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SPECIAL ISSUE ON MARINE MAMMALS

Toxicology of Marine Mammals: New Developments and Opportunities

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Abstract It is widely recognized that marine mammals are exposed to a wide variety of pollutants, with a weight of evidence indicating impacts on their health. Since hundreds of new chemicals enter the global market every year, the methods, approaches and technologies used to characterize pollution levels or impacts are also in a constant state of flux. However, legal and ethical constraints often limit the type and extent of toxicological research being carried out in marine mammals. Nevertheless, new and emerging in vivo, in vitro as well as in silico research opportunities abound in the field of marine mammal toxicology. In the application of findings to population-, species-, or habitat-related risk assessments, the identification of causal relationships which inform source apportionment is important. This, in turn, is informed by a comprehensive understanding of contaminant classes, profiles and fate over space and time. Such considerations figure prominently in the design and interpretation of marine mammal (eco)-toxicology research. This mini-review attempts to follow the evolution behind marine mammal toxicology until now, highlight some of the research that has been done and suggest opportunities for future research. This Special Issue will showcase new developments in marine mammal toxicology, approaches for exposure-effect research in risk assessment as well as future opportunities.

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Toxicology of marine mammals is a relatively small, but indispensable topic within the area of marine mammal sciences. It is a topic that has gained interest over the years due to the increased awareness of the toxic effects of pollutants in several organisms and the usually elevated levels of pollutants detected in marine mammal species (e.g. Tanabe et al. 1994; Ross 2000; Aguilar et al. 2002; Houde et al. 2005; Law et al. 2010). Despite being in contaminated habitats, marine mammals get the bulk of their body burdens through their diet rather than directly from their environment (Gray 2002). It is because of their top position in the trophic chain, the biomagnification process and the persistence of several pollutants, that marine mammals can accumulate high levels of pollutants. For biologists, marine mammal toxicology might be a highly theoretical and complex topic that sometimes seemingly abandons all connections with conservation and management. The ultimate goal in marine mammal toxicology, however, is to find minimally invasive and non-destructive tools or approaches that help to understand the causal link between pollution and its effects in marine mammals in order to (1) assess the past and current situation in terms of toxicology for marine mammals and to use that to (2) inform legislation for providing a healthier environment for these animals. This is a goal that will be valid for years and possibly decades to come and that fits seamlessly within any effort for conservation and management.

Since hundreds of new chemicals enter the global market every year, the methods, approaches and technologies used to characterize pollution levels or impacts

are generally also in a constant state of flux. This is also true for the methods, approaches and techniques used in marine mammal toxicology, despite the legal and ethical constraints when working with these animals. New and emerging in vivo, in vitro as well as in silico research opportunities abound in the field of marine mammal toxicology, both in exposure studies as well as in effect studies. A comprehensive understanding of contaminant classes, profiles and fate over space and time can have a profound influence on the design and interpretation of marine mammal effect studies. This paper will provide a brief overview of past and current in vivo, in vitro and in silico research thereby stimulating future research opportunities in this topic (Fig. 1).

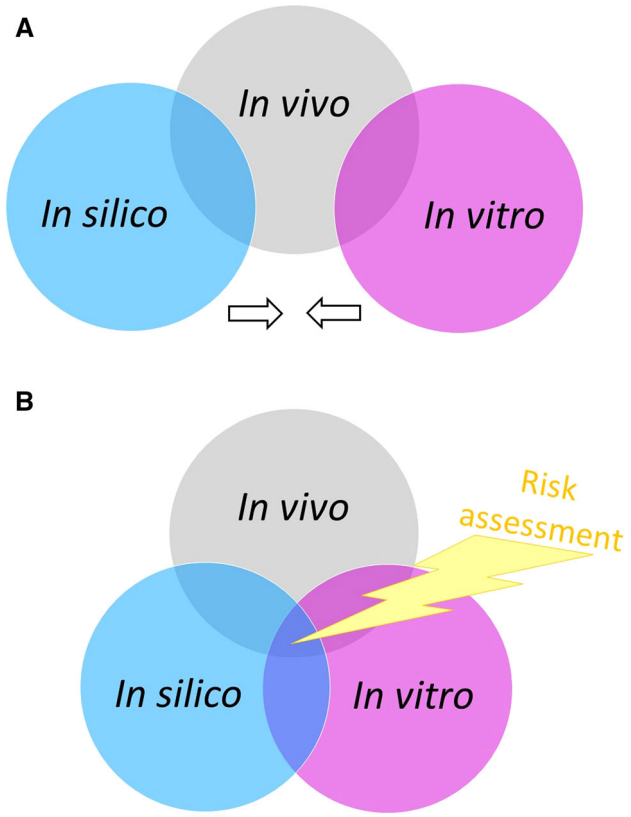

Fig. 1 Conceptual diagram of the different types of research that are underlying risk assessment in marine mammals. **a** represents the current situation in which results of in vitro research are difficult to implement in in silico models. **b** represents the ideal scenario in which all research types complement each other thereby facilitating (I) a thorough interpretation and understanding of current and past risks, as well as (II) an educated prediction and identification of risks in the future

Overview of In Vivo, In Vitro and In Silico Research Directions in Marine Mammal Toxicology

In Vivo Research

Following the protective guidelines and legislation for marine mammals, in vivo research is uncommon in marine mammals these days and is restricted mainly to collecting samples in a minimally invasive to non-invasive way. In the past, experiments using animals held in captivity were performed in a limited number of occasions (Tillander et al. 1972; Ramprashad and Ronald 1977; Van de Ven et al. 1979; Reijnders 1986; Ross et al. 1996a, b; De Swart et al. 1996; Thomas et al. 2005). These studies differ in a number of ways such as the administration type (e.g. fish from contaminated regions, spiked oils/fish), administered dose which was more realistic in the more recent studies (e.g. Reijnders 1986; Ross et al. 1996a, b) compared to the older ones (e.g. Tillander et al. 1972; Ramprashad and Ronald 1977) and general outcomes [fatalities were recorded in Ramprashad and Ronald (1977)]. Nevertheless, effects on the immune (Ross et al. 1996a, b; De Swart et al. 1996), sensory (Ramprashad and Ronald 1977) and reproductive systems (Reijnders 1986) were found thereby providing evidence that pollutants could be associated with adverse effects. To our knowledge, similar experiments were never performed using marine mammal species other than pinnipeds and were not performed for any marine mammal species in the last decade.

These days, in vivo research in marine mammals refers mainly to sampling techniques rather than to exposure experiments on animals in captivity. Blood and biopsy sampling can be done in a minimally to non-invasive manner both in animals in captivity as well as wild animals. Because blood and biopsy samples are often very fresh, they are ideal samples for studies involving health effect as well as chemical analysis. However, the majority of the biomonitoring studies, i.e. studies focussing on chemical analysis only, are still done using tissues of animals that were found dead on the beach or in fishing nets or that had died naturally. Those studies can investigate several types of pollutants in a wide array of tissues, but are sometimes also perceived as biased and untruthful with respect to the state of the population they are drawn from. Although dead or stranded animals are not necessarily ill, studies using tissues from traditionally harvested marine mammals can target specific animals regarding age class, gender or health status. Such studies, however, are obviously not classified as minimally to non-invasive (e.g. Tilbury et al. 2002; Brown et al. 2014).

Nevertheless, in addition to capture-and-release studies, studies using harmless sampling methods in live marine mammals are definitely gaining momentum. Tissues that qualify for harmless sampling are hair, skin, biopsies, faeces, urine and blood. A thorough review about the use of these matrices to study the physiology, and toxicology to a smaller extent, of larger marine mammals is provided by Hunt et al. (2013). Faeces and urine are typically hard to obtain for marine mammals in general, especially for cetaceans that never come to the land. Goldstein et al. (2009) collected sea lion (*Zalophus californianus*) faeces for domoic acid analysis, and Roman and McCarthy (2010) investigated humpback whale (*Megaptera novaeangliae*) fecal plumes to test the whale pump hypothesis which refers to the recycling process of nitrogen in the marine environment. A similar approach was done by Lavery et al. (2010) for sperm whales (*Physeter macrocephalus*) that stimulate carbon export to the atmosphere by producing iron-rich faeces. Marine mammal faeces were also investigated by Marcus et al. (1998) for elucidating harbour and grey seal (*Phoca vitulina* and *Halichoerus grypus*) diets, for revealing the gut microbial diversity of polar bears (*Ursus maritimus;* Glad et al. 2010), and for investigating nutritional stress in killer whales (*Orcinus orca*; Ayres et al. 2012).

Needless to say that faeces or urine of marine mammals have not been the subject of many toxicological studies so far. Metals were analysed in hair samples of pinnipeds (e.g. Wenzel et al. 1993; Habran et al. 2013) as well as in hair of polar bears (e.g. Born et al. 1991; Jaspers et al. 2010). Among all tissues that can be obtained in a minimally to non-invasive manner from marine mammals, skin and blubber biopsies and blood are probably the most popular. Several health points (e.g. vitamin A, hormones) can be measured in these matrices. Also, a variety of pollutants have been measured in skin and blubber biopsies (e.g. Newman et al. 1994; Hall et al. 2003; Foltz et al. 2014) as well as in blood (e.g. Newman et al. 1994; Hall et al. 2003; Das et al. 2008; Weijs et al. 2009), but the most important reason for using these tissues is their usefulness in ex vivo/in vitro experiments.

In Vitro Research

The development of in vitro techniques has allowed for the evaluation of toxicological effects without the use of live animals. An early in vitro study performed with marine mammal cells focused on the effect of heavy metals on steroid production in grey seals (Freeman and Sangalang 1977). That study outlined how selenium (Se) and arsenic (As) could induce, at relatively low concentrations (0.45 μg/g), an alteration in steroid hormone synthesis, thereby impairing the correct gonadic functionality.

Present studies focus mainly on the evaluation of enzymatic induction by different organic pollutants (e.g. Fossi et al. 2006, 2008; Marsili et al. 2008; Spinsanti et al. 2008) and on the evaluation of immunotoxicity and metabolic activity for pollutant breakdown or biotransformation (e.g. Boon et al. 1998; De Guise et al. 1998; Kim et al. 1998; White et al. 2000; Nakata et al. 2002; Levin et al. 2004, 2007; Mori et al. 2006; Wilson et al. 2007; Camara Pellissò et al. 2008). Several of these studies use blood samples collected from animals held in controlled environments that are trained for blood sampling. This minimizes the stress due to capture and constraint procedures that can alter immune responses. Levin et al. (2007) reported that captive and wild sea otters (*Enhydra lutris*) differ in their in vitro response to different organochlorine mixtures, with wild animals being more sensitive to contaminants compared to captive animals. The effect of capture stress and of exposure to pollutants in the wild are reported as possible explanations for observed differences.

Most studied species are those more commonly held in captivity, i.e. bottlenose dolphins (*Tursiops truncatus*), beluga whales (*Delphinapterus leucas*) and pinnipeds, while studied compounds are organohalogenated, i.e. PCBs (polychlorinated biphenyls), organochlorine and brominated compounds (De Guise et al. 1998; Levin et al. 2004, 2007; Mori et al. 2006). Nevertheless, there are also studies reporting immunotoxicity in relation to heavy metal exposure (e.g. Camara Pellissó et al. 2008; Dupont et al. 2013). Studies on wild individuals have been extensively performed in seals, which are somewhat easier to capture with respect to cetaceans, allowing sampling (blood, skin, etc.) for in vitro studies (e.g. Mori et al. 2006; Das et al. 2008; Frouin et al. 2008; Kakuschke et al. 2011; Dupont et al. 2013).

Most in vitro studies in marine mammal toxicology use cells derived from biopsies, blood samples or tissues originating from freshly dead animals, and these cells are then exposed to single contaminants or mixtures in order to evaluate the induced effects. Such cell systems are not perfect animal surrogates as they lack the multi-organ effect as well as important processes that ensure a realistic pollutant kinetics (Gauthier et al. 1999; Fossi et al. 2000). However, they allow to perform mechanistic and empirical studies, to investigate toxic effects at the cellular and molecular level, and to identify species-specific differences in toxic impacts which are impossible to study in vivo (Fossi et al. 2000). Apart from these studies, an additional step forward in in vitro studies has been the development of biosensor systems, which use engineered cells (i.e. bioassays) to be applied to marine mammals and concepts like 'effect-driven approach' (EDA), 'adverse outcome pathway' (AOP) and 'toxicity pathway' (Yordy et al. 2010; Burgess et al. 2013; Jin et al. 2013, 2015; Simon et al.

2013). These developments allow to screen marine mammal tissue samples with respect to specific endpoints and, depending on the study design, can be used as an initial step to explore the influence of contaminant mixtures.

Several studies have underlined how mixtures can have a stronger effect on immune system than individual compounds (De Guise et al. 1998; Levin et al. 2004, 2007; Mori et al. 2006). In addition, Levin et al. (2004) have found that bottlenose dolphins are more sensitive to organochlorine mixtures than beluga whales thereby pointing towards a species-specific sensitivity. Various studies have also shown that organochlorine compounds can have an AhR-independent mechanism of action, thereby indicating that the sensitivity of marine mammals could not be predicted from results obtained from other species (Levin et al. 2004, 2007; Mori et al. 2006). As bioassays are based on specific cell lines derived from other species like rodents or humans, one can argue that the observed responses to contaminant mixtures are not necessarily the same as the responses experienced by marine mammals in the wild. Despite this, the toxicity pathways highlighted by bioassays are usually well conserved pathways across species. Together with the possibilities to screen marine mammal tissue samples and to point towards the need for detection of novel, emerging contaminants, bioassays can provide important topics and opportunities for future research.

Overall, in vitro studies have been performed mainly using hepatic cells (e.g. Boon et al. 1998; Kim et al. 1998; White et al. 2000), but in recent times, fibroblast and integument cells are used as substitutes for liver cells. These tissues can be obtained from skin biopsies which can be collected from wild animals with little damage to the animal (e.g. Fossi et al. 2000, 2006; Marsili et al. 2000; Wilson et al. 2007). Fibroblasts can be cultured and preserved in liquid nitrogen, thus reducing the need of continuous sampling from freshly dead animals, as requested with hepatic microsomal preparations. Integument cells also can be used for metabolic activity evaluation, as many studies have proven that cytochrome P450 is expressed in all cell types of integument, and that expression can be induced by various contaminants (Wilson et al. 2007). Therefore, fibroblast and integument cell cultures can be used to study differences in pollutant metabolism thereby providing rapid and simple alternatives for biomarker research in live animal investigations.

In Silico Research

Modelling allows to interpret and observe biomonitoring data from several different angles and provides a whole-body approach that in vivo nor in vitro research can offer. Models come in all sizes and shapes and are, in marine mammal toxicology, highly dependent on the availability of data (i.e. concentrations measured in tissues) and parameters (i.e. species-specific and compound-specific constants, rates and equations). In the pharmaceutical industry, models are compulsory and cost-effective tools; they are required to make sure that a specific drug is capable of reaching the target site and that the administered dose is sufficient for its purpose. The best way to know this is by gaining information about all the processes that are involved in the kinetics of the drug of interest, namely the absorption, distribution, metabolism and excretion pathways. Models in marine mammal toxicology follow the same principles, but face probably more challenges as the biology and physiology of most marine mammal species is often scarcely known and biomonitoring data is usually focussed on just a few tissues. Nevertheless, several types of marine mammal models are available for cetaceans and pinnipeds (reviewed in Weijs et al. 2014) and polar bears (Sonne et al. 2009; Pavlova et al. 2014).

The first bioaccumulation model for a marine mammal species was published in 1999 by Hickie et al. (1999). This model was developed for marine mammals and applied to the sum of PCBs in beluga whales. The Hickie et al. (1999) model is the only model based on the fugacity approach in which the thermodynamic equilibrium between phases is responsible for the distribution and partitioning of pollutants. A second model for the lifetime bioaccumulation of the sum of PCBs in beluga whales was published shortly after that, though this was not based on the fugacity approach but on the approach that involves concentration fluxes and chemical potential (Hickie et al. 2000). Other models that followed were for PCBs in harbour seals from San Francisco, Arctic ringed seals (*Phoca hispida*), bottlenose dolphins from Florida, killer whales (*Orcinus orca*) from the northeastern Pacific Ocean region, polar bears from Greenland, long-finned pilot whales (*Globicephala melas*) from Australia and harbour porpoises (*Phocoena phocoena*) from the Black and North Sea (reviewed in Weijs et al. 2014). Other POPs (persistent organic pollutants), such as pesticides and PBDEs (polybrominated diphenyl ethers) were modelled in polar bears, harbour porpoises, and killer whales (reviewed in Weijs et al. 2014). As can be seen in this list, models were only made for POPs so far and models for the lifetime bioaccumulation of metals have yet to be developed in any marine mammal species.

For some species it is more difficult to find suitable parameters than for others. In vitro experiments in marine mammal toxicology usually use single pollutants and known doses. This would be an ideal scenario for developing models. However, all models for marine mammals so far, were validated against real-life values obtained through biomonitoring studies. Diets of wild animals are often poorly characterized, conditions in the wild are by

definition uncontrollable and wild animals are always exposed to pollutant mixtures which means that results of in vitro experiments cannot straightforwardly be used for modelling purposes (Fig. 1a). It is also because of these issues that several parameters had to be estimated in the existing marine mammal models leading to the addition of statistical methods for more reliable parameter estimation (Weijs et al. 2014).

Conclusions and Research Needs

Toxicological studies in marine mammals are hardly straightforward due to the protective guidelines that aim to protect marine mammals (inter)nationally. Although there is no doubt about the necessity and usefulness of these conservation guidelines, they put constraints on the toxicological work that can be done for marine mammals. This explains the knowledge gaps that still exist, the careful interpretation of research outcomes as well as the methods and techniques that are employed in marine mammal toxicology.

In this Special Issue, we have tried to showcase the current knowledge, novelties and opportunities in marine mammal toxicology. The field of marine mammal toxicology is broad and diverse, which is evidenced by the different topics, techniques, and species. Out of 14 studies in total, seven are biomonitoring studies, four studies combine chemical analysis and health effects (in vitro) and three are modelling studies (in silico). All three modelling studies have studied POPs: PBDEs in the killer whale food chain (Alava et al. 2015), PCBs and OCPs in the beluga whale (Cadieux et al. 2015) and PCBs in polar bears (Pavlova et al. 2015).

Brown and Ross (2015) have focussed on the transplacental transfer of PCBs, PBDEs and OCPs in ringed seal mother-fetus pairs. Noel et al. (2015) and McHuron et al. (2015) have investigated the influence of biological factors (e.g. age, gender, location) on the bioaccumulation of total mercury (THg) in hair/whiskers (Noel et al. 2015) and hair/blood (McHuron et al. 2015) of harbour seals and California sea lions, respectively. Peterson et al. (2015) have studied the relationship between THg levels in blood and hair in four different pinniped species. Kakuschke and Griesel as well as Hansen et al. (2015) have analysed a battery of trace elements in marine mammals: in blood of harbour seals (Kakuschke and Griesel 2015) and in liver of 16 cetacean Species (Hansen et al. 2015). Furthermore, although it is often very difficult to obtain feces samples of cetaceans, Lundin et al. (2015) have managed to obtain and analyse feces samples of killer whales.

Results of these biomonitoring studies can be compared to toxicity thresholds or previously reported effect levels, however, they do not have the same direct correlations between exposure and effect as the effect-studies in this Special Issue. Reiner et al. (2015), Lehnert et al. (2015), Bogomolni et al. (2015) and Dupont et al. (2015) have investigated the correlations between different toxic endpoints and the levels of POPs (Reiner et al. 2015; Lehnert et al. 2015; Bogomolni et al. 2015) or trace elements (Lehnert et al. 2015; Dupont et al. 2015) in tissues of pinnipeds. These endpoints range from vitamin A and E measurements (Reiner et al. 2015) to cellular/molecular biomarkers and haematology (Lehnert et al. 2015; Bogomolni et al. 2015; Dupont et al. 2015). In addition to this, Bogomolni et al. (2015) have ventured a step further and have investigated whether exposure increased the likelihood to Phocine Distemper Virus.

The discipline of toxicology in marine mammals has come a long way, for in vivo, in vitro as well as in silico research, but we are not quite there yet. Knowing that the highest levels of pollutants were detected in killer whales and polar bears, that pollutants are causing immunotoxicity in several marine mammal species or that the elimination half-life of pollutants can be longer than the entire lifetime of a marine mammal, is obviously very useful. All those findings, and many more, have prompted toxicologists for decades to continue investigating marine mammals and to be increasingly creative in solving important questions.

However, for a streamlined approach to conserve and manage marine mammal populations, studies have to be combined and results need to complement each other as has been proposed earlier by Ross (2000). It is the interface between in vitro, in vivo and in silico research that is of great importance for future conservation and management purposes (Fig. 1b). Unfortunately, it is also that interface that is the most challenging, especially in an ever-changing environment. Exposure has changed over time and new compounds are becoming more and more important, even if ‘old’ pollutants still have an important role in marine mammal toxicology. Future research should take this into account and also focus on more novel and emerging compounds. Pollution differs spatially, meaning that more site-specific knowledge about dietary and ecological factors should be integrated in toxicological research to thoroughly understand the degree of exposure and impact on marine mammal populations. Finally, new biomarkers need to be developed and implemented in addition to the more ‘traditional’ ones parallel with the analysis of novel and emerging compounds in order to streamline conservation efforts for the next decades.

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