Seals are swallowing a mouthful

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The situation resembles an episode of CSI, except that in this case, the actors in white coats are investigating the poisoning of large marine mammals in the North Sea. Grey and harbour seals are the focus of attention here because, due to their position at the top of the food chain, they are more exposed than other animals to the harmful effects of many pollutants such as mercury or persistent organic pollutants that act as endocrine disruptors, attack the immune system and impair reproduction. But many questions still remain unanswered. Assessing the threats associated to these pollutants and establishing the extent to which they are responsible for various diseases is a particularly complex operation. For the last fifteen years or so, researchers at the University of Liege, in collaboration with many other universities in Belgium and abroad, have been attempting to get to the bottom of this global phenomenon. In order to help them achieve their goal, they have called upon expertise from disciplines as varied as veterinary medicine, oceanology, ecotoxicology, chemistry, cellular biology and the study of biomarkers as attested to by several recent studies (1).
Many organic and inorganic pollutants present in our oceans represent a major ecological challenge. Mercury and cadmium are often referred to as heavy metals but this is not strictly true from a chemical point of view. The term "trace elements" would be more accurate. Unfortunately, many other pollutants can accumulate in marine animals. Persistent organic pollutants are long molecules assembled around carbon chains and are usually the result of human activity. This is the case with certain pesticides or PCBs. These industrial compounds which were banned more than thirty years ago are so stable that they have remained present and active up to the present day. Finally, the pollutant that is most familiar to the wider public is mercury. "It exists in the natural state," explains Krishna Das, a senior research associate at the FNRS and a member of the Oceanography Laboratory of the University of Liège, "But human activity has resulted in an almost three-fold increase in atmospheric emissions as compared with the pre-industrial era. This raises an obvious question". Mercury, under the influence of bacterial activity can become bonded to other compounds to form methylmercury. It is an organic molecule and, like many other pollutants, is very toxic. The entire trophic chain is affected by it in fact. In order to understand these levels of poisoning and quantify their consequences for the health and behaviour of animals, research that includes sea campaigns, laboratory studies, in vitro culture or analysis of biomarkers is necessary. The evolution of animal health is actually so dependent on a variety of factors with various causes that it is difficult to associate a given evolution to one phenomenon or another. The way animal life responds to its environment can only be understood in a global context. To understand this, multiple approaches are required.

Undesirable exposure to pollutants

This is the mammoth task that has been undertaken by Krishna Das and her collaborators for the last fifteen years, focussing on marine mammals like dolphins, humpback whales and more particularly, grey and harbour seals. The latter two species are at the top of the food chain in the North Sea. As predators, they are the most exposed to persistent organic pollutants such as polychlorobiphenyls or PCBs. These industrial pollutants are integrated and assimilated by all the species in the trophic chain and are only a small amount of them are eliminated by the kidneys and in fecal matter. Because they are lipophilic, they typically accumulate in adipose tissues. An efficient transfer of prey towards the tissues of predators has been observed, from the plankton to the seal. "We estimate that the concentrations of several pollutants are multiplied by around ten at each trophic level, a process called biomagnification". This process does not fully explain their high concentration of pollutants. As seals are mammals they need to maintain a body temperature around 37°C and they must do so in an environment where there is a high level of heat dissipation. In order to compensate for this heat loss, they must eat large quantities of food, more than 6 % of their body weight per day (this can vary from species to species, from season to season, and in accordance with their growth stage and their physiological status). These mammals can weigh as much as 200 kg. They possess a thick layer of blubber which favours the accumulation of lipophilic pollutants. This is an unfortunate combination of factors from the seals' point of views but it means that they make fascinating toxicological models.

The accumulation of toxicological clues

"At the beginning of the 2000s, we observed an increase in the beaching of porpoises and seals on Belgian coasts", recalls Krishna Das. "The scientists sought to understand the causes of death. I had the benefit of access to samples taken by Thierry Jauniaux, from the Faculty of Veterinary Medicine who had been studying the causes of death in these marine mammals and I began to observe the concentrations of trace elements. I wanted to understand what led an animal to be contaminated or not. The age of the animal, for
example, its diet, its state of health or even whether it was possible to establish a link between the diseases observed and the levels of concentration of the pollutants. This epidemiological-type approach, where we establish statistical correlations between toxic elements and diseases was the subject of my doctoral thesis”. It was an interesting first research component. The beaching of these mammals provided access to an enormous quantity of tissues without the need to capture large mammals in their natural environment. But this approach soon showed its limitations. “By only studying the beached porpoises and whales, we had no measurements from a control population. We could only study sick individuals. And these statistical correlations did not necessarily signify a connection between cause and effect. Other proofs were necessary and therefore other methods to complete this research”. There was yet another major difficulty. The traditional toxicological approach requires a control group and a test group in the laboratory to whom pollutants would be administered. The development of diseases would be in line with a directly observable causality report. But what works for rats in a laboratory does not work for large wild animals that evolve in a fully natural environment and showing significant inter-individual differences. Such an approach would encounter many obstacles and raise important ethical questions. Therefore another type of approach needed to be developed. Each approach had its weaknesses and its limits but it also led to new research possibilities and revealed new clues all of which provided proof.

From beaching to capture campaigns

A complementary approach studying beached individuals therefore results in the capture of animals in their natural environment which is only possible by developing an international network. In the case of harbour seals, the species endured several epizootic diseases. This phenomenon encouraged German researchers (Professor Ursula Siebert, Institute for Terrestrial and Aquatic Wildlife Research (ITAW) at the University of
Veterinary Medicine Hannover) to focus their interest on these animals in their environment. "Twice a year, the German team organizes capture campaigns in the North Sea. The infrastructure and logistical means deployed are colossal. The seals captured are then weighted and measured, and blood, hair and saliva samples are taken from them. This is a somewhat holistic approach whose main challenge is to understand the state of the population. The blood samples enable cell culture and a relatively in-depth analysis of the pollutants in direct relation to the life of the animal. Studying the hair enables observation of the evolution of health over a much longer time-scale which is important because trace elements are integrated during the entire growth of the hairs which sometimes represents a period of several months". On the other hand, harbour seals are particularly fearful animals. All the precautions taken to limit the invasive character of the operation were taken and the captures took place in May and September and avoided calving periods. When panicked, the mother could abandon the calf and flee.

In contrast, the capture of grey seals, organized in Scotland with the University of Saint Andrews (Dr. Paddy Pomeroy, Sea Mammal Research Unit, Scottish Oceans Institute, University of St Andrews), concerned only females at the moment when they return to land to suckle their young. "They are slightly less fearful than harbour seals. As for the males, they are large and quite aggressive so we avoid approaching them! But from a scientific point of view, these studies are complementary to the capture of harbour seals and are particularly interesting from the point of view of understanding the transfer of energy between the mother and her calf. When suckling, the mothers fast for three weeks and produce milk which is almost 50% fat. Many pollutants are then passed to the new-born. In addition, among many species of marine mammals, we can observe a decrease in the concentrations of the pollutants in females (in accordance with the number of pregnancies and lactation events) so these levels do not stop increasing in males. This transfer from mother to young is at a maximum in the case of the first-born".

The hair, blood and milk samples taken from both the mother and the new-born makes it possible to quantify the transfer of pollutants associated with lipids and therefore very present in the milk. But even before suckling for the first time, the calf has already been exposed to pollutants for a long time. "While taking blood samples from young newly-born animals, we also noticed that they were already strongly contaminated by certain pollutants, notably from mercury. This signifies that there is an efficient transfer of certain trace elements from the placenta. On also study samples of lanugo a soft layer of downy hairs that the calves synthesize in the uterus and which they lose very quickly. This lanugo contains significant concentrations of toxic elements".
The effect of mercury on the white blood cells

In order to better understand the immunotoxic effect of methylmercury, different *in vivo*, approaches were attempted in collaboration with scientists in Germany (prof. Ursula Siebert) and Professor Marie-Claire Gillet (Mammalian Cell Culture Laboratory, ULg). The *white blood cells*, mainly T lymphocytes from harbour seals were isolated and placed in culture in order conduct an *in vitro* study of their immune response to exposure to toxins. In culture, these cells do not generally survive longer than 72 hours, which is very short, but they are the workers of the immune system. "*During a doctoral project*, explains Krishna Das, "*We focused more specifically on the effects of methylmercury. Present in fish, this methylmercury causes strong levels of contamination in seals. The objective was not to determine the acute toxicity of methylmercury which has been known for a long time: the molecule seriously affects the nervous system and embryo development. We were interested in exposing the lymphocytes to concentrations that reflected those in the animals of the North Sea in order to observe the severity of adverse effects related to this level of poisoning*".

Observations by electron microscope confirmed the suspicions of the two researchers. The pollutant greatly affected the *mitochondria* of the common seals, a highly toxic contact which caused apoptosis or cellular death of the lymphocytes. "*At the same time, we had to remain prudent in our conclusions. An in vitro study does not reflect what is happening in the entire seal. Once it enters the blood, methylmercury follows a complex path and ends up in the liver where, astonishingly, it is no longer in methylated form. This observation, which was made as far back as the 1980s, tends to show that the seal is capable of partly neutralizing the toxicity of mercury thanks to selenium*".
The search for biomarkers

Biomarkers are the responses of an organism to stress. In science, the objective is to identify them and determine their causal link to a disease. The interest here is twofold. Not only is this kind of approach less invasive (a blood sample is sufficient to make a diagnosis), but these responses often appear very early, sometimes well before a disease is identified. "This type of approach was developed in hospitals", explains the researcher. "A sizeable part of the fight against cancer, in particular, has contributed to the identification of predictive biomarkers in blood samples. This is the case with certain proteins whose deficiency or increased presence in terms of concentration increases the possibility of contracting certain types of cancers". The method was developed in clinics in order to complete the arsenal of weapons used in other disciplines such as ecotoxicology. "With regard to the question of poisoning in seal populations, this research component remains at the preliminary stage but is showing promise. German colleagues (including K. Lehnert as first author) have, for example, established a relationship between certain trace elements with a variation in the secretion of interleukin 10, an anti-inflammatory cytokine, a process which disrupts the immune response. We still need to further develop the identification of these biomarkers and an understanding of the mechanisms involved".

Feeding habits outside of the poisoning issue

More globally, another question the researchers are interested in is the position of seals in the food chain. "The question is important", says the oceanographer, "Particularly in terms of the management of stock levels. Seals are animals that eat enormous quantities of food and their food, which is mainly made up of fish, varies according to age, sex and the place they live. The evolution of their demographic can have important consequences for the profitability of commercial fishing". But this is not the only interest of better understanding their dietary behaviour. "Several scientists have recently observed new behaviour in grey seals which could be the result of emerging competition from other marine mammals. Several studies point to the possibility that grey seals are likely to attack harbour seals and porpoises"

The violent death of seals had already been observed, but it was presumed that boat propellers were the cause. For two years, there has been growing proof of the fact that their main rivals are behind these deaths. "Film footage showing grey seals attacking harbour seals exists. A subsequent autopsy on the victims showed that they had been peeled like apples (see article: When grey seals become killers). Importantly, some seals particularly like the skin and blubber of their prey, the parts which are richest in energy". Traces of grey seal DNA have been identified in wounds by Thierry Jauniaux. A doctoral project begun in October 2015 and financed by the FNRS (France Damseaux) aims to use stable isotopes and trace elements to understand the role of grey seals and other species of marine mammals in the trophic food webs of the North Sea. "Analysis of isotopic ratios is one of the specialities of the Oceanology Laboratory of the University of Liege. In accordance with what is eaten by the organism, the variation of stable carbon isotopes and nitrogen can be observed in the skin or hair of the animals studied. This means that certain species can be identified by means of this study alone, but also the evolution of their dietary habits can be observed, without having to kill an individual to see what is contained in its stomach". According to the tissues in question, we can now go further back in terms of analysing the diet of the specimen. Comparing the isotopic ratios of a maximum number of grey seals will make it possible to determine if the attack on harbour seals is becoming a collective habit or whether it remains the speciality of a few more aggressive and adventurous individuals.
The phenomenon seems new in the North Sea but had already been identified in Canada, "Was this down to lack of attention on our part or is it a recent development in our waters? Is it linked to an increase in the population which would mean a reduction in levels of prey and which is driving them into competition? We can also ask the question as to whether it is isolated behaviour or if it is more common. In summary, there are enough unanswered questions to merit our full attention". This story, which seems so far removed from poisoning by trace elements and persistent organic pollutants has what it takes to arouse the curiosity of Krishna Das and his collaborators. "Up to now, we know that the dietary habits of seals are confined mainly to fish. If it emerges that grey seals are specialising in the capture of marine mammals, this would mean that they are by-passing a trophic level and will be even more contaminated by lipophilic pollutants. In these areas of research, everything is connected", says the researcher. "There are still so many aspects that we don't understand such as certain aspects of the mercury cycle or the cocktail effect of pollutants. For the moment, we are studying them separately. But they can act in synergy in the body of the animal. It is one of the many aspects that we wish to research more fully".

(1) Xenobiotic and Immune-Relevant Molecular Biomarkers in Harbor Seals as Proxies for Pollutant Burden and Effects?
> Effects of Methylmercury on Harbour Seal Peripheral Blood Leucocytes In Vitro Studied by Electron Microscopy
> Seasonal Variation of Harbor Seal's Diet from the Wadden Sea in Relation to Prey Availability
> Relationships between in vitro lymphoproliferative responses and levels of contaminants in blood of free-ranging adult harbour seals (Phoca vitulina) from the North Sea
> Changes in trace elements during lactation in a marine top predator, the grey seal
> Selective transfer of persistent organic pollutants and their metabolites in grey seals during lactation
> Concentrations of chlorinated and brominated contaminants and their metabolites in serum of harbour seals and harbour porpoises
> Marine mammals from the southern North Sea: feeding ecology data from delta C-13 and delta N-15 measurements
> Inter-species differences for polychlorinated biphenyls and polybrominated diphenyl ethers in marine top predators from the Southern North Sea: Part 1. Accumulation patterns in harbour seals and harbour porpoises
> Biomagnification of naturally-produced methoxylated polybrominated diphenyl ethers (MeO-PBDEs) in harbour seals and harbour porpoises from the Southern North Sea
> Mercury immune toxicity in harbour seals: Links to in vitro toxicity
> Tissue distribution of perfluorinated chemicals in harbor seals (Phoca vitulina) from the Dutch Wadden Sea