflect upon for the entire international scientific community. In addition, new and more recent information on the state of contamination of the coastal area of our territories is important information for the protection of public health and animal products, as well as for the verification of the state of health of the ecosystem and the acquisition of new data also on the state of health

of the *Caretta caretta* populations in the Mediterranean basin. Data on the presence or absence of this substance in the blood of sea turtles in Italy are completely absent. Therefore, this project aims to start a data collection that will allow, in the future, to evaluate the possible exposure and the degree of the specimens that swim and visit the coast of Romagna, thus making a significant initial contribution to scientific research. On the other hand, the existence of some recent, albeit sporadic, studies on these chemical compounds will allow a comparison of the data obtained and the relative conclusive considerations.

MATERIALS & METHODS

We collected 25 serum samples from *Caretta caretta* (Tab.1,2), taken from the animal's jugular vein on the same day it was stranded and admitted to the Cetacea Foundation. The samples were individually labeled and stored in the freezer until laboratory analysis. Freshwater samples were taken in two different points of the Savio river course (Fig.13,14) and of the surface waters of the Adriatic Sea in front of the Cesenatico coast; samples were individually labeled and kept until further analysis.

name	Sex	Lenght (cm)	weight (kg)	Stranding cause	Date of sampling
ANTONIO	M	79	63,45	/	22/01/2021
NASNADA	F	76	51,7	Drowining	23/03/2021
FLAMINIA	F	80	60	hypotermia	22/01/2021
FORTUNA	F	61	27,7	Hypotermia	25/03/2021
VALENTINO	M	65	33,45	Drowining	20/01/2021
VIRGILIA	F	64	31,8	Drowining	27/08/2021
MAGICA	F	40	8,40	Hypotermia	23/03/2021
MATTEO	M	19	0,896	Hypotermia	23/03/2021
MARGHERITO	M	63	29,3	Drowining	14/04/2021
FAZIO	M	37	6,50	Drowining	28/04/2021
CAROLINA	F	22	1,400	Hypotermia	03/07/2021
MIRKA	F	63	28,55	Drowining	23/04/2021
MARIO	M	/	/	/	21/06/2021
BEATRICE	F	70	39,45	Drowining	27/03/2021
ZANNA	M	61	29	Drowining	17/03/2021
MARCO	M	34	4,25	Trauma	03/07/2021
MICHELA	F	17	0,750	Hypotermia	13/05/2021
ILENIA	F	35	5,6	Hypotermia	22/04/2021
JENNY	F	69	36,90	Drowing	08/04/2021
ALBERTO	M	77	45	Pneumonia	14/06/2021
LUCIA	F	20	?	Trauma	18/07/2021
FRANCESCA	F	61	26,4	Hypothermia	23/04/2021
MALO	M	/	/	/	28/04/2021
MILVA	F	72	43,75	Drowining	28/04/2021
JULIO	M	68	37	Drowining	28/04/2021

Tab.1 Data sheets of the turtles admitted and sampling date

Fig.1 Savio river sampling site n°1

Fig.2 Savio river sampling site n°2

Samples preparation and assay procedure

Water and blood samples were thawed and centrifuged for 15 minutes at 1000 RMP. Then samples were tested for Atrazine using an antigen-antibody chromatographic method: the ELISA (Enzyme-linked immunosorbent assay) method (ABNOVA, www.abnova.com) The kit was tested for serum and validated by the manufacturers specifically for this study. The standard blood and water used and placed in each well was 50 µL. Then we carefully added 50 µL of standard (Positive Calibrator containing 10 µg/mL of atrazine) and water and serum samples to the bottom of each well. We slightly tapped the side of the strip holder to evenly distribute the sample and added 100 µL of ATZ-ALP conjugate to each well. Slightly tapped the side of the strip holder to properly mix the sample and enzyme conjugate. We incubated at room temperature for 40 minutes. After incubation, we disposed the solution in the wells by inverting and shaking. We washed microtiter wells 3 times with wash buffer to remove the non-bound conjugate. After that we added 100 µL of pNPP substrate to

each well and incubated at room temperature for 20 min. Then we added 50 µL of Stop Solution to each well and tapped the strip holder for proper mixing. At the end we read absorbance at 405 nm using an ELISA reader.

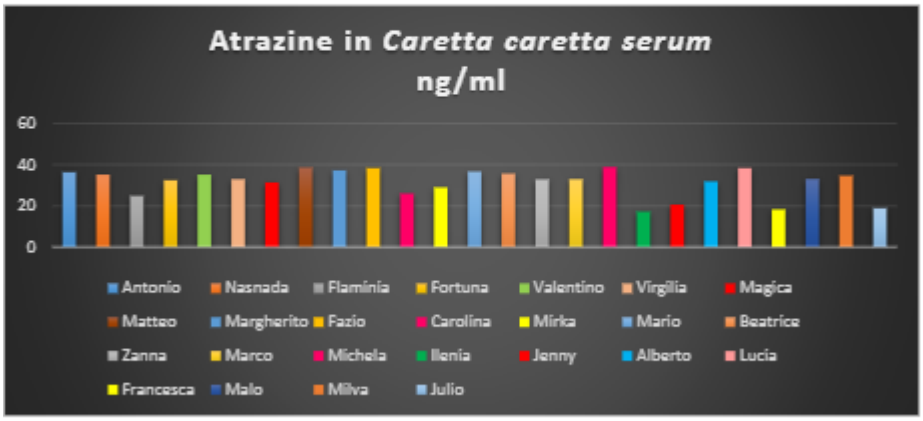
RESULTS & DISCUSSION

The results obtained from this study are shown in Table 3 and show an evident positivity of all test subjects and the detection of traces of atrazine in the surface water samples included in the analyses.

subject	Atrazine ng/ml serum
ANTONIO	36,4
NASNADA	35,2
FLAMINIA	24,9
FORTUNA	32,5
VALENTINO	35,2
VIRGILIA	33,0
MAGICA	31,4
MATTEO	38,7
MARGHERITO	37,3
FAZIO	38,4
CAROLINA	26,0
MIRKA	29,0
MARIO	36,7
BEATRICE	35,7
ZANNA	32,9
MARCO	33,0
MICHELA	38,9
ILENIA	17,2
JENNY	20,6
ALBERTO	31,9
LUCIA	38,4
FRANCESCA	18,3
MALO	33,1

MILVA	34,7
JULIO	18,9

Table 3 Atrazine concentrations (ng/ml) in plasma samples from *Caretta caretta* turtles

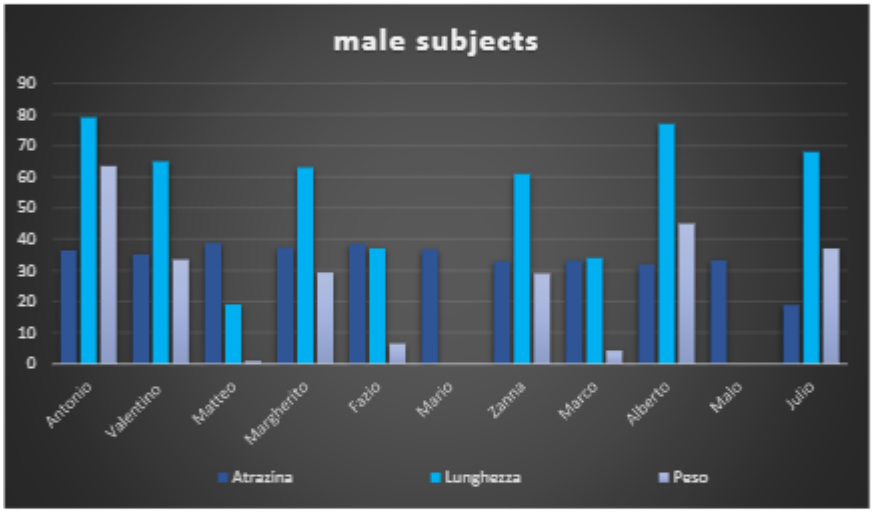


Grafic 1: Results in *Caretta caretta* serum

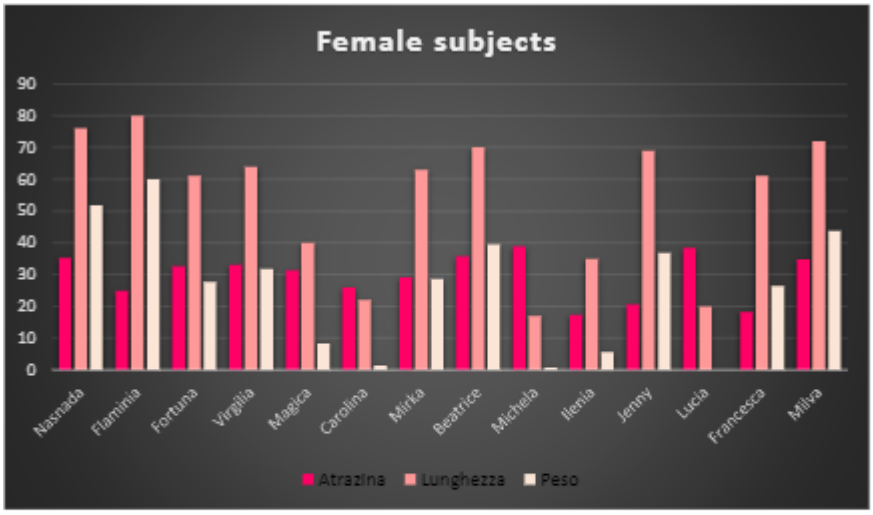
As we exclude the possibility of human error during the analytical procedure, and as this ELISA test's sensitivity and specificity parameters are guaranteed for the substance to be tested and the matrices used, our results are surprising and worrying. Even though only a few studies are looking for the presence of atrazine and estimating its quantity both in the environment and in wild species, it was possible to compare our results with those obtained from research carried out in the Gulf of Mexico on Kemp's ridley sea turtles (Lepidochelys kempii) (Montes et al.; 2020). In this study, several chemical compounds were sought in the blood of these endangered animals, including atrazine. Of all the contaminants considered, atrazine reached the highest percentage levels in plasma (69.62%). In addition, a considerable statistical difference was observed between the results derived from the year 2015 and those derived from the year 2016 (Fig.19), which saw a considerable rise in the mean, minimum and maximum values in the most recent period ((2015: Median 2.40, Min. 2.40 and Max. 65.35 ng/ml), 2016: (Median 39.26, Min. 11.42 and Max. 83.71 ng/ml).The concentrations of atrazine found in the blood of common turtles analyzed in our study (Tab.3) show levels very similar to the average concentrations found in 2016 in L. kempii turtles from Mexico. The highest value found in our study is 38.9 ng/ml, which is not an insignificant level of contamination considering that in our country, the use of

substances containing atrazine has been banned since 1992, while in Mexico, its use is still permitted. Therefore, the values found are the result of a high environmental persistence of the contaminant examined, which extends over time. It should be stressed that the use of blood samples as a research matrix always and only allows us to analyze a 'snapshot' condition, i.e., to photograph a precise and fleeting moment of the contaminant's pharmacokinetics within the body, as the blood simply acts as a means of transport for the pesticide. Although atrazine concentrations are high compared with those found in sea turtle serum from countries where atrazine is still legal, we cannot predict the future of the substance in our study subjects. Because we were unable to analyze other tissues that typically act as storage sites for the chemicals, such as liver, kidney, or muscle, we cannot determine whether and how much of the atrazine found in the animals' circulatory streams was eliminated or accumulated and thus posed a potential risk to their survival. In addition, it should also be noted that, unlike the research carried out on the Mexican specimens, the animals we assessed were not in an optimal state of health but were all defecated, stranded in a severe condition, underweight, parasitized or suffering from trauma and infection. Therefore, it is reasonable to assume that some of the atrazine found in the circulatory stream may have been released from storage organs in response to the body's mobilization of lipid energy reserves in distress. This evaluation is very important because, considering this hypothesis, the presence of atrazine in the serum we analyzed may not necessarily be recent but simply represent the movement of a previous accumulation of contaminant in the body that has not yet been disposed of. This could be true, at least partly, considering that the presence of atrazine also found in the water samples analyzed raises the suspicion of current exposure to the compound.When analyzing the data obtained from the turtle sera, the question was asked whether there was any correlation between the sexes and the concentrations of atrazine found and whether there was any correlation between the length of the carapace, and thus between an approximate estimated age of the subjects by this measurement, and the serum levels of the pesticide. Taking male and female subjects separately and evaluating their atrazine levels and estimated age, it can be observed in the graphs below (graphs 2 and 3) that it is not possible to identify a correlation between the age of the subject and the serum concentration of atrazine that we detected. Even though there is a considerable variation between the ages of some specimens, such as the turtles Antonio and Alberto, who are the oldest, and other much younger ones, the most important of which is Matteo, the levels of atrazine are very similar. The same observation can be made by comparing the subjects' weights, proportional to their ages, where Antonio is the

heaviest, Matteo the lowest, and the atrazine levels, which show only a few points of difference. The comparison between the two sexes and atrazine levels also shows no particular correlation, although atrazine is described in the literature in turtle eggshells analysed. However, a fundamental question is whether this finding is related to maternal elimination of the compound, affecting the composition of the eggs, or whether the eggs, once laid and porous, underwent subsequent environmental contamination. If the latter is the case, then even after reaching adulthood and spawning, female subjects would not necessarily have significantly lower levels of atrazine than sub-adults or juveniles.



Graf. 2: Male subjects: sex and weight ratio to atrazine



Graf.3 Female subjects: sex and weight ratio to atrazine

The values found in the surface water of the Savio river could raise concerns as they exceed the limit value for drinking water (0.1 µg/L), whereas this is not the case for the levels measured in seawater (Tab.4).

Savio river and Adriatic sea suface's sampling sites	Atrazine (µg/L)
Staz. 1 Fiume Savio	0,35
Staz. 2 Fiume Savio	0,32
Acqua marina, Cesenatico	0,02

Tab.4 Atrazine concentration in surface water sampling sites

A comparison can be made with a study carried out by Ispra on the presence of atrazine in surface and groundwater in the River Po, in which no problems were found in the analyses for surface water, in which the measured levels of atrazine did not exceed the limit value, contrary to the values at some groundwater sites. The same study also shows that the presence of atrazine has been decreasing since 2007. However, a rapid disappearance of the substance is not possible due to its chemical and physical characteristics. In addition to the dangers of atrazine as an endocrine disruptor, we are unaware of the risks of possible mixtures of contaminants in the environment that may affect the entire ecosystem. Therefore, it is essential to consider that even low concentrations can cause damage to living organisms and the environment. The following studies do

not show a natural exposure to atrazine. Instead, the animals were treated artificially through injections into the eggs or contaminated soil where the eggs were incubated. In both cases, experiments were carried out to demonstrate the potential effects of atrazine on different species. The structure of reptile eggs consists of a shell that acts as a physical barrier and membrane to protect the embryos inside from exposure to microbiological or anthropogenic factors such as pesticides. For this reason, the actual concentrations of atrazine reaching the tissues inside are not known, as it is possible that due to the protective role played by the natural organization of the eggs, the levels of contaminants to which the embryos are subjected are lower. Consequently, the effects on reptiles from exposure to atrazine are small compared to those seen in other vertebrate species. However, there is some evidence that turtle eggs laid in the soil absorb contaminants from the ground. In the United States, eggs of Chelydra serpentina snapping turtles were tested and treated with two different concentrations of atrazine (0.02 mg and 0.4 mg), reflecting actual contamination levels. The injection only occurred following gonadal sex determination to avoid any possible impact on the sex ratio of the animals (Russart et al.; 2016). The results obtained from this experiment showed that no alterations of any kind occurred 24 hours after atrazine treatment. In contrast, one week and six months after the same experiment, C. serpentina turtle embryos showed considerable alterations in gene expression in the hypothalamus. More specifically, increases in prolactin (PRLH) and aromatase gene expression were detected in both sexes following treatment with the lowest dose of atrazine. According to a 2011 study (Hayes et al.), atrazine is responsible for de-masculinization and feminization of the male genders in several vertebrate species such as fish, amphibians, reptiles, and mammals. De-masculinization is characterized by a reduction in the size of the testes of male subjects, a reduction in the production and secretion of androgens, which are necessary for the differentiation and development of germ cells, and a consequent decrease in germ cell production, with the subsequent emptying of the seminiferous tubules or filling them with detrital cellular material, and a progressive decrease in Sertoli cells. These manifestations lead to an interruption of testicular development, with consecutive lesions of the target tissue. Sometimes, in addition to the lack of sperm production, it is possible for female germ cells, oocytes, to develop within the testicles, giving rise to testicular oocytes and thus to partial female differentiation. At the same time, in other cases, one speaks of complete ovarian differentiation. As noted above, atrazine can induce over-regulation of the aromatase enzyme, which is responsible for the conversion of testosterone to oestradiol, leading to overproduction of estrogen and partial

or total feminization of the male gonads of exposed individuals. Another study (Willingham et al.; 2005) looked at the relationship between temperature and the effects of atrazine, in this case, specifically on red-eared tortoises (Trachemys scripta elegans). It is known that for many reptiles, including turtles, sex determination during the embryogenesis process is influenced by temperature, which plays a key role: at incubation temperatures below 29.4°C, most births will be male, whereas at higher temperatures, the majority will be female, with 100 % above 31°C. The eggs used in the experiment were treated with levels of atrazine commonly found in the environment; the concentration applied is 0.5 ppb, which is among those approved for drinking water. Once the eggs hatched, after incubation at a temperature of 29.4°C, which predicts mixed-sex turtle hatchlings, a significant majority of female hatchlings were observed, probably due to atrazine interfering with the normal process of gonadal differentiation by temperature. A further difference was found when measuring the platelet length of turtles incubated at 26°C, which was unchanged from that of turtles incubated at 29°C. This demonstrated that the combination of low temperatures with exposure to atrazine produced the same effects as raising the temperature alone. Several studies have highlighted further anatomical changes in different species of turtles exposed to atrazine. One of the most recent (Carneiro et al.; 2021) examined embryos of the tortoise *Podocnemis expansa*. Subjects were divided into three groups, and each was treated with different levels of atrazine during the incubation period. It was possible to observe bone changes, far from the normal physiology of the species examined; abnormal calcified structures were found between the scleral ossicles and the empty spaces in the embryos subjected to 2 mg/L and 200 mg/L of atrazine. This could have harmful consequences for the various life stages of the animals, as the sclerotic rings, by providing support to the ciliary muscle of the lens, play an important role in improving vision. Turtles may have difficulty finding food and escaping from predators with inadequate eyesight, making them less likely to survive. Another study demonstrating structural abnormalities in the North American river turtle species *Graptemys ouachitensis* and *G. pseudogeografica* was carried out in 2011 (Neuman-Lee et al.), where a thorough morphological analysis revealed irregularities in the carapace, specifically in the number of scutes, but no direct association with the levels of atrazine used to treat the eggs. In contrast, the various post-hatching effects recorded were linked to atrazine exposure, particularly at the lowest exposure levels (0.1 mg/L). The contaminant significantly reduced embryo survival. Not only that, but even pups at one year of age suffered significant losses, even though all external environmental factors that generally affect animal life in nature were eliminated in this study.

Therefore, it is likely that the high mortality was due to physiological abnormalities and diseases caused by debilitation following treatment. It was also possible to observe that treated animals showed less active behavior than untreated animals. The natural event of the turtle hatchlings emerging from the shell and emerging from the ground was simulated. A nest-like enclosure was used, and it was found that subjects exposed to atrazine were less able to escape, which could affect the survival of the species in the wild, as they would not be able to leave the nest and die. Subjects treated with the highest levels of atrazine (100 mg/L) showed more similar effects to the untreated group, demonstrating that high concentrations are not necessary to cause severe damage to exposed populations. As if that were not enough, the mere fact that they were treated with atrazine only once and yet showed adverse effects on their survival raises concerns about the biological impacts on the ecosystem that all categories of contaminants, not just atrazine, can have. Another negative aspect is that the early mortality of these individuals means that any chronic consequences of atrazine exposure cannot be detected. In 2016, a study was carried out (Soltanian et al.) on the effects of atrazine on Trachemys scripta tortoises. In this experiment, the researchers wanted to use an administration technique, intraperitoneal injection, so that the levels of contaminant absorbed by the test subjects were safer and more accurate than if the exposure had been in the water and absorbed through the skin. Again, the atrazine concentrations used for the treatments were chosen from a range of values relevant to the natural environment. It was observed that the dose equivalent to 1 ng/g body mass caused the most significant alterations. Following the analysis of several parameters, several alterations induced by exposure to atrazine were identified. The contaminant led to a reduction in the total number of leucocytes in the turtles. It increased the heterophil/lymphocyte index (H/L), synonymous with a state of stress in the animals. Phagocytic activity, lysozyme activity, and complement activity differed from the untreated group. In particular, the first two, which belong to a fundamental defense mechanism against bacterial pathogens, suffered a significant reduction, while complement activity was even wholly inhibited. Combining these changes means that the turtles' ability to fight off pathogens has changed dramatically, making them more susceptible to infection and more likely to die in the wild. Thus, atrazine has been confirmed to be responsible for immunosuppression and immunomodulation in red-eared turtles.

CONCLUSIONS

In the current study, the data obtained show that significant concentrations of atrazine are still persistent in the Mediterranean basin, both in the environment and in the marine fauna that is part of it. However, this substance was legally banned in the 1990s. The hematological investigations carried out are essential. They reflect the conditions under which local populations of common sea turtles live, helping to acquire important information for individual individuals and implementing knowledge essential for formulating predictive plans for the survival of the species. It is possible to raise public awareness, collaborate with international research groups, and provide reliable data to implement environmental and wildlife protection measures. Specifically, given the initial results obtained in this study, it would be appropriate to implement the research by repeating it on a larger sample of subjects and interfacing with other research groups, making the study interdisciplinary by making use of different research techniques such as tagging or genetics, for example, to acquire data relating to the movements of the animals and their origin to understand which geographical areas they frequent and thus be able to correlate the toxicological data obtained. In addition, our first investigation sheds light on the need to continue monitoring atrazine concentrations in the water system and marine species in the coming years to assess and monitor the decrease of this compound in the marine ecosystem. This study could also be an excellent example of how it is essential to carefully study chemistry and environmental behavior of the new substances we now use for commercial purposes to avoid the uncontrolled release of potentially harmful substances into nature in the future, with persistence of decades. Specifically with regard to loggerhead turtles, it has not been demonstrated that these substances are responsible for actual population declines. So further studies are needed to understand their effects. With the launch of this project, the hope is that a future assessment of the exposure and degree of exposure of turtles to pollutants can be obtained so that the health of the animals and the risks they face can be assessed. Conservation and protection programs for these species can be optimized, including mitigation actions to reduce exposure to these and other contaminants.

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Paper n.3: # Metal Concentration in Muscle and Digestive Gland of Common Octopus (*Octopus vulgaris*) from Two Coastal Site in Southern Tyrrhenian Sea (Italy)

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Article

Metal Concentration in Muscle and Digestive Gland of Common Octopus (*Octopus vulgaris*) from Two Coastal Site in Southern Tyrrhenian Sea (Italy)

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Abstract: *Octopus vulgaris* constitute an important part of most suitable marine resources for human consumption, however, they can represent a source in chemical contaminants intake such as heavy metals. In this scenario, the aim of the study was the evaluation of the concentration of lead (Pb), cadmium (Cd) and mercury (Hg) in the muscle and digestive gland of octopus caught from two different locations along Campania coast (Castellammare di Stabia and Napoli) and the estimation of their weekly human intake derived from the ingestion of octopus. Analysing 38 samples showed a higher concentration of Pb in the muscle of octopus in Castellammare di Stabia than in Napoli. No statistical differences were reported for Cd, Pb and Hg concentrations in the digestive gland of octopus between two sampling sites. Differences were observed between the two tissue types, with a higher level of Cd and Pb observed in the digestive gland compared with the muscle. Noteworthy, the consumption of muscle from Castellammare di Stabia could increase Pb intake in heavy consumers of local octopus. In conclusion, the present work determines that it is important to improve strategies to minimize environmental pollution sources in these areas.

Keywords: lead; cadmium; mercury; cephalopods

1. Introduction

Regular dietary fishery products intake is recommended by nutritionists since they contain high concentrations of functional nutrients, including omega-3 fatty acids, useful in decreasing the risk of cardiovascular diseases [1]. Fishery products consumption in Italy has increased from 16 kg/year per person in 2016, to 25 kg/year in 2018, with good prospects of further growth [2]. Cephalopods constitute an important part of the marine resources most suitable for human consumption. Common octopus (*Octopus vulgaris*) is mainly consumed in Southern European countries such as Italy and Spain; consumption in Italy has a range from 1.5 to 5.1 kg per capita/year [3]. However, this species can represent a source for chemical contaminants intake. The levels of heavy metals in tissues of marine organisms is mainly influenced by biotic and abiotic factors [4]. *O. vulgaris* is a benthic species, living

in direct contact with the seabed, which constitutes a possible pathway for trace element accumulation, and can therefore represents a source of human exposure to toxic elements [5,6].

In the Campania region (Italy) several areas exist where the pollution of soil, marine water, and groundwater is extremely severe and represents a serious hazard to public health. Moreover, these sites are located near highly urbanized and populated areas and usually represented by ex-industrial areas or lands nearby illegal waste dumps [7].

European Regulation 1881/06 [8] established maximum levels of contaminants in foodstuffs, fixing specific limit for heavy metals in Cephalopods (without viscera). The Joint FAO/WHO Expert Committee on Food Additives [9] revised its risk assessment on heavy metals in fish and adopted a PTWI of 4μg/kg b.w. week for mercury, 7 μg/kg b.w. for cadmium and 25 μg/kg b.w. for lead. Therefore, the objectives of the present study were: firstly to evaluate lead, cadmium and mercury levels in the muscle of common octopus (*Octopus vulgaris*) collected at two different sites along the Southern Tyrrhenian Sea coast (Italy) and estimate the weekly human intake (WHI) of heavy metals deriving from the ingestion of octopus compared with the PTWI; secondly to investigate the geographical variation of the same metals in muscle and digestive gland.

2. Results and Discussion

Mean concentrations of heavy metals in samples of the muscle and digestive gland of Octopus vulgaris are summarized in Figure 1. Results showed significantly higher concentration of Pb ($p < 0.001$) in the muscle of *O. vulgaris* in Castellammare di Stabia (mean value 0.537 μg g^{-1}) versus Napoli (mean value 0.046 μg g^{-1}). Levels of Cd and Hg were very low in all samples of octopus muscle. No statistical differences were reported for Cd and Hg concentrations in the muscle of octopus between both sampling sites. In the digestive gland Cd was found at a mean concentration of 2.643 and 2.969 μg g^{-1}, Pb was found at a mean concentration of 1.511 and 0.763 μg g^{-1} and Hg of 0.040 and 0.101 μg g^{-1}, in Castellammare di Stabia and Napoli, respectively. No statistical differences were reported for Cd, Pb and Hg concentrations in digestive gland of octopus between Castellammare di Stabia and Napoli (Figure 1).

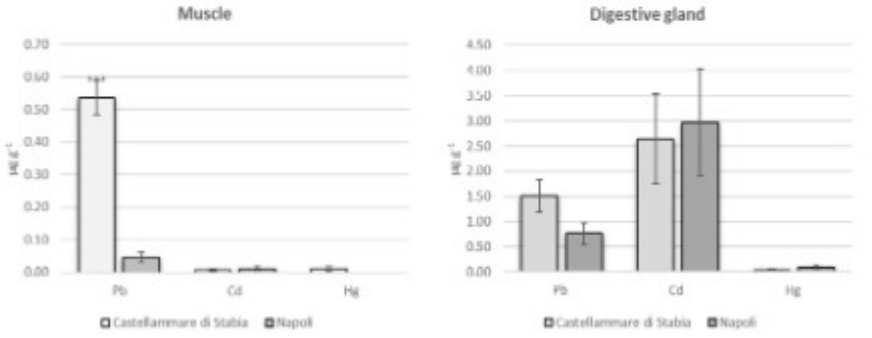

Figure 1. Mean concentration (μg g^{-1} wet weigth) ± SEM of Pb, Cd and Hg in *O. vulgaris* muscle and digestive gland from Napoli and Castellammare di Stabia. Probability levels for significant differences from sampling sites: $p < 0.001$(***).

Metal concentrations in tissues of *O. vulgaris* captured along the Campania coast were compared to the values reported for *O. vulgaris* in other coastal waters.

In the present study, Hg concentrations in muscle of *O. vulgaris* were lower than levels found in samples from the Portuguese coast (0.49 μg g^{-1}) [10]. Also, other works reported higher levels of Hg in the muscle (0.13–0.76 μg g^{-1}) and digestive gland (0.36–7.4 μg g^{-1}) [11,12].

Lead concentration in the in muscle was higher than levels reported in samples from the Portuguese coast, ranging from 0.04 to 0.09 μg g^{-1} [10]. However, other studies showed higher levels of Pb (range value 1.5–7.2 μg g^{-1}) in the digestive gland of *O. vulgaris* than those reported in the current study [13,14].

2.1. Metal Concentration versus Sampling Sites and Tissue Type

Data analysis using multivariate tests allowed an estimation of how tissue type and sampling sites influence heavy metals concentration (Table 1).

Table 1. Factorial analysis of variance (ANOVA) testing the effect from the collection site (Napoli versus Castellammare di Stabia), the accumulation organ type (muscle versus digestive gland) on the concentration of heavy metals (Pb, Cd and Hg) in Octopus vulgaris. df = degree of freedom. Probability levels for significant effects: $p < 0.001$ (***); $p < 0.01$ (**). MS = mean squares; F = F-ratio.

Source of Variation	Dependent Variable	df	Mean Square	F
Site	Pb	1	7.292	10.08 **
	Cd	1	0.521	0.06
	Hg	1	0.012	1.15
Organ	Pb	1	13.583	18.77 ***
	Cd	1	148.532	16.08 ***
	Hg	1	0.081	7.68

The tissue type had a highly significant ($p < 0.001$) influence on the accumulation of Cd and Pb, leading to higher accumulation in the digestive gland (mean values of 2.81 and 1.137 μg g^{-1}, respectively) than in muscle (Figure 2A). Mercury was accumulated at same concentration in both organs. Significant site-specific differences were detected for Pb ($p < 0.01$) only. Concentration of lead were significatively higher in octopus collected in Castellammare di Stabia (1.024 μg g^{-1}) versus that in Napoli (0.405 μg g^{-1}) (Figure 2B).

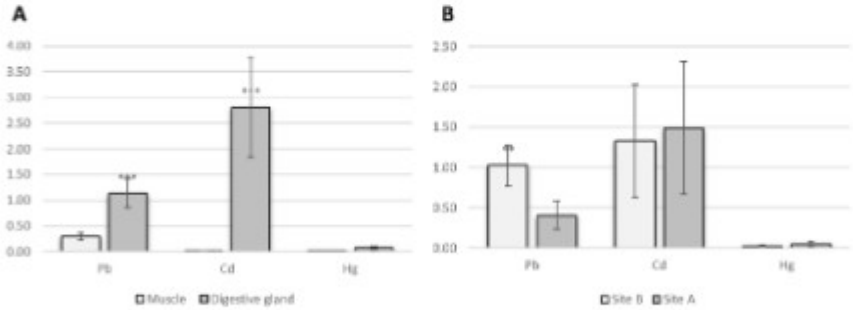

Figure 2. Concentration of Pb, Cd and Hg in *O. vulgaris* depending on (**A**) organ type: muscle versus digestive gland; (**B**) sampling sites: Napoli versus Castellammare di Stabia. Vertical bars represent average concentration (μg g^{-1} wet weigth) ± SEM. Probability levels for significant differences: $p < 0.001$(***); $p < 0.01$ (**).

All digestive gland samples of octopus showed the highest Cd and Pb concentration, confirming the primary role of this district in the bioaccumulation and detoxification processes of Cd and Pb and confirming the presence of these metals at both sampling areas [6,15]. Also, a similar distribution of Hg in the two different tissue has already been described in other studies [15,16].

Cadmium concentrations in muscle and digestive gland in the present study were comparable with those previously reported by other authors [10,17].

2.2. Metal Concentration versus Biological Parameters

The analyzed individuals varied in size and weight ranges, including males and females. The multiple regression analyses indicate that there was no correlation between weight, gender and concentration of Pb, Cd and Hg ($p > 0.05$). The lack of relations between heavy metals concentration in muscle and digestive gland of *O. vulgaris* and weight suggest that, within the range of weight of the studied specimens these parameters had minor effect on metal accumulation. These observations were already described in other studies on *O. vulgaris* captured along Portuguese coast [13]. The absence of relation between Pb, Cd and Hg levels and gender agrees with other studies on cephalopods [13,18].

2.3. Concern for Public Health

Levels of Cd and Hg in present study were low in all samples of muscle and were below the legal limit for human consumption. Anyway, the average concentrations of Pb in samples of muscle from Castellammare di Stabia were above the maximum concentration level of 0.3 μg g^{-1}, leading to the exclusion of this product for human consumption [8].

To establish possible human health implications related to consumption of octopus, the Pb estimate weekly intakes (EWI) were subsequently compared with the provisional tolerable weekly intake (PTWI) of 25 μg/kg of body weight [9]. Estimating a weekly consumption of 100 g of *O. vulgaris* muscle, EWI values were found to be 53.7 μg/week. This value accounted for 3.06% of the tolerable weekly intake set by EFSA. Considering the level of Pb, the consumption of octopus muscle from Castellammare di Stabia may increase Pb intake, but it would not contribute significantly to the PTWI. In contrast, it may contribute greatly to high EWI values in heavy consumer of octopus, when all other main contributors to dietary Pb intake and professional exposure were included in the exposure assessment.

3. Materials and Methods

3.1. Sampling

Thirty-eight samples of common octopus (*Octopus vulgaris*) were fished directly from two different locations along Campania coast (Italy) in autumn of 2016 (Figure 3).

Figure 3. Map showing locations of the sampling sites: Napoli (site A) and Castellammare di Stabia (site B).

Once captured, the octopus were weighed, their total length were measured and then they were immediately sealed in individual polyethylene bags, frozen at −20 °C and kept at the same temperature until dissection. Sex was also determined for each individual (Table 2).

Table 2. Number of individuals (n), weight (g), size (mm) and sex of *O. vulgaris* captured along the Campania coast.

Sites	Geographic Coordinates	*n*	Weight Range (g)	Total Lenght Range (cm)	Sex
Napoli	40° 49′ 39″ N; 14°14′ 47″ E	19	845 ± 143	71.7 ± 18.3	15 ♀ 4 ♂
Castellammare di Stabia	40° 41′ 46″ N; 14°27′ 53″ E	19	740 ± 229	67.6 ± 21.5	8 ♀ 11 ♂

In the laboratory, the digestive gland of each organism was totally removed under partially defrost conditions without rupture of the outer membrane. Subsequently, the digestive gland was treated separately from the remaining tissues and an interior portion was sampled for metal analysis. Arms and mantle were dissected including the skin. Each tissue was subsequently homogenized by means of a laboratory mixer and stored at −20 °C until further analyses.

3.2. Chemical and Instrumental Analysis

Glassware and laboratory equipments were decontaminated before use with diluted ultrapure 65% HNO_3 (Romil UpA, Cambridge, UK) and rinsed with Milli Q water (Millipore, Bedford, MA, USA). Aliquots of each sample (0.50 ± 0.02 g) were digested in 5 mL of ultrapure 65% HNO_3 and 2 mL of 30% H_2O_2 (Romil UpA, Cambridge, UK) in a microwave digestion system (Milestone, Bergamo, Italy). The final volume was obtained by adding Milli-Q water. Metal concentrations in the digested samples were determined with an atomic absorption spectrometer (Aanalyst 600, Perkin-Elmer, Madrid, Spain) equipped with a graphite furnace and a L'vov platform for Pb and Cd. A flow injection analysis hydride system (FIAS 100, Perkin-Elmer) was used for the determination of the total Hg. The equipment was calibrated with standard solutions (Perkin-Elmer), resulting in a calibration curve with three concentrations for Pb and Hg and four concentrations for Cd.

Recovery of the metals was determined by adding known amounts to metal-free samples, which were then subjected to the same digestion procedure. The resulting solutions were analyzed for metal concentrations. Recovery of metals from spiked samples ranged from 85 to 120%. Concentrations of each heavy metal were expressed as milligrams per kilogram wet weight [19].

3.3. Quality Assurance

Quality was monitored through analysis of procedural blanks, duplicate samples, and standard solutions. Standard solutions of analytes were prepared from certified stock solutions of Cd, Pb, and Hg with a relative matrix modifier (atomic spectroscopy standard, Perkin Elmer). Concentrations for each set of samples were determined in the medium range of the calibration curve.

The performance of the method was assessed through participation in interlaboratory studies organized by FAPAS (Food Analysis Performance Assessment Scheme, Sand Hutton, UK). The FAPAS studies were conducted with fish tissue. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated by determining the standard deviation of 10 independent blanks spiked at 1, 2, 4 and 8 $\mu g\ g^{-1}$ for Cd and 25, 50 and 100 $\mu g\ g^{-1}$ for both Pb and Hg, with an external standardization curve [20–22].

3.4. Statistical Analysis

All metal concentrations were expressed in wet weight as mean ± SEM (standard error from mean) [16]. Factorial analysis of variance was used to test statistical significance of the influence of the sampling site (Napoli versus Castellammare di Stabia), the target tissue for metal accumulation (muscle

versus digestive gland) on the concentration of heavy metals. Moreover, ANOVA and Mann-Whitney test was used to detect differences between metal concentration in muscle and digestive gland and sampling area. Statistical significance between concentration of metals and the variables (total weight and gender) were analysed using multiple regression.

The normal distribution of data was confirmed by the One-Sample Kolmogorov-Smirnov Test. Statistical analyses were performed using MedCalc for Windows, version 18.11.3 (MedCalc Software, Ostend, Belgium). The result, of $p < 0.05$, was considered significant.

4. Conclusions

The present study provided data on heavy metals concentrations in Octopus vulgaris from the Southern Tyrrhenian Sea (Italy). The concentration of Cd and Pb found in tissues of octopus sampled at Castellammare di Stabia and Napoli witness for their presence in the environment. Mercury occurred only at trace concentrations at both sampling area. The results also indicate that there was no correlation between weight, gender and concentration of heavy metals. Instead, large variations in Cd and Pb concentration existed across muscle and digestive gland of this species.

Considering Hg and Cd intake, the consumption of octopus muscle from both sampling sites does not contribute significantly to the PTWI of 4 µg/kg b.w. week and 7 µg/kg b.w. week, respectively. In contrast, the consumption of muscle from Castellammare di Stabia could increase Pb intake in the heavy consumer of local octopus, but it would not contribute significantly to the PTWI of 25 µg/kg b.w. week.

The capability of digestive gland to accumulate higher levels of Cd than muscle, as reported by Roldán-Wong et al. [23] could provide a new tool for the monitoring of the geographical distribution of this metal, even when present at negligible levels in the edible part. The presence of Pb at a higher concentration at Castellammare di Stabia in both muscle and digestive gland was probably due to a major anthropogenic pressure in this site.

Monitoring studies on heavy metals in a greater number of samples of Octopus vulgaris and other species of the marine food chain, as suggested by Sangiuliano et al. [24], will provide more detailed information of the human exposure to metals in these areas. Although monitoring of levels of chemical contaminants in marine food resources is fundamental for human health and ecological approach, it is needful to improve strategies to minimize environmental pollution sources in these areas.

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Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Not available.

PAPER N° 4 Heavy metals in the muscle and hepatopancreas of red swamp crayfish (*Procambarus clarkii*) in Campania (Italy)

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Heavy Metals in the Muscle and Hepatopancreas of Red Swamp Crayfish (*Procambarus clarkii*) in Campania (Italy)

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Simple Summary: Heavy metals can represent a serious threat to marine and freshwater fauna through exposure, bioaccumulation and biomagnification processes. The aim of this study was to evaluate the presence of non-essential and essential elements in freshwater crayfish (*Procambarus clarkii*) edible tissues to establish the healthiness of this product and to evaluate the pollution status of the sampling sites from Campania region (Italy). The results suggest that crayfish were safe for human consumption and indicated mild contamination of heavy metals of the sampling areas.

Abstract: The aim of this study was to carry out a quali-quantitative analysis of the presence of non-essential and essential trace elements in freshwater crayfish (*Procambarus clarkii*) edible tissues to establish the healthiness of this product and to evaluate the pollution status of the sampling sites included in the present study. *P. clarkii* is one of the most common species of freshwater crustaceans in Italy, regularly consumed by local people. Moreover, the crayfish, due to its trophic position and diet, can be considered as an excellent bioindicator of the health status of the ecosystem. We collected sixty crayfish samples from two different sites in Campania (Italy): Villa Literno and Sessa Aurunca. Concentrations of trace elements were determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). Our data showed low concentrations of Cd, Hg and Pb, with values below the European Commission MRL (Commission Regulation (EC) 1881/2006). We suggest that data obtained from this study showed that crayfish collected from Villa Literno and Sessa Aurunca were safe for human consumption. Furthermore, the results of this research indicated mild contamination of heavy metals of the sampling sites, indicating a good health status of the area's aquatic ecosystem.

Keywords: heavy metals; crustaceans; bioindicator; anthropogenic pollutant

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1. Introduction

Trace elements are classified by the scientific community as non-essential and essential. Non-essential trace elements have no biological role in animal organisms and represent a serious threat to aquatic fauna. Heavy metals and metalloids such as arsenic, lead, cadmium and mercury originate from natural sources and human activities (mining, metal production, combustion of fossil fuels, sewage sludge and waste incineration) [1,2] and are spread worldwide in fresh and salty waters, becoming one of the major causes of persistent aquatic pollution. Trace elements enter the food chain through bioaccumulation and biomagnification processes, contributing to compromising the balance of the food chain for a long time [3]. Adverse effects linked to acute or chronic exposure to metals

include damages to the immune system, helping the onset of infectious diseases, and interference with the endocrine system, leading to reproductive alterations. Among the freshwater fauna, crustaceans are one of the most sensitive macroinvertebrate species to suffer negative effects of exposure to metals due to their diet, way of feeding with direct contact with sediments, and life span [4–6], and they easily accumulate trace elements in the hepatopancreas, the target organ for metals investigation [7–10]. Since crustaceans are extremely sensitive to metal effects, are widely spread in aquatic ecosystems and are regularly consumed by humans, they represent an optimal bioindicator to gain information about the health status of the ecosystem and to determine safety and quality of food intended for human consumption. In our study, we focused on the red swamp crayfish *Procambarus clarkii* (Girard, 1852), which is common in the sampling areas we included in the study and usually consumed by local people. Moreover, *P. clarkii* is considered by the scientific community as an optimal bioindicator for trace elements contamination [11,12]. Indeed, the red swamp crayfish has been used as an indicator species to monitor the environmental quality and the contamination of biological habitats in previous studies [7,13–17]. Nowadays, the red swamp crayfish is listed in Italy as an invasive species. It originates from the United States and Mexico and arrived in Europe during the last century, for aquaculture purposes [18]. Unfortunately, most of the Italian farmers failed to take adequate precautions in their cultivation methods to prevent the crayfish escape from farm enclosers. Soon after, the red swamp crayfish established wild stable populations in many lakes and ponds across Italy and rapidly became the dominant freshwater crayfish [19,20]. Regarding the sampling areas, we focused our attention on geographic areas of the Campania region (Italy) which are well known to be characterized by high pollution of soil, fresh, salty water and groundwater. These sites represent ex-industrial areas and are located nearby illegal waste dumps [21]. Specifically, since the 1980s Naples and Caserta have been exploited as illegal landfills of toxic waste. Such operations and the accumulation of toxic products have had a serious impact on the ecosystem of the coast and the hinterland, influencing health and future development of the local fauna and human population [22]. In the present study, we performed a quali-quantitative analysis of trace elements in samples of hepatopancreas and abdominal muscle of *P. clarkii* collected in two different Italian sampling sites, selected for their potential high level of metal contamination. We sought to identify sources of pollution in the study area, to assess public health risk linked to consumption of crayfishes and to improve the current knowledge about the use of *P. clarkii* as a bioindicator of heavy metal pollution in freshwater ecosystems.

2. Materials and Methods

2.1. Sampling

Sixty samples of red swamp crayfish were collected during summer 2017. Crayfishes were captured using baited traps placed at Villa Literno (ViL), near the Volturno River, and at Sessa Aurunca (SeA), near the Garigliano River (Figure 1) in the Campania region. No data is at present available concerning pollution of the two areas, apart from one study reporting trace elements concentration in the blood of dogs from Sessa Aurunca [23]. Specimens were then transferred alive in refrigerated boxes (4–8 °C) to the laboratory. In our facility, crayfishes were weighed and sexed. Furthermore, we measured each carapace length using a caliper (Absolute Digimatic caliper, Mitutoyo, Japan) (Table 1), from the tip of the rostrum to the edge of the carapace. Crayfishes were euthanized by thermal shock (−80 °C for 30 min). Subsequently, the abdominal muscle and the hepatopancreas were removed under partially defrosting conditions and stored in Falcon tubes at −20 °C until further analyses.

Figure 1. Map showing locations of the sampling sites: Villa Literno (ViL) and Sessa Aurunca (SeA).

Table 1. Number of individuals (n), weight (g), size (mm) and sex of Procambarus clarkii captured at Villa Literno and Sessa Aurunca.

Sites	*n*	Mean Weight (g) ± SD	Mean Total Lenght (cm) ± SD	Sex
Villa Literno (ViL)	30	28.19 ± 4.43	9.58 ± 0.67	17 ♀ 13 ♂
Sessa Aurunca (SeA)	30	27.81 ± 3.51	9.32 ± 0.59	16 ♀ 14 ♂

2.2. Chemical and Instrumental Analysis

Each sample was homogenized and 0.5 ± 0.2 g of tissue was added to 5 mL of 65% HNO_3 and 2.0 mL of 30% H_2O_2. Microwave-assisted digestion was performed with a specific mineralization program for 25 min at 190 °C. Samples were cooled at 32 °C and the digested mixture was transferred into a 50.0 mL flask and the final volume was obtained by adding Milli-Q water [24].

Trace elements detection and quantification were determined by ICP-OES technique using a Perkin Elmer Optima 2100 DV instrument coupled with a CETAC U5000AT. Subsequently, both metals quantification and quality assurance procedure were performed as described by Zaccaroni et al. [23]. LODs values (limit of detection values) as wet weight were: 0.024 μg g^{-1} for As; 0.0002 μg g^{-1} for Cu; 0.006 μg g^{-1} for Zn; 0.001 μg g^{-1} for Cr; 0.0018 μg g^{-1} for Cd; 0.011 μg g^{-1} for Pb; 0.001 μg g^{-1} for Hg. The performance of the method has been defined by interlaboratory studies organized by FAPAS (Food Analysis Performance Assessment Scheme, Sand Hutton, York, UK).

2.3. Statistical Analysis

Results are reported in wet weight as mean ± SEM (standard error) [25]. Statistical significance of the influence of sampling sites (ViL Vs. SeA) and statistical significance in concentrations of trace elements in target organs (muscle vs. hepatopancreas) were tested using factorial analysis of variance. Furthermore, we apply the ANOVA test to highlight differences between trace element accumulation in the hepatopancreas and the muscle and between the sampling areas. Multiple regression was used to discover statistical significance between trace element concentration and intrinsic variables (as total weight and gender of specimens). One-Sample Kolmogorov–Smirnov Test confirmed the normal distribution of our data. All our statistical analyses have been performed using MedCalc for Windows, version 18.11.3 (MedCalc Software, Ostend, Belgium). Significant value has been established at $p < 0.05$.

3. Results

Mean concentrations of As, Cu, Zn and Cr in abdominal muscle (AbM) and hepatopancreas (Hep) of *P. clarkii* are summarized in Figure 2.

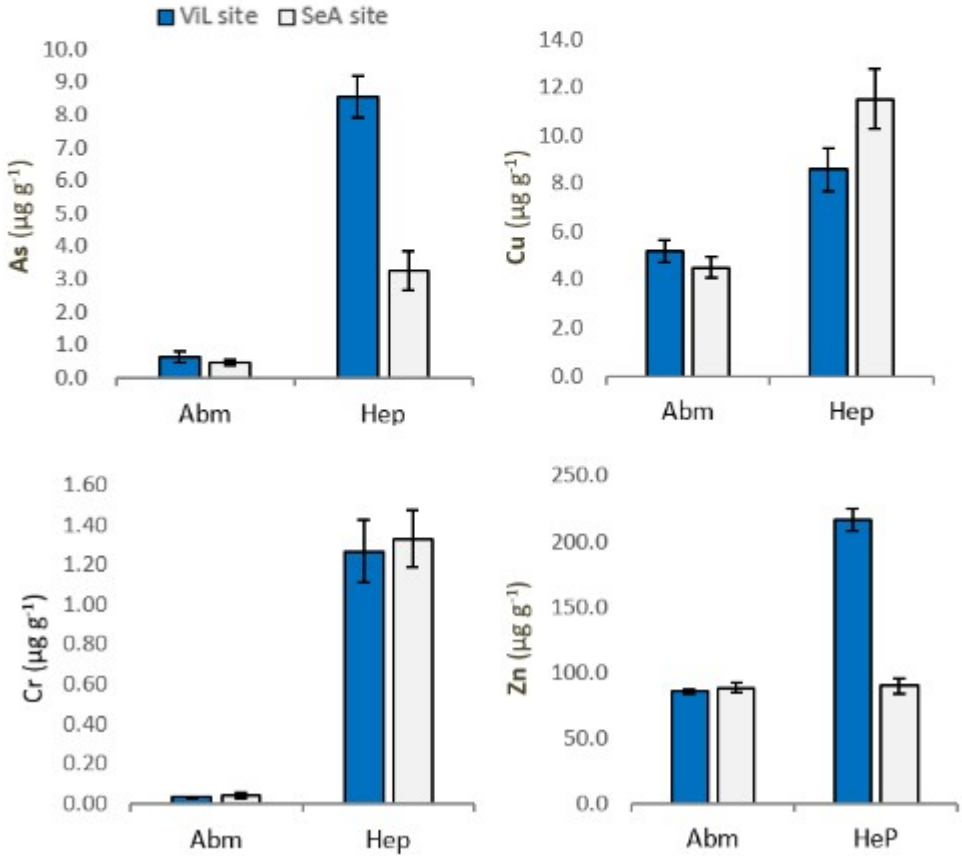

Figure 2. Concentrations of As, Cu, Zn and Cr in Procambarus clarkii abdominal muscle (AbM) and hepatopancreas (Hep) from Villa Literno (ViL) and Sessa Aurunca (SeA). Vertical bars represent average concentration (µg g^{-1} wet weigth) ⊥ SEM.

Our results show a variability in the concentration of two trace elements in *P. clarkii*, depending on sampling sites. Specifically, the levels of As and Zn were significantly higher ($p < 0.01$) in *P. clarkii* tissue from ViL site. Significant differences in organ accumulation of As, Cr, Cu and Zn have been highlighted. Indeed, trace elements concentration was significantly higher in hepatopancreas than in muscle (Table 2.). In Hep, Arsenic was found at a mean concentration of 8.534 and 3.248 μg g^{-1}, while in AbM mean values were 0.627 and 0.456 μg g^{-1}, in ViL site ($p < 0.01$) and SeA site ($p < 0.01$), respectively. Our data show, both in samples from SeA site and Vil site, significant differences ($p < 0.01$) between Cu concentration in Hep and AbM. In addition, significant differences ($p < 0.01$) were found for Zn between Hep and AbM at ViL site. Finally, higher concentrations of Cr were found in the crayfish Hep compared AbM at both sampling sites ($p < 0.01$).

Table 2. Mean concentration (μg g^{-1} wet weigth) ± SEM of trace elements (As, Cu, Zn, Cr, Cd, Pb and Hg) in Procambarus clarki abdominal muscle (AbM) and hepatopancreas (Hep) from Villa Literno (ViL) and Sessa Aurunca (SeA).

Trace Elements (μg g^{-1} Wet Weight)	AbM ViL Site	Hep ViL Site	AbM SeA Site	Hep SeA Site
As	0.627 ^{A} ±0.173	8.534 ^{B} ±0.628	0.456 ^{A} ±0.092	3.248 ^{B} ±0.605
Cu	5.172 ^{a} ±0.450	8.577 ^{b} ±0.896	4.518 ^{A} ±0.461	11.512 ^{A} ±1.239
Zn	85.553 ^{A} ±1.788	216.643 ^{B} ±8.225	87.961 ±3.753	89.617 ±6.091
Cr	0.031 ^{A} ±0.002	1.265 ^{B} ±0.157	0.042 ^{A} ±0.016	1.328 ^{B} ±0.144
Cd	<dl	0.020 ±0.002	<dl	0.018 ±0.002
Pb	<dl	0.015 ±0.002	<dl	0.012 ±0.001
Hg	<dl	<dl	<dl	<dl

Probability levels for significant differences depending on organ type: AbM versus Hep: A, B: $p < 0.01$, a, b: $p < 0.05$.

Results showed negligible levels of Cd and Pb in all samples of the crayfish AbM. In the Hep, Cd was found at a mean concentration of 0.020 and 0.018 μg g^{-1}; Pb was found at a mean concentration of 0.015 and 0.012 μg g^{-1} in Villa Literno (ViL site) and Sessa Aurunca (SeA site), respectively. Mercury was found under the detection limit (dl) in all analyzed samples (Table 2).

The analyzed individuals varied in size and weight ranges, including males and females. The multiple regression analyses indicate that there were no correlations between weight, gender and concentration of all analyzed trace elements ($p > 0.05$).

4. Discussion

The absence of a relation between trace elements and gender agrees with other published studies on *P. clarkii* [13,16,26]. Moreover, we did not appreciate a significant link between trace element concentration in the analyzed tissues and the weight of specimens, suggesting that these parameters have a minor effect on metal accumulation in subjects inside the weight range considered in this study [24].

Arsenic concentrations found in the crayfish muscle are comparable to results obtained by Bellante et al. (0.537 μg g^{-1} w.w.). In the same study the concentration of As in hepatopancreas was lower than those found in the present study (1.128 μg g^{-1} w.w.) [16]. Comparable levels of As in muscle were found by Gedik et al. in crayfish from Lousiana [14]. Devesa et al. [27] report arsenic concentration ranging from 9.2 to 12 μg g^{-1} in muscle

and from 2.5 to 2.6 μg g^{-1} in hepatopancreas of crayfish from Southern Spain, higher than those found in the present study. On the contrary, Mistri et al. [28] and Tan et al. [29] report mean As concentration in both Hep and AbM lower than those detected in present study.

Regarding essential trace element concentrations, previous studies reported variable values of copper and zinc levels in the crayfish tissues. Among them, Bellante et al. [16] reported Cu levels in crayfish hepatopancreas and muscle ranging from 1.149 to 48.3 μg g^{-1} (mean value 12.3 μg g^{-1}) and from 1.34 to 12.72 μg g^{-1} (mean value 5.19 μg g^{-1}) w.w., respectively. These data agree with the results of the present study. Similarly, Kuklina et al. [30] and Mistri et al. [28] report comparable Cu concentrations in both tissues. Despite this, another study conducted in Lousiana established a range for Cu and Zn concentrations in the crayfish muscle ranging from 23.8 to 44.2 μg g^{-1} and from 41.3 to 55.8 μg g^{-1}, respectively [31]. Moreover, a recent study conducted in Central Italy showed Cu levels that varied from 23 to 1031 μg g^{-1} in Hep and from 27 to 187 μg g^{-1} in AbM [15]. Cu levels in the hepatopancreas and muscle reported by those authors were higher than those detected in the present study, while Zn levels in Hep and AbM were lower than those found in ViL and SeA sites.

Regarding Cr concentrations, Bellante et al. [16] reported levels in crayfish hepatopancreas and muscle of 0.915 μg g^{-1} and 0.24 μg g^{-1} w.w., respectively. Mancinelli et al. [13], reported Cr in muscle tissue of *P. clarkii* (0.20–0.29 μg g^{-1}) at higher concentrations than those found in AbM in ViL and SeA. Kuklina et al. [30] and Tan et al. [29] report similar Cr concentrations in the Hep to those detected in present research, while levels detected in AbM are higher in Campania samples with respect to these two studies.

Detection of Cd and Pb has been widely explored in crayfish. The levels of Cd in AbM of ViL site and SeA site, respectively, are generally comparable with those found in the muscle of *P. clarkii* from Preola Lake (<dl–0.01 μg g^{-1} d.w.) and Gorgo Medio Lake (<dl–0.03 μg g^{-1} d.w.) in Sicily, Italy [16], and lower than those reported in crayfish muscle from Trasimeno Lake (0.05 μg g^{-1} and 2.2 μg g^{-1}) and Bolsena Lake (0.03 μg g^{-1}) in Central Italy [13,15]. The levels of Pb accumulated in AbM and Hep determined in our research are also lower than concentrations measured in other areas [15–17].

Cadmium concentrations found in Hep of ViL site and SeA site are comparable to those measured in hepatopancreas of *P. clarkii* from Preola Lake and Gorgo Medio Lake in Sicily, Italy [16], but lower than the ones reported by other authors [7]. In 2016, Goretti et al. [15], detected Cd (mean value 8.2 μg g^{-1} unpolluted area; 28.2 μg g^{-1} polluted area) and Pb (mean value 8.5 μg g^{-1} unpolluted area; 3.2 μg g^{-1} polluted area) in the hepatopancres of *P. clarkii* from Trasimeno Lake (Cental Italy) at higher levels than those found in ViL and SeA sites. Same results were reported for both Cd and Pb by Tan et al. [29], Mistri et al. [28] and Kuklina et al. [30].

The general evidence was that crayfishes from ViL and SeA accumulated higher levels of metals (As, Cu, Zn and Cr) in Hep than in AbM, in accordance with those reported in literature [7,14–16]. Almost all studies on the distribution of trace elements in crayfish tissues showed that the hepatopancreas is the target organ of storage and detoxification of heavy metals [7–10]. However, in the present study, no statistical differences were reported for Cd and Pb concentrations in AbM and Hep of *P. clarkii*, probably due to the negligible concentrations of these non-essential trace elements in the aquatic environment of both sampling sites.

Concern for Public Health

Even though no European or Italian regulation for As, Cu, Zn and Cr concentration in crustaceans and food products exists (because they are considered as essential trace elements, necessary for specific physiological functions), some tolerable upper intake levels have been proposed by both American and EU governmental and research entities (National Institutes of Health, U.S., Department of Health and Human Services; German Federal Institute for Risk Assessment, BfR; Scientific Committee on Food, European Commission, SCF; EFSA).

Copper is easily found in the environment and is essential for normal growth and metabolism [32]. Additionally, it is a component of the respiratory metalloprotein hemocyanin in crustaceans [33]. Therefore, relatively high copper amounts may be found in crayfish tissues, mainly in the hepatopancreas [7,34]. The role of Cu in crayfish metabolism and its great variability in data reported by other studies make comparison difficult, but the concentrations of Cu found in the present study are generally similar or higher than those reported in nine crayfishes captured both in polluted and unpolluted study areas [16]. Detected levels of copper are well above the recommended dietary allowances for toddlers and for adults (0.14–0.15 μg g^{-1} respectively) set by NIH [35] and of 0.08 μg g^{-1} for adults defined by BfR, SCF and EFSA [36–38].

The concentrations of Zn were higher than concentrations found by other authors in polluted and unpolluted areas [16,31,39]. Our results are indicative of high Zn levels, especially in the ViL site. These levels exceed the tolerable upper intake level (UL) defined by the SCF of 0.41 μg g^{-1} for adults [40].

Anyway, it should be noted that crayfish consumption is not that common among the Italian population, and the quantity of flesh usually consumed is generally reduced, so for both Cu and Zn a reduced exposure, and consequent health risk, is expected.

Chromium levels detected in Hep and AbM are comparable or lower than those reported by other authors [13,16]. Furthermore, Cr concentrations in AbM are below the threshold concentration suggested by FDA [41] of 1.089 μg g^{-1} w.w. for human consumption. Anyway, it is important to remember that also an excess of these metals can potentially cause harmful effects in organisms [10,42]. No UL has been defined for Chromium, but the WHO suggested to not exceed a 250 μg/day supplementation, equivalent to a daily dose of 4.16 μg g^{-1}, if using a standard weight of 60 kg [43,44].

Although our results are suggestive of higher levels of As in Hep, especially in the ViL site, concentrations of As in AbM are comparable to those reported in the literature and considered concentration responsible for low risk for human consumption [14]. No UL has been set for As at present by any governmental institution, but a maximum concentration of 50 μg L^{-1} has been defined [35], well below the mean concentrations detected in present study. Anyway, it should be remembered that the substantial portion of arsenic present in fish and mollusks is in the organic form and, as stated by Trumbo et al. [35] as well, these forms are less toxic than inorganic form (for whom the assessment is done). Consequently, any increased health risk from food products such as fish and mollusks is unlikely.

Regarding non-essential trace elements, The European Union legislation (Commission Regulation (EC) 1881/2006 and its amendment (Commission Regulation (EU) 420/2011) on food safety clearly establish the MRLs for total Cd, Pb and Hg which can be detected in the muscle of crustaceans (0.5 μg g^{-1} w.w. for Cd; 0.5 μg g^{-1} w.w. for Pb and 0.5 μg g^{-1} w.w. for Hg) intended for human consumption [45,46]. The results obtained in the current study show lower levels of Cd, Pb and Hg in AbM and Hep from ViL and SeA sites than the MRLs reported by EU regulations. Furthermore, our data are largely below the established MRLs, suggesting a limited Cd, Pb and Hg contamination of the aquatic environment of the study areas, and good food safety of aquatic products derived from these geographic areas.

5. Conclusions

The accumulation of trace elements in *P. clarkii* tissues reflects the concentrations of metals in the surrounding environment [5] and our data suggest that *P. clarkii* could be considered a good bioindicator for metal pollution. The higher Cu and Zn concentrations found in *P. clarkii* tissues, especially for Zn from ViL site, could be related to higher anthropic activity in these areas, as already proved by a paper by Zaccaroni et al. [23]. However, these results must be evaluated with caution because of the small number of samples collected and the lack of legal limits for the detection of some trace elements concentration in crustaceans and other fish products. The higher As concentrations in crayfish Hep, especially from ViL site, must be further clarified in order to identify possible

sources of contamination in these areas. Further studies are also needed in determining the percentage of organic and inorganic arsenic in crayfish tissues.

Ongoing studies on metals in a greater number of *P. clarkii*, in other biological and environmental samples and in other geographical areas, will provide more useful information to confirm this species as indicator of environmental contamination.

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Institutional Review Board Statement: Ethical review and approval were waived for this study, since *P. clarkii* is considered an invasive species in Italy which should be eradicated. Its collection is thus allowed without any special permission.

Informed Consent Statement: Not applicable.

Data Availability Statement: Detailed data supporting results are available on request at the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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PAPER N° 5 Ecotoxicity in *Hyriopsis bialatus* of copper and zinc biocides used in metal-based antifouling paints

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RESEARCH ARTICLE

Ecotoxicity in *Hyriopsis bialatus* of copper and zinc biocides used in metal-based antifouling paints

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Abstract
Biofouling is a costly burden for the shipping industry. Metal-based antifouling paints are widely used to protect submerged surfaces, but the release of metals from coatings and the recoating of hulls can leach large amounts of copper and zinc into aquatic environments, posing a risk for aquatic ecosystems and biodiversity. With this study, we studied the time-course metal accumulation and oxidative stress in the digestive gland and the gills of *Hyriopsis bialatus*, an Asian freshwater mussel, exposed to sublethal concentrations of cuprous chloride (50 and 5 µg/L) and zinc sulfate (1000 and 100 µg/L). Time-dependent accumulation was observed after exposure to copper, but zinc uptake was negligible. Integrated biomarker response (IBRv2) and statistical analysis of individual biomarker levels showed a greater biomarker response in the digestive gland and the gills after exposure to the higher concentration of CuCl and $ZnSO_4$. Both compounds elicited a biochemical response, especially in the digestive gland. Glutathione peroxidase activity was increased after exposure to both metals at both concentrations, suggesting a powerful defense against lipid peroxidation. The biological impact of zinc was less than that of copper, suggesting mitigated ecological pressure.

Keywords Cuprous chloride · Ecotoxicity · Oxidative stress biomarkers · Pearl mussel · Tissues · Zinc sulfate

Introduction

The shipping industry has long battled biofouling on ship hulls. The growth of bacteria, plankton, and animals on submerged surfaces increases the friction between the hull and the water surface, resulting in higher fuel consumption and faster hull deterioration, with considerable economic loss (Ytreberg et al. 2017). The most commonly used means to minimize biofouling is by applying antifouling biocides. Until 12 years ago, tributyltin compounds were the preferred biocides thanks to their broad-spectrum effectiveness against biofouling organisms (Antizar-Ladislao 2008). In 2008, the International Maritime Organization (IMO) (https://www.imo.org/) banned the use of tributyltin because of the devastating toxicological and endocrine effects it had on non-target animals, including mussels, fish, marine mammals, and birds (Antizar-Ladislao 2008). The search for effective but more ecofriendly antifouling biocides that meet both industrial demands and environmental sustainability has gained momentum.

Copper (Cu) and zinc (Zn) are frequent components in ecofriendly biocides in antifouling paints because of their efficacy against biofouling (Soroldoni et al. 2018). Both metals are essential trace elements for many organisms but can be toxic depending on concentration, physicochemical characteristics of water, and duration of exposure (Elia et al. 2017; Soroldoni et al. 2018). Nowadays, toxicity data of copper-based compounds on mussels are still limited and based on metal bivalent form (Amara et al. 2018). A previous study on larvae of *Crassostrea gigas* showed that the exposure to 10 µg/L of $CuCl_2$ led no effects, whereas higher

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concentration (50 µg/L) caused a disruption of embryonic development (Alzieu et al. 1980).

Copper can modulate the levels of glutathione and glutathione-dependent enzymes in *Mytilus galloprovincialis* lowering the concentration of thiol and increasing the activity of glyoxalases (Regoli and Principato 1995). In brown mussel *Perna perna*, CU affected the level of phospholipid hydroperoxide glutathione peroxidase (PHGPx) which plays a key defense role against lipid peroxidation (de Almeida et al. 2004). Moreover, Cu exposure can boost the activity of glutathione S-transferase in *M. galloprovincialis* (Canesi et al. 1999) and *Perna viridis* (Goswami et al. 2014).

There is also little information on the toxicity of Zn in antifouling paints in bivalves (Soon et al. 2019). In *Mytilus edulis*, the 48hEC_{50} of zinc pyrithione (ZnPT) was 2.54 µg/L and can affect the embryonic development even at lower concentrations (Bellas et al. 2005). On the other hand, embryotoxicity was assessed at higher zinc concentration (75 µg/L) in *C. virginica* (Calabrese et al. 1973), and even higher (500 µg/L) in *Ostrea edulis* exposed to Zn salts (Alzieu et al. 1980).

Metals in antifouling paints can enter aquatic ecosystems by either direct release from the coating of the hull or accidental dispersion into the water during removal and repainting of the hull (Chambers et al. 2006). Although the release of biocides from paint coatings is designed to be slow, periodical reapplication to maintain its biocidal efficiency can result in antifouling paint particles (APPs) leaching large amounts of Cu and Zn into aquatic environments (Soroldoni et al. 2017). General guidelines on waste collection and its correct disposal in ship maintenance are issued by the shipbuilding and the repair industry (British Marine Federation 2005; Environment Canada 1995). Nonetheless, disposal methods are often inadequate due in part to the lack of clarity of guidelines (Srinivasan and Swain 2007). The potential increase in metal contamination from poor maintenance procedures in areas where waste generation and disposal are loosely regulated poses a risk for aquatic ecosystem stability and a threat to endangered species and biodiversity.

The pearl mussel *Hyriopsis bialatus* (Simpson 1900) is a freshwater species of the family Unionidae. It is native to Thailand, where it is widely distributed in the bottom of reservoirs and rivers in the center, north, and northeast areas of the country. Like all sessile feeders, the pearl mussel is ecologically important since it siphons nutrients from the water column and maintains freshwater ecosystems. It also has economic value for the nacreous shell employed in making pearl-inlaid furniture, ornaments, kitchen utensils, and souvenirs. Finally, it is a good protein source and its well-being can be interpreted as an indication of the health of its habitats (Kovitvadhi et al. 1999). *H. bialatus* has a rapid reproductive cycle and can generate offspring all year round. However, its relatively low survival rate in the field due to poor settlement of the larvae on their settling sites (gills of the fish host) besides excessive harvesting, increased commercial use, and water pollution have contributed to its alarming decline (Supannapong et al. 2008).

Antifouling compounds can act as neurotransmission blockers, quorum sensing inhibitors, inhibitors of transmembrane transport, adhesive production/release inhibitors and enzyme/protein inhibitors, biofilm inhibitors. Many of them are also recognized as oxidative stress inducers (Chen and Qian 2017). Oxidative stress is the biological condition in which level of free radicals increases due to xenobiotic exposure, following modulation of detoxifying defense against oxidative pressure (Ji 1995). Antioxidant and detoxifying biomarkers are useful tools to investigate the oxidative stress mounted by xenobiotics (Elia et al. 2006, 2010, 2017, 2020). Metallothioneins (MT) are ubiquitously expressed proteins and their function is closely related to their ability to bind heavy metals by cysteine residues. They act as homeostatic buffers against metallic micronutrients (Zn, Cu) and as detoxifiers against toxic metals such as cadmium (Cd). Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and reduced glutathione (GSH) are involved in defense mechanisms against the oxidative damage exerted by reactive oxygen species (ROS) (Elia et al. 2017). SOD dismutates superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2), which is then converted into H_2O by CAT and GPx. GR is an NADPH-dependent oxidoreductase that converts oxidized glutathione (GSSG) into GSH, a powerful biological antioxidant. The phase II enzyme glutathione S-transferase (GST) catalyzes the conjugation of GSH to the electrophilic centers of a wide range of substrates by sulfhydryl groups, preventing their interaction with biomolecules. Malondialdehyde (MDA) is a by-product of lipid peroxidation following the failure of antioxidant defenses.

The investigation of multiple biomarkers allows to infer the biological effects of xenobiotics when a single mussel species is considered; however, translation of this meaning in a potential ecological risk can be limited without a broader integrated system (Cravo et al. 2012). In this scenario, integrated biomarker response (IBR) is a simple but reliable tool to better categorize the severity of stressors on the health status of organisms and to summarize biomarker responses (Beliaeff and Burgeot 2002). Originally devised by Beliaeff and Burgeot (2002), IBR is a recognized methodology to assess holistic responses of individual biomarkers in field and laboratory studies (Serafim et al. 2012). The original mathematical approach was strongly dependent on the arrangement of biomarkers on a star plot and did not properly take into account fluctuations in biomarker levels (Sanchez et al. 2013). The IBR calculation was later modified (Sanchez et al. 2013) to obtain a novel integrated

biomarker response version 2 (IBRv2) that overcomes the weakness of the original method.

Considering the large release in aquatic environment of the two metals from the inadequate removing of antifouling paints from ship hulls, the aim of this study was to gain insight into the time course of metal accumulation and related stress response by the Asian freshwater mussel *Hyriopsis bialatus* exposed to high concentrations of cuprous chloride (CuCl 50 and 5 µg/L) and zinc sulfate ($ZnSO_4$ 1000 and 100 µg/L). *H. bialatus* was chosen as experimental model due to its ecological relevance in freshwater ecosystems. The specimens were exposed to the two compounds for 16 days and the levels of selected oxidative stress biomarkers were measured on day 1, 4, 8, 12, and 16. This study is the first to investigate the oxidative stress effects in mussels of cuprous ion, still poorly investigated only in fish (Elia et al. 2017). Moreover, this study is the first to investigate the potential ecotoxicological effects of Cu and Zn on the pearl mussel; it is a pivotal step in protecting this vulnerable species and maintaining biodiversity.

Materials and methods

Chemical preparation and rationale

Cuprous chloride (CuCl, purity 99.9%, code 651,745) and zinc sulfate ($ZnSO_4$, purity 99.9%, code 307,491) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For this study, we evaluated the effects of high concentrations of CuCl and $ZnSO_4$ on *H. bialatus*. To set high but not lethal concentrations for *H. bialatus*, we searched the literature for the environmentally relevant concentrations for dissolved copper (1–5 µg/L, Ferreira et al. 2008) and zinc (1.3–14.6 µg/L, Sprang et al. 2009), and the LC_{50} for both compounds found no data on acute toxicity caused by CuCl and $ZnSO_4$ on *H. bialatus*. So, we set the higher concentration of CuCl and $ZnSO_4$ to below the median 96 h LC_{50} for invertebrates (240 and 1200 µg/L, respectively) (http://gestis-en.itrust.de). Concentrations of 50 µg/L CuCl and 1000 µg/L $ZnSO_4$ were tested for lethality prior to the experiment. Once the higher concentrations were determined, we set up the experiment in which specimens were exposed via a bath-mediated exposure to two concentrations of both compounds: 50 and 5 µg/L CuCl and 1000 and 100 µg/L $ZnSO_4$. Primary stock solutions of CuCl and $ZnSO_4$ were prepared at 0.005 g/L and 0.1 g/L, respectively.

Experimental design

A single lot of 245 specimens of *H. bialatus* was purchased from a local dealer for aquarists. The pearl mussels were acclimated for 15 days in 8-L tanks filled with oxygenated filtered artesian well water (20 °C ± 1, pH 7.5 ± 0.3, average dissolved oxygen 8.7 mg/l) at the Experimental Station of the Department of Agricultural, Forest, and Food Sciences (DISAFA), University of Turin (Italy). The specimens were fed daily with a mixture of *Chlorella* sp., *Chlorococcum* sp., and *Scenedesmus* sp. in equal amounts at a total concentration of 10^3 cells/mL (Supannapong et al. 2008). After acclimation and prior to the experiment, the lethality of 50 µg/L CuCl and 1000 µg/L $ZnSO_4$ was checked. Twenty mussels were placed in two tanks (10 specimens each), one for each compound, at the same conditions described above. Assessment ran for 16 days. Water was changed every day and compounds re-added daily. Mortality was checked daily. Since no mortality was recorded, the final experiment was conducted using 50 and 5 µg/L CuCl or 1000 and 100 µg/L $ZnSO_4$. The lower concentration of both compounds was set at tenfold lower than the higher concentration. The remaining 225 mussels were placed in 15 tanks (15 specimens per tank) at the same acclimation conditions. Three tanks were used for each group: control, CuCl (50 and 5 µg/L), and $ZnSO_4$ (1000 and 100 µg/L). The experiment was carried out for a total of 16 days. Mortality was checked daily and no mortality was recorded. Every 4 days, 9 mussels from each group (3 from each tank) were sampled. We carried out a trial of 2 weeks and 5 endpoints spaced 24 h and then 96 h apart to monitor both the early effect and the effects over time of biocides on *H. bialatus*. The digestive gland and the gills were dissected, placed in labeled test tubes, and stored at − 80 °C until chemical and biochemical analysis.

Metal accumulation

Determination of Cu and Zn was performed on the digestive gland and the gills by inductively coupled plasma-optic emission spectrometry (ICP-OES, Perkin Elmer Optima 2100 DV, PerkinElmer, Inc., Shelton, CT, USA) following the protocol reported in (Pastorino et al. 2020). Briefly, samples were homogenized and microwave-digested in a Milestone ETHOS ONE oven (Milestone, Sorisole, Italy) using 4 mL HNO_3 and 1 mL H_2O_2. All reagents were from Merck (Darmstadt, Germany); acids were of Suprapur grade. Results are presented as mg/kg wet weight (w.w.). For each session, instrument performance was checked with blank reagents by processing certified reference material (HISS-1). Limit of detection (LOD) was 0.0003 mg/kg and 0.0007 mg/kg for Cu and Zn, respectively. Percentages of recovery ranged from 103 to 113% for Cu and Zn, respectively.

Oxidative stress biomarkers

Oxidative stress biomarker levels were measured in triplicate by spectrophotometry (Varian Cary 50, Santa Clara, CA, USA) at 25 °C on the cytosolic fraction of the digestive

gland and the gills. Metallothionein levels were measured in three pooled samples (three specimens each) of the digestive gland or the gills. Tissues were homogenized (1:4) in a 0.02 M TRIS/HCl buffer, added with 0.5 M sucrose, 0.1 mg/ml bacitracin, 0.008 TIU/ml aprotinin, 87 µg/ml phenylmethylsulfonylfluoride (PMSF), and 0.1 µl/ml α-mercaptoethanol. Homogenates were centrifuged at 14.500 × *g* to obtain the cytosolic fraction. The supernatants were purified in a chloroform/ethanol solution and then in HCl/ethanol to obtain the metallothionein fraction. Pellets were washed in an ethanol:chloroform:TRIS/HCl (87:1:12) solution and resuspended in 0.25 M NaCl. Destabilizing solution (1 N HCl + 4 mM EDTA) and Ellman's reagent (DTNB: 5,5 dithiobis-2-nitrobenzoic acid) were added in each sample. Sulfhydryl residue contents (−SH) were quantified at 412 nm. The absorbance of each sample was related to a reduced glutathione calibration curve (1 mg/ml GSH) to obtain the metallothionein concentration.

Total glutathione (GSH + 2GSSG) was assessed according to a method previously used in invertebrates (Dörr et al. 2020). Thiol concentration was measured in 100 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 unit of GR, 4 mg ml − 1 NADPH, and 1.5-mg ml − 1 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), both dissolved in 0.5% NaCO3. The digestive gland and the gill tissues (0.1–0.2 g) were homogenized in 5% sulfosalicylic acid, 4 mM EDTA, and centrifuged at 30,000 × *g* for 30 min (4 °C). Absorbance was recorded at 412 nm and glutathione disulfide was used as reference.

Analysis of MDA was conducted according to the method previously standardized in mussels (Magara et al. 2021). Briefly, the digestive glands or the gills of three pooled samples of three specimens each were homogenized (1:5) in TRIS/HCl 20 mM buffer pH 7.4 with 0.5 M butylated hydroxytoluene (BHT) and centrifuged. The supernatant was derivatized in 1-methyl-2-phenylindole, HCl, and TRIS/HCl pH 7.4, sample or MDA standard. After incubation at 45 °C for 60 min, the samples were centrifuged at 15,000 × *g* and read spectrophotometrically at 586 nm.

Enzymatic assays were conducted on the digestive gland and the gills. Tissues were homogenized in 100 mM KP buffer pH 7.5 with 0.1 mg/ml bacitracin, 0.008 TIU/ml aprotinin, and 2.5% sodium chloride (NaCl) and centrifuged at 30,000 × *g*. The supernatant was divided into aliquots for each enzymatic assay. The analytical methods for SOD, CAT, GPx, GST, and GR in mussels are described in detail elsewhere (Magara et al. 2021). Briefly, SOD activity was evaluated in 50 mM Na_2CO_3 buffer pH 10 with 0.1 mM EDTA, 500 mM cytochrome C, 1 mM hypoxanthine, and xanthine oxidase. CAT was determined following the consumption of H_2O_2. GPx was assessed following the oxidation of NADPH at 340 nm using H_2O_2 as substrate. GST levels were measured following the formation of thioether with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. GR activity was determined following the oxidation of NADPH at 340 nm. In order to normalize enzyme activity, the cytosolic total protein concentration was determined according to Lowry et al. (1951).

Integrated biomarker responses version 2

The integrated biomarker responses version 2 index was calculated following the original method of Beliaeff and Burgeot (2002) later modified by Sanchez et al. (2013). In this method, a value is obtained that summarizes the response of a representative panel of biomarkers. The ratio between the data of each biomarker (Xi) and the mean reference data ($X0$) was calculated and then log transformed to reduce variance:

$$Yi = \log(Xi/X0)$$

The Yi values are related to the general mean (μ) and standard deviation(s) of each biomarker and standardized:

$$Zi = (Yi - \mu)/\sigma$$

The mean standardized biomarker response (Zi) and the mean of the reference biomarker data ($Z0$) were used to determine the biomarker deviation index (A). This allowed us to assume the mean of the control group as the baseline value and then present in a star plot the biomarker variations we observed in the groups accordingly:

$$A = Zi - Zi$$

Finally, A values for each biomarker in each group were summed.

$$\text{IBRv2} = \sum |A|$$

Statistical analysis

Normality of biochemical data was tested with the Shapiro–Wilk test. Statistical analysis was performed using two-way ANOVA with concentration, time, and concentration × time interaction as independent variables, followed by Bonferroni's post hoc test ($p < 0.05$). Data are reported as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

Metals accumulation

An increase in copper load was observed on day 12 in the digestive gland of mussels exposed to the higher concentration of CuCl (1.5-fold) and on day 16 (2.5-fold) after exposure to both CuCl doses (Fig. 1A). A significant increase in Cu (1.5-fold) in the gills was recorded only on day 16 after exposure to 50 µg/L CuCl (Fig. 1B), whereas the increase in Zn (onefold) was early and transient (Fig. 1D).

Oxidative stress biomarkers

Cuprous chloride

Table 1 presents the results of two-way ANOVA of concentrations, time, and interaction (concentrations × time)

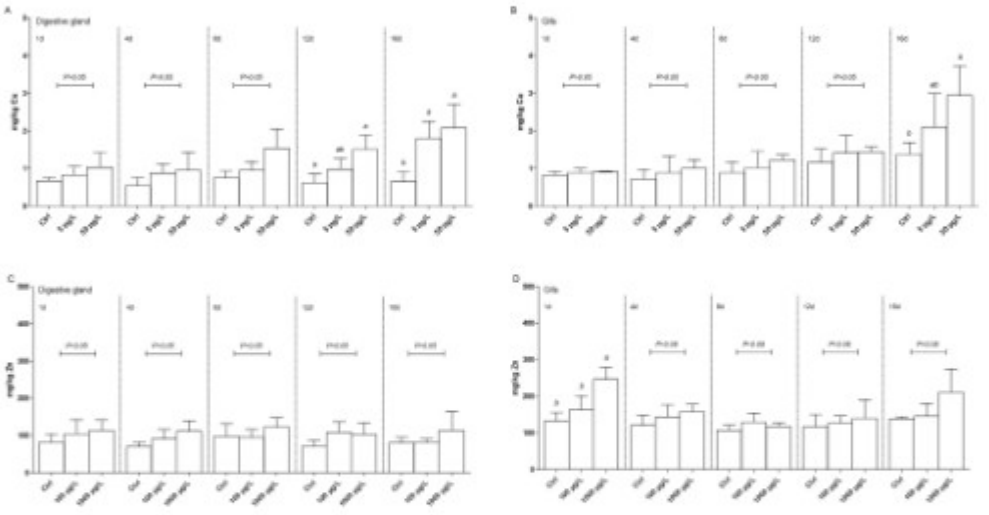

Fig. 1 Copper (**A**, **B**) and zinc (**C**, **D**) concentration (mg/kg) in the digestive gland (**A**, **C**) and the gills (**B**, **D**) of *H. bialatus* exposed to cuprous chloride (5 and 50 µg/L) and zinc sulfate (100 and 1000 µg/L). Ctrl, control. The letters (a, b) indicate statistical significant differences ($p < 0.05$) between the groups at the same time point

Table 1 The results of two-way ANOVA of time, treatment and interaction (time × treatment) on oxidative stress biomarkers in digestive gland and gills of *H. bialatus* exposed to CuCl

Cuprous chloride (CuCl)									
	DFn, DFd			FC		*F* time		*F* interact	
	C	Time	Interact	DG	Gills	DG	Gills	DG	Gills
MT	2, 6	4, 24	8, 24	2152.10^{***}	32.19^{***}	134.06^{***}	31.43^{***}	90.89^{***}	25.41^{***}
Glut	2, 6	4, 24	8, 24	12.39^{**}	318.20^{***}	27.37^{***}	1.07	12.75^{***}	2.01
MDA	2, 6	4, 24	8, 24	4.72	1.29	5.32^{**}	4.23^{**}	2.70^{*}	0.95
SOD	2, 6	4, 24	8, 24	83.66^{***}	52.95^{***}	1.96	18.97^{***}	0.95	7.12^{***}
CAT	2, 6	4, 24	8, 24	430.41^{***}	74.74^{***}	5.13^{**}	1.58	7.12^{***}	1.19
GPx	2, 6	4, 24	8, 24	216.46^{***}	671.60^{***}	374.69^{***}	17.26^{***}	92.83^{***}	9.47^{***}
GST	2, 6	4, 24	8, 24	331.02^{***}	24.85^{**}	0.06	44.95^{***}	1.05	17.38^{***}
GR	2, 6	4, 24	8, 24	15.96^{**}	2.21	25.05^{***}	7.06^{***}	4.68^{**}	1.90

F indicates the *F* values following two-way ANOVA test. *MT*, metallothioneins; *Glut*, total glutathione; *MDA*, malondialdehyde; *SOD*, superoxide dismutase; *CAT*, catalase; *GPx*, glutathione peroxidase; *GST*, glutathione S-transferase; *GR*, glutathione reductase. Significant code $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$

in the digestive gland and the gills after exposure to 50 and 5 µg/L CuCl. A late increase (up to 2.5-fold) in MT levels in the digestive gland was observed after exposure to both copper concentrations (Fig. 2A). The higher metal concentration led to lower levels of GSH + 2GSSG than in the control group (up to 70%) until day 4; an opposite trend (up to onefold) was measured from day 12 in both groups (Table 2). On day 16, the MDA concentration was decreased by 70% (Table 2); the SOD, CAT, and GST levels were lower than in the control group (up to 70%) (Table 2). GPx activity was significantly higher after exposure to both concentrations of CuCl (up to threefold) until day 12 (Table 2), whereas GR levels were lower than in the control group until day 8 (Table 2).

The MT levels were increased (up to 80%) in the gills until day 8 after exposure to the higher CuCl concentration (Fig. 2B). Conversely, thiol levels after exposure to both copper concentrations were consistently lower (up to 80%) (Table 2). SOD, GST, and GR activity was significantly increased (up to 2.5-fold) at the last endpoint (Table 2). The CAT levels were decreased (up to 60%) after exposure to both copper concentrations during the whole experiment (Table 2), whereas GPx activity was consistently elevated (up to threefold) after CuCl exposure (Table 2).

Zinc sulfate

Table 3 presents the results of two-way ANOVA of concentrations, time, and interaction (concentrations × time) on the digestive gland and the gills after exposure to 1000 and 100 µg/L $ZnSO_4$. An early and transient increase in MT levels (onefold) was observed in the digestive gland after exposure to both $ZnSO_4$ concentrations (Fig. 2C). The higher zinc concentration negatively affected thiol levels (up to 50%) on days 4 and 8 (Table 4). The MDA concentration was decreased up to 50% on days 12 and 16 (Table 4). SOD and CAT levels were lower than the control (up to 70%) (Table 4). An opposite trend was observed for GPx activity, which was increased up to threefold (Table 4). GST levels were decreased after 4 days of exposure and remained low (up to 80%) until the end of the experiment (Table 4). In contrast, GR activity was decreased (up to 60%) by $ZnSO_4$ on days 1 and 4, after which enzyme activity returned (Table 4).

Exposure to the higher zinc concentration increased SOD and GPx levels (up to fourfold) in the gills during the whole experiment (Table 4), whereas CAT activity was lower than the control group (up to 80%) on days 8, 12, and 16. A late increase in GST levels (up to onefold) was observed (Table 4), whereas GR activity was increased (up to 1.5-fold) in a dose-dependent manner starting from day 4 (Table 4).

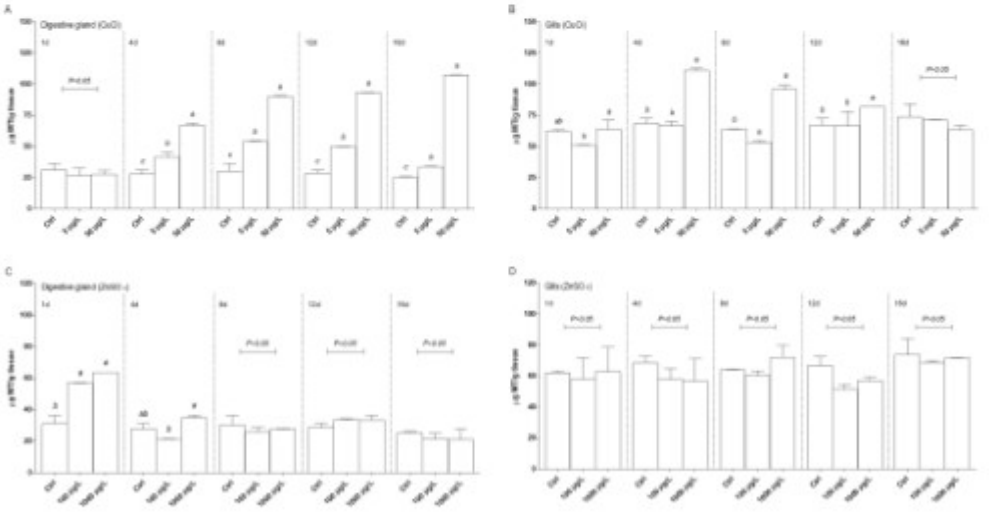

Fig. 2 Metallothionein concentration in the digestive gland (**A**, **C**) and the gills (**B**, **D**) of *H. bialatus* exposed to cuprous chloride (5 and 50 µg/L) and zinc sulfate (100 and 1000 µg/L). Ctrl, control. The letters (a, b, c) indicate statistical significant differences ($p < 0.05$) between the groups at the same time point

Table 2 Oxidative stress biomarkers in digestive gland and gills of *H. bialatus* exposed to cuprous chloride (CuCl)

Cuprous chloride (CuCl)							
		Digestive gland			*Gills*		
		Ctrl	*5 µg/L*	*50 µg/L*	*Ctrl*	*5 µg/L*	*50 µg/L*
Glut	1 d	205.64 ± 12.40 a	115.68 ± 18.41 a	77.34 ± 7.67 b	244.02 ± 49.64 a	63.40 ± 7.34 b	47.35 ± 11.97 b
	4 d	246.03 ± 10.74 a	155.55 ± 10.63 a	91.14 ± 15.34 b	197.88 ± 21.46 a	83.66 ± 15.52 b	69.32 ± 10.56 b
	8 d	245.42 ± 16.90 a	157.08 ± 10.74 a	155.55 ± 35.37 a	236.30 ± 43.44 a	93.06 ± 18.25 b	77.52 ± 23.39 b
	12d	224.05 ± 14.49 b	254.00 ± 65.74 b	371.78 ± 1.54 a	253.57 ± 37.34 a	90.87 ± 1.10 b	55.72 ± 13.58 b
	16d	238.36 ± 23.16 b	223.33 ± 57.27 b	462.57 ± 123.53 a	207.00 ± 34.66 a	127.82 ± 25.67 b	70.87 ± 20.74 b
MDA	1 d	10.71 ± 1.28 a	12.32 ± 3.78 a	12.75 ± 3.07 a	11.81 ± 3.56 a	9.64 ± 1.38 a	11.51 ± 1.38 a
	4 d	15.58 ± 1.05 a	14.43 ± 1.68 a	14.55 ± 4.50 a	10.40 ± 3.20 a	12.25 ± 2.77 a	10.12 ± 1.74 a
	8 d	14.03 ± 3.33 a	12.10 ± 4.46 a	14.69 ± 5.01 a	12.40 ± 0.27 a	14.38 ± 0.72 a	12.11 ± 3.16 a
	12d	14.73 ± 0.58 a	12.15 ± 1.82 a	9.94 ± 1.16 a	13.25 ± 1.97 a	15.55 ± 2.33 a	15.05 ± 2.83 a
	16d	15.45 ± 1.96 a	6.65 ± 2.17 b	4.75 ± 1.71 b	11.06 ± 2.28 a	14.73 ± 1.19 a	14.40 ± 3.71a
SOD	1 d	7.09 ± 1.16 a	2.81 ± 0.68 b	2.52 ± 0.74 b	6.52 ± 0.79 a	7.02 ± 1.11 a	5.88 ± 0.36 a
	4 d	5.33 ± 1.24 a	2.85 ± 0.48 b	2.14 ± 0.30 b	6.04 ± 0.30 a	7.35 ± 0.45 a	8.17 ± 2.06 a
	8 d	6.59 ± 1.36 a	2.66 ± 0.76 b	1.90 ± 0.55 b	4.90 ± 1.54 a	7.32 ± 1.03 a	8.36 ± 0.80 a
	12d	5.99 ± 0.83 a	1.64 ± 0.51 b	1.68 ± 0.14 b	6.03 ± 0.61 a	6.97 ± 0.22 a	8.09 ± 0.43 a
	16d	7.04 ± 1.88 a	2.18 ± 0.59 b	2.41 ± 0.19 b	5.19 ± 1.28 b	13.84 ± 3.01 a	15.72 ± 2.55 a
CAT	1 d	74,18 ± 3.96 a	58.64 ± 7.30 b	23.97 ± 2.87 c	22.49 ± 2.20 a	11.77 ± 1.75 b	12.38 ± 2.81 b
	4 d	77,99 ± 1.87 a	41.80 ± 1.78 b	28.67 ± 3.45 c	20.27 ± 4.54 a	12.28 ± 2.02 b	11.54 ± 1.34 b
	8 d	71.20 ± 8.75 a	35.79 ± 5.08 b	29.03 ± 1.94 b	23.83 ± 3.35 a	16.03 ± 1.59 b	12.31 ± 1.67 b
	12d	79.37 ± 8.82 a	27.46 ± 3.27 b	25.03 ± 2.90 b	22.17 ± 1.96 a	12.31 ± 2.04 b	13,19 ± 2.46 b
	16d	71.96 ± 7.43 a	27.00 ± 3.04 b	28.34 ± 7.18 b	24,24 ± 4.81 a	9.47 ± 1.26 b	10.67 ± 2.33 b
GPx	1 d	48.54 ± 2.69 c	159.46 ± 6.64 b	182.99 ± 3.81 a	32.03 ± 3.83 b	65.39 ± 4.94 a	65.49 ± 4.75 a
	4 d	45.88 ± 3.03 c	144.36 ± 6.64 b	180.78 ± 13.57 a	26.23 ± 3.00 c	59.47 ± 4.21 b	87.39 ± 8.19 a
	8 d	47.02 ± 5.81 b	70.66 ± 7.70 a	76.16 ± 12.11 a	28.99 ± 2.23 c	61.53 ± 4.82 b	96.72 ± 6.08 a
	12d	42.53 ± 4.68 b	59.14 ± 4.47 a	58.85 ± 4.16 a	28.75 ± 3.09 c	68.19 ± 3.64 b	92.36 ± 2.56 a
	16d	45.87 ± 5.11 a	50.41 ± 6.61 a	53.19 ± 1.62 a	33.82 ± 4.99 c	79.40 ± 1.79 b	100.71 ± 6.29 a
GST	1 d	114.19 ± 9.20 a	70.66 ± 8.95 b	59.70 ± 2.59 b	81.65 ± 3.21 a	75.98 ± 6.61 a	77.58 ± 8.43 a
	4 d	106.94 ± 13.36 a	73.51 ± 11.38 b	70.13 ± 11.80 b	84.46 ± 4.54 a	73.21 ± 3.03 a	79.74 ± 7.00 a
	8 d	119.51 ± 14.82 a	67.91 ± 7.20 b	57.35 ± 9.30 b	80.28 ± 6.49 a	70.37 ± 8.39 a	75.10 ± 6.26 a
	12d	116.46 ± 15.90 a	78.22 ± 5.66 b	52.68 ± 9.23 c	81.82 ± 4.49 a	74.55 ± 0.32 a	82.76 ± 5.16 a
	16d	112.99 ± 5.38 a	77.87 ± 10.13 b	58.69 ± 3.09 b	74.31 ± 4.28 b	129.89 ± 7.44 a	127.70 ± 8.27 a
GR	1 d	22.66 ± 4.48 a	7.71 ± 1.10 b	8.04 ± 0.58 b	35,59 ± 6.84 a	33.71 ± 5.00 a	32.09 ± 2.85 a
	4 d	24.78 ± 0.65 a	10.37 ± 2.99 b	9.78 ± 1.99 b	37.11 ± 3.47 a	39.60 ± 8.11 a	40.03 ± 4.77 a
	8 d	27.08 ± 3.65 a	22.75 ± 4.66 a	22.08 ± 5.02 a	35.99 ± 5.24 a	34.52 ± 2.40 a	35.56 ± 1.24 a
	12d	20.15 ± 3.77 a	24.71 ± 5.57 a	20.81 ± 3.82 a	37,24 ± 3.46 a	32.17 ± 2.47 a	32.31 ± 6.11 a
	16d	25.92 ± 4.88 a	26.06 ± 2.89 a	24.09 ± 4.66 a	36.46 ± 3.97 b	53.28 ± 12.45 a	61.03 ± 19.02 a

Data are reported as mean and standard deviation. Different letters (a, b, c) indicate statistically significant differences ($p < 0.05$) between control and treated groups at the same experimental endpoint. *Glut*, total glutathione; *MDA*, malondialdehyde; *SOD*, superoxide dismutase; *CAT*, catalase; *GPx*, glutathione peroxidase; *GST*, glutathione S-transferase; *GR*, glutathione reductase; *Ctrl*, control

Integrated biomarker responses version 2

Figure 3 presents the results of IBRv2 on the digestive gland and the gills following exposure to CuCl (50 and 5 µg/L) or $ZnSO_4$ (1000 and 100 µg/L). Increased or decreased deviation indices indicate an increase or a decrease in biomarker levels. The sensitivity of each biomarker for each metal in the digestive gland and the gills is depicted in star plots and compared to 0 value, corresponding to the control group. GPx activity was consistently higher in both tissues exposed to Cu and Zn, whereas opposite trend was found for CAT (Fig. 3A, B, C, D). The MT concentration was higher than the control in the gills exposed to the higher CuCl concentration (Fig. 3B) and in the digestive gland exposed to both concentrations (Fig. 3A). SOD activity in the gills was generally higher than in the control after exposure to both CuCl

Table 3 The results of two-way ANOVA of time, treatment and interaction (time x treatment) on oxidative stress biomarkers in digestive gland and gills of *H. bialatus* exposed to $ZnSO_4$

Zinc sulfate ($ZnSO_4$)									
	DFn, DFd			FC		F Time		F Interact	
	C	Time	Interact	DG	Gills	DG	Gills	DG	Gills
MT	2, 6	4, 24	8, 24	73.83***	6.02	83.94***	3.35	17.25***	0.82
Glut	2, 6	4, 24	8, 24	9.34*	10.00*	4.55**	2.22	3.51**	0.49
MDA	2, 6	4, 24	8, 24	5.51*	8.53*	3.88*	2.47	1.44	0.46
SOD	2, 6	4, 24	8, 24	55.67***	56.62***	18.98***	4.59**	3.82**	2.56*
CAT	2, 6	4, 24	8, 24	932.36***	375.65***	6.96***	8.98***	4.31**	4.78**
GPx	2, 6	4, 24	8, 24	289.58***	586.96***	3.08*	169.38***	4.10**	51.55***
GST	2, 6	4, 24	8, 24	842.53***	14.83**	37.43***	40.58***	10.90***	22.01***
GR	2, 6	4, 24	8, 24	60.81***	304.98***	13.67***	24.93***	3.03*	12.16***

F indicates the *F* values following two-way ANOVA test. *MT*, metallothioneins; *Glut*, total glutathione; *MDA*, malondialdehyde; *SOD*, superoxide dismutase; *CAT*, catalase; *GPx*, glutathione peroxidase; *GST*, glutathione S-transferase; *GR*, glutathione reductase. Significant code $^{***}p<0.001$; $^{**}p<0.01$; $^{*}p<0.05$

and $ZnSO_4$ (Fig. 3A, C). There was an increase in GR levels in the gills after zinc exposure (Fig. 3C) where the IBRv2 values were higher after exposure to the higher metal concentration. Furthermore, the IBRv2 was lower in the gills than in the digestive gland: 5 µg/L CuCl: 8.59 (digestive gland) and 7.32 (gills); 50 µg/L CuCl: 11.65 (digestive gland) and 9.38 (gills); 100 µg/L $ZnSO_4$: 8.06 (digestive gland) and 7.81 (gills); 1000 µg/L $ZnSO_4$: 12.10 (digestive gland) and 9.11 (gills).

Discussion

With this study, we investigated the effects of cuprous chloride and zinc sulphate, two antifouling compounds, on freshwater species *H. bialatus*, and results are related to freshwater environment. We measured metal accumulation and related oxidative stress in the gills and the digestive gland, two tissues with high potential for ROS production. Time-dependent accumulation was observed in both the gills and the digestive gland after exposure to copper, while early and transient metal accumulation of zinc was noted only in the gills. To our best knowledge, no mechanistic study of Cu and Zn uptake in the pearl mussel has been published to date.

Copper bioaccumulation in biological tissues is known to be related to the redox status of the metal (Georgopoulos et al. 2001). Accordingly, we assume that our observation of late copper bioaccumulation, which is shared by a previous study on rainbow trout *Oncorhynchus mykiss* exposed to cupric and cuprous ion (Elia et al. 2017), can be related to a copper speciation process. Among Cu species, only cupric ion is able to coordinate with endogenous organic ligands. In water, however, the reservoir of cupric ion can be ensured by the bivalent form itself and also from a disproportionation of cuprous ion (Georgopoulos et al. 2001). In the present study, it is likely that the addition of CuCl led to the transformation of cuprous ion to cupric ion, which later coordinated with biological ligands following bioaccumulation. Furthermore, dose-dependent elimination mechanisms, with a more efficient elimination rate on exposure to higher copper levels, have been recently shown in the zebra mussel *Dreissena polymorpha* (Yen Le et al. 2021). This mechanism corroborates our findings and can slow down further the bioaccumulation of copper.

In contrast, almost no zinc bioaccumulation was observed in either the gills or the digestive gland. The marked discrepancy in the ability of *H. bialatus* to bioaccumulate Cu and Zn may be surprising. A recent study on glochidia of diverse species in the order Unionida showed, however, that the binding affinities (log *K* values) of Cd, Co, Cu, Ni, Pb, or Zn to ligands at the mussel cell surface were higher than the other metals for Cu (range, 7.2–7.5) and lower for Zn (range, 6.0–6.2) (Markich 2017). Furthermore, although the Zn concentration was not increased in the body of the green mussel *Perna viridis*, subcellular distribution of the metal was altered and the proportion of Zn associated with the metal-rich granules was increased (Blackmore and Wang 2002). Besides the pivotal physiological role Zn plays in living organisms, this suggests a change in the Zn pattern and a scenario of potential metal redistribution and remodeling of biochemical metabolisms (Turkmen et al. 2008).

In the present study, both CuCl and $ZnSO_4$ affected the oxidative stress biomarker levels. It is sometimes difficult to summarize a concise information from a panel of biomarker responses to address a potential risk driven by xenobiotics (Cravo et al. 2012). IBRv2 has been successfully applied to understand biological responses following contaminant exposure in laboratory studies; consistent relationships have been established between IBRv2 values and exposure conditions (Vieira et al. 2018). It can discriminate toxicity among diverse compounds and concentrations according to IBRv2 values and thus provide a broader integrated view

Table 4 Oxidative stress biomarkers in digestive gland and gills of *H. bialatus* exposed to zinc sulfate ($ZnSO_4$)

Zinc sulfate (ZnSO4)							
		Digestive gland			*Gills*		
		Ctrl	*100 µg/L*	*1000 µg/L*	*Ctrl*	*100 µg/L*	*1000 µg/L*
Glut	1 d	205.64 ± 12.40 a	157.59 ± 31.48 a	146.35 ± 50.68 a	244.02 ± 49.64 a	169.24 ± 35.57 a	200.35 ± 49.75 a
	4 d	246.03 ± 10.74 a	190.31 ± 54.03 ab	117.31 ± 6.90 b	197.88 ± 21.46 a	152.49 ± 37.97 a	189.40 ± 20.93 a
	8 d	245.42 ± 16.90a	230.69 ± 13.81 a	140.21 ± 12.27 b	236.30 ± 43.44 a	187.21 ± 47.05 a	261.62 ± 44.58 a
	12d	224.05 ± 14.49 a	217.77 ± 51.53 a	238.36 ± 42.94 a	253.57 ± 37.34 a	205.82 ± 29.30 a	252.17 ± 67.04 a
	16d	238.36 ± 23.16 a	184.17 ± 40.50 a	224.05 ± 50.43 a	207.00 ± 34.66 a	199.71 ± 47.57 a	191.76 58.74 a
MDA	1 d	10.71 ± 1.28 a	9,15 ± 0.70 a	8.52 ± 2.11 a	11.81 ± 3.56 a	13.32 ± 1.55 a	12.67 ± 2.97 a
	4 d	15.58 ± 1.05 a	11.45 ± 1.89 a	10.68 ± 0.68 a	10.40 ± 3.20 a	11.27 ± 1.56 a	11.34 ± 4.21 a
	8 d	14.03 ± 3.33 a	13.23 ± 3.65 a	9.51 ± 2.79 a	12.40 ± 0.27 a	15.25 ± 3.75 a	17.34 ± 2.10 a
	12d	14.73 ± 0.58 a	8.52 ± 2.72 b	8.86 ± 2.87 b	13.25 ± 1.97 a	16.96 ± 2.09 a	14.75 ± 1.66 a
	16d	15.45 ± 1.96 a	9.72 ± 4.72 b	9.76 ± 2.73 b	11.06 ± 2.28 a	15.54 ± 5.49 a	16.71 ± 5.32 a
SOD	1 d	7.09 ± 1.16 a	5.07 ± 0.98 a	3.65 ± 0.79 b	6.52 ± 0.79 b	11.66 ± 2.79 a	7.80 ± 2.18 a
	4 d	5.33 ± 1.24 a	2.74 ± 0.15 b	2.28 ± 0.16 b	6.04 ± 0.30 b	14,96 ± 3.57 a	15.07 ± 1.26 a
	8 d	6.59 ± 1.36 a	2.55 ± 0.42 b	2.23 ± 0.45 b	4.90 ± 1.54 b	12.38 ± 1.67 a	14.01 ± 2.02 a
	12d	5.99 ± 0.83 a	3.55 ± 0.68 b	1.45 ± 0.36 b	6.03 ± 0.61 b	17.14 ± 2.83 a	13.62 ± 2.27 a
	16d	7.04 ± 1.88 a	8.74 ± 1.46 a	4.83 ± 0.91b	5.19 ± 1.28 b	14,29 ± 1.95 a	12.80 ± 3.02 a
CAT	1 d	74,18 ± 3.96 a	52.48 ± 5.84 b	32.62 ± 4.23 c	22.49 ± 2.20 ab	22.54 ± 4.29 a	16.05 ± 3.63 b
	4 d	77,99 ± 1.87 a	29.41 ± 4.02 b	23.92 ± 4.74 b	20.27 ± 4.54 a	17.48 ± 2.61 a	15.98 ± 3.38 a
	8 d	71.20 ± 8.75 a	36.68 ± 2.09 b	24.69 ± 2.56 c	23.83 ± 3.35 a	14.46 ± 2.44 b	5.53 ± 0.71 c
	12d	79.37 ± 8.82 a	26.26 ± 3.15 b	26.77 ± 3.08 b	22.17 ± 1.96 a	9,17 ± 1.23 b	7.68 ± 0.61 b
	16d	71.96 ± 7.43 a	29.42 ± 3.36 b	21.16 ± 1.76 b	24,24 ± 4.81 a	8.80 ± 0.86 b	7,36 ± 1,48 b
GPx	1 d	48.54 ± 2.69 b	156.78 ± 13.12 a	158.14 ± 10.75 a	32.03 ± 3.83 b	36.21 ± 4.20 b	48.43 ± 4.18 a
	4 d	45.88 ± 3.03 c	113.65 ± 5.57 b	165.95 ± 14.51 a	26.23 ± 3.00 b	29.38 ± 3.30 b	49.55 ± 4.53 a
	8 d	47.02 ± 5.81 b	147.31 ± 13.87 a	163.10 ± 12.00 a	28.99 ± 2.23 b	47.62 ± 5.21 a	50.33 ± 5.43 a
	12d	42.53 ± 4.68 c	145.49 ± 12.17 b	184.00 ± 21.27 a	28.75 ± 3.09 c	64.34 ± 1.96 b	103.53 ± 6.18 a
	16d	45.87 ± 5.11 c	136.41 ± 4.46 b	175.67 ± 16.62 a	33.82 ± 4.99 c	84.62 ± 6.23 b	146.85 ± 10.31 a
GST	1 d	114.19 ± 9.20 a	104.71 ± 11.16 a	106.79 ± 16.92 a	81.65 ± 3.21 a	72.42 ± 4.75 a	87.71 ± 10.40 a
	4 d	106.94 ± 13.36 a	47.26 ± 4.30 b	27.17 ± 4.36 b	84.46 ± 4.54 a	79.66 ± 6.80 a	75.34 ± 4.62 a
	8 d	119.51 ± 14,82 a	53.93 ± 5.04 b	25.04 ± 4.23 c	80.28 ± 6.49a	65.60 ± 4.36 a	66.53 ± 6.14 a
	12d	116.46 ± 15,90 a	50.92 ± 8.30 b	27.34 ± 6.25 c	81.82 ± 4.49 b	77.55 ± 9.62 b	113.98 ± 12.31 a
	16d	112.99 ± 5.38 a	24.60 ± 1.56 b	29.61 ± 4.36 b	74.31 ± 4.28 c	105.53 ± 8.44 b	159.26 ± 20.76 a
GR	1 d	22.66 ± 4.48 a	13.39 ± 1.37 b	10.86 ± 2.59 b	35,59 ± 6.84 a	46.08 ± 1.76 a	40,62 ± 4.43 a
	4 d	24.78 ± 0.65 a	16.69 ± 3.89 b	9.31 ± 0.98 b	37.11 ± 3.47 c	60.13 ± 5.58 b	79.04 ± 6.79 a
	8 d	27.08 ± 3.65 a	20.06 ± 4.54 a	20.42 ± 3.21 a	35.99 ± 5.24 c	54.21 ± 7.05 b	81.55 ± 3.26 a
	12d	20.15 ± 3.77 a	19.63 ± 3.93 a	19.34 ± 3.05 a	37,24 ± 3.46 c	57.36 ± 5.52 b	90.22 ± 2.72 a
	16d	25.92 ± 4.88 a	29.49 ± 3.53 a	26.94 ± 4.54 a	36.46 ± 3.97 c	59.66 ± 5.68 b	91.96 ± 7.68 a

Data are reported as mean and standard deviation. Different letters (a, b, c) indicate statistically significant differences ($p < 0.05$) between control and treated groups at the same experimental endpoint. *Glut*, total glutathione; *MDA*, malondialdehyde; *SOD*, superoxide dismutase; *CAT*, catalase; *GPx*, glutathione peroxidase; *GST*, glutathione S-transferase; *GR*, glutathione reductase; *Ctrl*, control

coupled with a statistical analysis of the responses of individual biomarkers. In the present study, the digestive gland was affected by Cu and Zn exposure. IBRv2 showed a more prominent biomarker response in both tissues exposed to the higher concentration of CuCl and $ZnSO_4$. Although severe responses were observed following exposure to both compounds, statistical analysis of individual biomarkers suggested a slightly more mitigated response to Zn.

Despite the difference in response patterns between the gills and the digestive gland, GPx activity was consistently increased in both tissues in each treatment group, indicating a powerful and effective antioxidant shield to prevent lipid peroxidation. Induced GPx activity was previously observed in *Venerupis philippinarum*, in which 40 µg/L of Cu^{2+} up-regulated the enzyme activity (Cong et al. 2016). Similar results have been found in gills and digestive gland

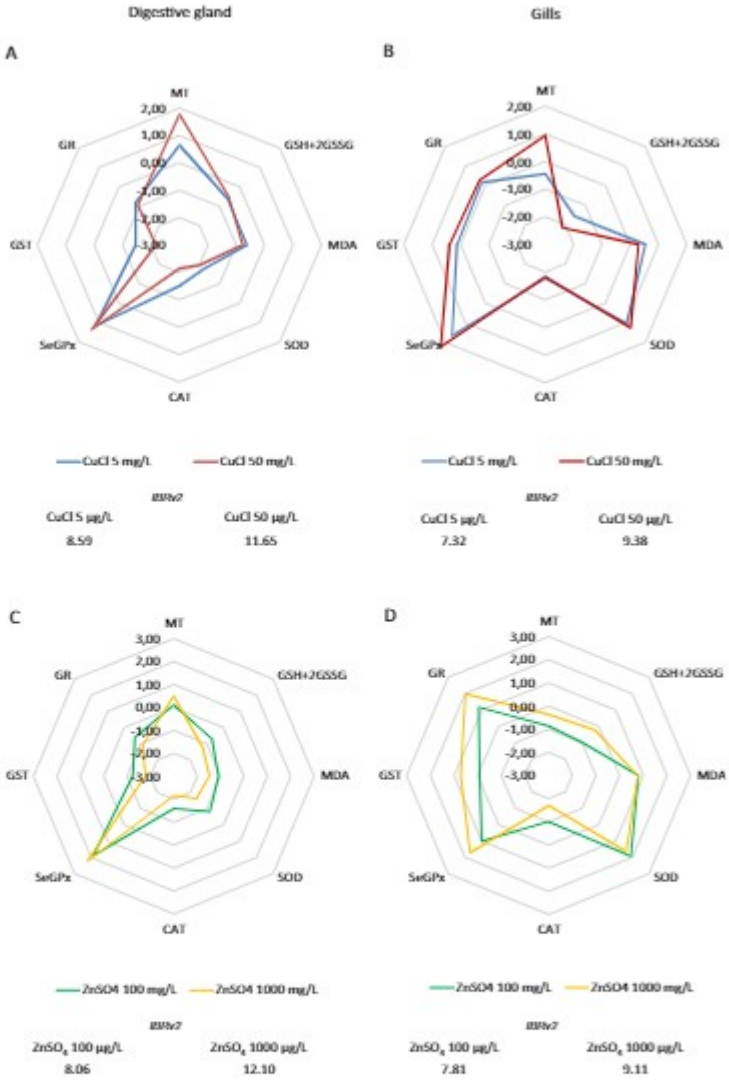

Fig. 3 Star plot and Integrated Biomarkers Response Index version 2 (IBRv2) in the digestive gland (**A**, **C**) and the gills (**B**, **D**) of *H. bialatus* exposed to cuprous chloride (5 and 50 µg/L) and zinc sulfate (100 and 1000 µg/L). MT, metallothioneins; GSH + 2GSSG, total glutathione; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GST, glutathione S-transferase; GR, glutathione reductase

of *M. coruscus* exposed to cupric ion and in gills of the same species exposed to zinc, suggesting a boost of antioxidant defense against metals (Huang et al. 2018; Qu et al. 2019). An unchanged or decreased MDA level in aquatic organisms exposed to Cu and Zn is quite peculiar; previous studies showed greater lipid peroxidation following exposure to these metals (Liu et al. 2017; Luo et al. 2020). Of note, however, decreased MDA levels were found in *Oreochromis niloticus* and *Tilapia zillii* exposed to 500 µg/l Zn (Saddick et al. 2017). This was attributed to Zn's antioxidant properties, which induced activity of the enzymatic and the non-enzymatic antioxidant shield and the subsequent scavenging of free radicals following lower lipid peroxidation (Saddick et al. 2017). Similar phenomena were found in *Megalobrama amblycephala*–fed diets supplemented with 9 or 25 mg/kg Cu (Shao et al. 2012) and in the Chinese horseshoe crab

Tachypleus tridentatus treated with 50 mg/kg Cu (Xu et al. 2020).

MT are metal-chelating proteins that prevent oxidative stress and lipid peroxidation. As shown by IBRv2 for both the gills and the digestive gland, MT acted as an active defense against oxidative stress driven by Cu, especially in the mussels exposed to the higher CuCl concentration. In the digestive gland, however, we observed late activation of MT concomitant with an early and transient decrease in glutathione levels. Previous studies showed that MT levels were not altered by Cu exposure in mussel *M. galloprovincialis* (Maria and Bebianno 2011; Peric et al. 2017; Peric and Buric 2019). The delayed increase in MT following Cu exposure may be rather unexpected; however, the ability of glutathione to act as a first defense line against cuprous ion toxicity has been well documented (Freedman et al. 1989; Elia et al. 2017). The Cu defense mechanism driven by glutathione is based on sequestration of the metal through the formation of a complex between the GSH and the monovalent ion ($GSH\text{-}Cu^{+}$), which is then oxidized to $GSSG\text{-}Cu^{+}$ and finally to $GSSG\text{-}Cu^{2+}$ (Freedman et al. 1989; Elia et al. 2017). In the present study, the decrease in total glutathione levels suggests activation of the metal defense mechanism driven by thiol. The method we used to measure total glutathione is based on the regeneration of GSH operated by the GR enzyme, excluding molecules complexed with Cu and revealing a significantly lower thiol concentration than that of the control group. The generation of copper-GSSG complexes may also be responsible for the reduction of GR activity. It is likely that complexed GSSG cannot be transformed into GSH by GR, and it is feasible that the mussels produced less enzyme to support the diminished free GSSG reservoir and devolved energy to other defense mechanisms. Differently, we observed an early and transient MT induction and a decrease in glutathione levels at days 4 and 8 in the digestive gland after exposure to the higher concentration of $ZnSO_4$. Although Zn is a recognized inductor of MT, especially in presence of other metals (Ganger et al. 2016); our results for the digestive gland and the gills suggest that in *H. bialatus*, these metal-trapping proteins may not mount a prompt defense against metal toxicity, as suggested by IBRv2. Moreover, the lower hepatic glutathione levels on days 4 and 8 indicate a transient weakening of the antioxidant shield against ROS. A previous study showed the ability of zinc to impair thiol homeostasis and inhibit GR activity (Trevisan et al. 2014). A plausible mechanism in the reduction of glutathione in tissues has been described in the mechanistic study by Gregus et al. (1992): zinc binding by glutathione is a defense against metal toxicity, similar to that reported for copper. In detail, glutathione can form Zn-glutathione complexes that can be excreted (Gregus et al. 1992). This may explain the general lack of Zn bioaccumulation we observed in the gills and the digestive gland in *H. bialatus*. GR and GSH play a crucial role in counteracting ROS damage. Low enzyme levels are deleterious to the cell, with impairment of the cellular reducing environment and the shield against oxidative pressure (Trevisan et al. 2014). A previous study reported failure of GR activity in the brown mussel *Perna perna* exposed to zinc chloride, which resulted in a more oxidized status in the gills (Trevisan et al. 2014). In our study, the higher GR levels in the gills after Zn exposure may counterbalance the early and transient decrease in hepatic GR, ensuring an effective oxidative defense against ROS.

Hepatic SOD activity after exposure to Cu and Zn was severely compromised during the entire experiment, as shown by IBRv2, and may have been caused by the interference of Cu or Zn ions in the enzyme structure. This outcome is in contrast with those reported in previous studies on mussels (Company et al. 2008; Huang et al. 2018; Li et al. 2009). Moreover, different SOD activities were measured in different tissues of mollusks exposed to copper; SOD levels were higher in gills than mantle in *Bathymodiolus azoricus* (Company et al. 2008), whereas on *Onchidium struma* the higher SOD activity was measured in digestive gland (Li et al. 2009). Increased SOD concentration was also observed in *Ruditapes philippinarum* and *M. coruscus* exposed to Zn (Marchi et al. 2017; Huang et al. 2018). Cytosolic SOD has a rather complex structure and contains one cupric and one Zn ion in each subunit. Furthermore, –OH can oxidize histidine, one of the Cu ligands, releasing Cu (Pedrajas et al. 1995) and thus inactivate enzyme function. Similarly, exposure to $ZnSO_4$ may have triggered a mechanism by which Cu was replaced in the SOD subunits. In contrast, the higher SOD levels in the gills especially after zinc exposure suggests a compensatory mechanism against ROS, where a marked decrease in CAT activity was found in both the gills and the digestive gland following exposure to both Cu and Zn. Inhibition of CAT activity associated with CuCl was reported in rainbow trout (Elia et al. 2017). The cuprous ion triggered a more severe enzymatic response than the cupric ion via displacement of iron from iron-sulfur clusters of CAT by copper; this hypothesis was based on data published by Macomber and Imlay (2009). In this study, we may suggest a similar mechanism on pearl mussels to explain the decreased levels of this crucial antioxidant enzyme. As regards Zn, the consistent decrease in CAT activity in the gills and the digestive gland is a peculiar outcome, as Zn is a recognized catalase enhancer in mussels (Trevisan et al. 2014). Our results suggest that exposure to high Zn concentrations may severely weaken this essential antioxidant defense probably because the threshold level of Zn is exceeded. Furthermore, the lower levels of SOD and CAT may be exacerbated by the oxidation of glutathione-Cu/Zn complexes that can generate superoxide radicals during oxidation (Freedman et al. 1989; Elia et al. 2017).

Finally, we observed a consistent and severe reduction in GST activity in the digestive gland after exposure to both metals during the entire experiment. The integrated results of IBRv2 suggest impairment of the metabolic panel against xenobiotics. This result is in line with a previous study on *M. galloprovincialis* and *edulis* exposed to copper (Mlouka et al. 2019) and on soft tissues of *Dreissena polymorpha* exposed to Zn^{2+} (Gagné et al. 2019). However, the high activity of GPx and the high late levels of glutathione indicate an effective antioxidant defense shield against oxidative pressure that efficiently counteracts ROS and lipid peroxidation.

Conclusion

We observed a time-related metal accumulation in *H. bialatus* exposed to CuCl, whereas slight $ZnSO_4$ uptake was noted in the digestive gland. The severe antioxidant response in the digestive gland suggests an oxidative challenge for this mussel species exposed to both metals. However, the effects of the two antifouling compounds were slightly different, with a less severe response after exposure to Zn than to Cu. The lower biological impact of Zn suggests mitigated ecological pressure than Co.

Author contribution Elia AC: conceptualization; investigation; writing—original draft; writing—reviewing and editing; Magara G: investigation; methodology; writing—original draft; writing—reviewing and editing; Pastorino P: data curation; writing—reviewing and editing; Zaccaroni A: investigation; methodology; writing—reviewing and editing; Caldaroni B: data curation; methodology; writing—reviewing and editing; Andreini R: methodology; Righetti M: methodology; Silvi M: data curation; methodology; Dörr AJM: data curation; methodology; writing—reviewing and editing; Prearo M: conceptualization; funding acquisition; writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval and consent to participate The procedures for the care and management of aquatic organisms were conducted in accordance with EC Council Directive 86/609 EEC (Council of the European Communities 1986) implemented by Italian Law (D. Lgs 116/1992) and approved by the Italian Ministry of Health (DGSAF 0024175-P-17/12/2013, D. Lgs. 306/2013-B).

Competing interests The authors declare that they have no conflict of interest.

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CONCLUSIONS

We conducted several ecotoxicology studies focused on different classes of chemical compounds: endocrine disruptors and immunotoxic substances. We took in exam different aquatic species occupying different levels of the trophic chain in the aquatic environment primarily because the xenobiotics included in our research share common characteristics: the ability to persist in the water, accumulate in the organisms, and biomagnify in the trophic web. Our investigations on chlorinated pollutants in yellow legs gulls (*Larus michahellis*) and on halogenated flame retardants in sperm whales (*Physeter macrocephalus*) focused on two marine species belonging to the apex of the trophic chain but profoundly different as they belong to two different taxa, a condition which includes profound differences in physiology, defense mechanisms, behavior, diet, and habits. Nevertheless, these two species share the enrolment of the top of the food chain of the same ecosystem. Moreover, these two species both belong to the Mediterranean basin and can cover large distances within the Mediterranean area. These common characteristics make the data obtained partly comparable. Above all, these shared characteristics allow us to gain important information on the state of contamination of the Mediterranean basin by exploiting the recognized role of sperm whales and seabirds as bio-indicators. This is an important consideration for numerous reasons. It is fundamental to have a reliable vision of the health status of the populations included in the study. Moreover, it is essential to gain reliable data fundamental to build predictive models on the species' future survival. Consequently, it helps scientists define new conservative strategies to be presented to governments. This is important to notice since obtaining reliable and in-depth data is a prerequisite for submitting to the relevant government bodies wildlife protection proposals that ideally should be both affordable and efficient and respectful of the protection of humans' economic activities. All these considerations and the stimulus to ask in-depth scientific questions would not have been possible without the vision of extensive teamwork involving the

active collaboration of several research groups and different specialist skills. The scientific world must be specifically stimulated to collaborate proactively to ensure that as much information is obtained, thus deepening each discipline's global and specific knowledge.

In conclusion, our study shows that not only older, well-known compounds may still pose a potential risk to aquatic wildlife and the natural ecosystem, but also that compounds traditionally considered less toxic and more modern, such as pyrethroids or atrazine, can accumulate in the trophic chain at different levels and potentially interact with the organism and other pollutants, leading to effects that are not yet fully understood by us, potentially affecting wildlife. This evidence highlights the urgency to continue monitoring wild species and further investigate the role of xenobiotics on their health status.

In our study, the choice to focus on different aquatic species belonging to different Phyla, different ecosystems and therefore characterized by different biology, physiology, and pathology, but which are all forced to face the aquatic pollution, is linked to the desire to have an overall view of the contamination of the entire food chain.

Moreover, including these wild species is related to other multiple factors. First, we wanted to apply the concept of one health to our studies. Over the past decades, environmental pollution has been recognized as a pressing global problem that affects nature and human beings in direct and indirect ways, affecting their health and playing an increasingly important active role in the development of diseases. The importance of acting and sponsoring the “one health” concept is essential to stimulate public awareness and educate people to behave and eat consciously. Moreover, it is crucial to relate to national and international institutions by providing reliable scientific data that incentivize governments to adopt the correct precautionary measures and protect humans and wildlife. The concept of one health has its precise origin in 2004 and was initially applied mainly in the emerging problem of antibiotic resistance and re-emerging infectious diseases. However, it is important to extend the meaning and

application of one health to other medical, scientific disciplines, such as toxicology, in compliance with the original concept of one health, which sees all the elements of the animal and plant kingdom interconnected and able to influence each other positively or negatively. This modern approach to scientific research that includes the One Health concept and multidisciplinary collaboration that insist on the same research theme is tough to reach in ecotoxicology. Although several studies are available to compare data, it is important to remember that one limitation in ecotoxicology studies is linked precisely to the different laboratory methods used to analyze the samples. Unfortunately, it is tough to standardize research methods both nationally and internationally. This is particularly true for emerging branches of research such as immunotoxicology or the study of endocrine disruptors. Many methods still need adequate validation processes for assessing these xenobiotics, and there are no well-defined scientific protocols, especially for aquatic species. In our research, for example, regarding trace element analyses, the detection method can be standardized between the various research groups that used an atomic absorption spectrometer to perform the detection. Nevertheless, the treatment of sample handling and its digestion may differ considerably between research groups, potentially affecting the comparison among results. Furthermore, regarding the possibility of comparing data with international researchers, there is often no standardization regarding the units of measurement in which the samples' weights are expressed, as they are conditioned by the pre-treatment method they have undergone. This also makes bibliographic comparisons more difficult and inquisitive as it requires the numerical conversion of sample weights, which is easily subject to error. We have chosen to collaborate with the same research group, analyzing the same contaminants, extending the research spectrum, and using the same sample preparation and laboratory analysis methods. All of this made it possible to standardize the overall working scheme considerably. Furthermore, ecotoxicology studies involving wildlife are complicated by the difficulty of obtaining samples. Often the samples obtained result from “occasional findings” and not always their state of conservation is adequate. Moreover, often the amount of

biological matrix collected is not adequate to allow a multidisciplinary investigation. It must be stressed that wildlife is placed under an important official and ethical protection; therefore even in cases where research is carried out on live animals, the sampling techniques must be as least invasive as possible. This translates into the need to adapt the research to the studied species and not *vice versa.* In conclusion, this thesis summarises several ecotoxicological studies conducted on aquatic species belonging to different marine and freshwater environments, occupying different levels of the trophic chain and characterized by different biology, ethology, and nutrition. Despite this, all the wild species under investigation represent excellent bio-indicators of the healthiness of the surrounding environment. Furthermore, they are constantly exposed to anthropic pressure, which results in the release of xenobiotics that can bioaccumulate and biomagnify and interfere with the endocrine and immunological systems of the species analyzed, representing a real risk for the survival of the current and future generations. This thesis also sought to draw attention to the limiting aspects of ecotoxicological research, offering food for thought and research alternatives that could circumvent the main obstacles affecting the field application of ecotoxicological concepts. Scientific data obtained confirm the urgency to continue monitoring both old and emerging contaminants that, as we have observed, can interfere with wildlife at different trophic levels, affecting multiple physiological systems and altering the normal balance of ecosystems, posing a potential threat not only to the survival of wildlife but also to the global economic and public health.

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