Chapter 5

Skin Biopsy Applications in Free Ranging Marine Mammals: A Case Study of Whale Skin Biopsies as a Valuable and Essential Tool for Studying Marine Mammal Toxicology and Conservation

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1. Introduction

The need to study, evaluate and understand the impacts of marine pollution on marine life is real and urgent. We depend on the ocean for food, transportation, economic gain, leisure and to enhance the quality of our lives. The residence times of pollutants in ocean water are short. The pollutants either settle to the bottom and attach to sediments or enter the food chain and accumulate in marine organisms. These outcomes mean that the only effective ways to assess marine pollution are to study the concentrations of pollutants in sediments or to study them in marine organisms. The average depth of the world ocean is 3,790 meters making it technically impractical to assess pollutants in sediments worldwide because of the great ocean depths under extreme pressures and vast amount of area. Thus, the best approach to assessing ocean pollution is to study it in marine organisms and, because of their relationship to humans (both biologically, culturally and inspirationally) and their ability to integrate air, water and prey, the best marine organisms to focus on are marine mammals.

There are many marine mammal species. Many of them are listed as endangered or threatened. A species that is considered to be one of the most endangered in the world is the North Atlantic right whale. Their population numbers only about 400 individuals [1]. This species suffers detrimental losses to their population through boat strikes and entanglements in fishing gear [1]. Regulations are being implemented to prevent extinction; however, boat strikes and entanglements may not be the only reason the population numbers remain so low. Other factors, perhaps pollutants, might be affecting the overall survival and reproductive ability of these animals [2].

Ocean pollution is a threat to marine mammals. Some pollutants cause obvious and direct harm to the animals such as plastics and other debris. Others can be less evident, such as, agricultural runoff and industrial wastes. Industrial wastes can include air pollution that the animals breathe in and pollutants that can be found at different levels of the water column. These types of pollutants can have long term and persistent exposures.

Whales are exposed to all environmental pollutants that reach the ocean. They spend time at the surface and travel throughout the water column so they are exposed to pollutants that remain at the surface and those that disperse through the water column. They experience dermal exposure through pollutants in the water. They feed at different depths so they get pollutants through ingesting animals that may have accumulated them. They all breathe air and, thus, are exposed to air pollutants. Consequently, they make excellent models to use for studying the threats and consequences of ocean pollution. The challenge, of course, is to develop an approach for studying the toxicology of marine pollution in marine mammals that provides species specific data along with an individual and population context.

Marine mammal research is difficult and expensive. There are laws that have been implemented to specifically protect marine mammals. Some species not only fall under this protection but also are protected by the Endangered Species Act. Thus, research with these animals is strictly enforced and regulated through various permitting agencies (eg, National Marine Fisheries Service (NMFS) and United States Fish and Wildlife Service, USFWS). These permits limit the amount, time and ways they can be studied. In addition to the permits, properly trained personnel and proper equipment are required. Whales in particular are difficult to study because they are typically found far from shore requiring extensive travel and vessel time. There is specialized equipment to aid in finding the whales while they are underwater but most of the search requires visual sightings of animals for the short period of time they are at the surface, requiring trained crew to be on watch during all daytime hours in a variety of environmental conditions. Because of these factors, researching whales is a particularly challenging task and one that requires careful use of resources and the ability to extract as much information as possible.

A unique and effective way to study marine mammals is through skin biopsies. The skin can be used to determine the genetics of an individual which can allow for gender identification as well as determining intraspecific relationships, genetic diversity of different subpopulations and tracking individual animals over time. Biopsies can provide important information about environmental pollutants and their effects. For example, they can be used to measure the levels of metals because many metals are known to accumulate in the skin. Through a skin biopsy, blubber is also collected which can be measured for a variety of organic pollutants. In addition, the interface between the skin and blubber can be used to create living cell lines. These cell lines can further be used to determine the toxicity of a pollutant by measuring levels of toxicity and DNA damage.

In this chapter we use chromium as an example for how skin biopsies of free ranging whales can be used to evaluate the environmental impact of a particular ocean contaminant. We chose to use chromium because it is a known carcinogen and a known reproductive and

developmental toxicant that is understudied in the marine environment [3]. Our approach is straightforward: 1) Determine if Cr exposure has occurred, 2) Determine if Cr is cytotoxic and genotoxic to cultured whale cells and 3) Compare data in cultured whale cells to levels in whale tissue to gain a toxic context. We exemplify this using biopsy samples from both sperm whales and North Atlantic right whales.

2. Materials and methods

2.1. Determining if Cr exposure has occurred in whales

2.1.1. Biopsy collection

Whale skin biopsies were collected using a specialized biopsy dart and crossbow according to standard methods [4]. The biopsy dart has a stainless steel tip that collects a skin sample that is about 25 mm long or less and 7 mm in diameter. A buoyant stopper located behind the tip prevents the biopsy dart from penetrating beyond the depth of the tip. The stopper causes the dart to bounce off and float for an easy retrieval with a net. Tips are stored in 70% ethanol until use. Upon retrieval, the tissue sample is removed from the tip using Teflon forceps and placed into a glass Petri dish.

2.1.2. Biopsy processing

The skin and blubber were separated leaving the interface to be used for skin fibroblast cell growth. The blubber and skin were used for analysis of genetics, levels of metals and organics. The interface, once isolated, was immersed in a tissue buffer (PBS with 20% penn/strep and 2% gentamicin) for 30 minutes to get rid of any bacteria that may have been present on the skin. Tissue was then placed in a Petri dish and cut into approximately 1 mm pieces. These pieces were transferred into two T-25 flask with 1 ml of medium (DMEM-F12, cosmic calf serum, L-glutamine, penicillin, streptomycin, sodium pyruvate) and placed upside down in a 33°C humidified incubator with 5% CO_2. After 24 h, 5 ml of medium was added and the flask was gently turned right side up and monitored for cell growth. Living cells typically plated out on the flask directly from the tissue explants within one week.

2.1.3. Measuring Levels in the Skin from the Biopsy

The whale skin biopsies were analyzed using inductively coupled plasma mass spectroscopy to determine the total chromium in the tissue according to published methods using a Perkin-Elmer/Sciex ELAN ICPMS. Samples were rinsed with deionized water and allowed to air dry in a laminar flow hood to minimize contamination. Approximately 0.1 g of tissue was placed in a digestion vessel, 2 ml of Optima grade nitric acid was added, the vessel placed in a hot block, and refluxed at 95°C for 4 h. The sample was cooled, 2 ml Optima grade hydrogen and deionized water (3:2 v/v) was added, heated until the effervescence subsided, cooled, and brought up to a final volume of 20 ml. Standard quality assurance procedures were employed (Table 1) and include the analysis of standard

reference materials, a duplicate sample and a pre-digestion spike. Instrument response was evaluated initially and after 10 samples, using commercially available calibration verification standards and a blank. All calibration verifications were within the acceptance criterion of 85-115% recovery and the preparation blank values were below 3x the limit of detection. Standard reference materials were used to assess method performance, where applicable. Interference check solutions were analyzed with all sample runs to check for matrix effects which might be interfering with sample analysis. The mean limit of detection (LOD) was the lowest analyte concentration likely to be reliably distinguished from the blank and at which detection is feasible. The LOD was previously determined by utilizing both the measured blank and test replicates of a matrix matched sample known to contain a low concentration of analyte. All samples were diluted 2x for analysis by ICP-MS. All data are presented as ug/g wet weight.

2.2. Determining if Cr is cytotoxic and genotoxic to cultured whale cells

2.2.1. Chemical treatments

There are two major biologically relevant valence states for chromium, hexavalent and trivalent, with the hexavalent forms considered more potent than the soluble forms. This study focused on the hexavalent form because the marine environment favors the hexavalent form [5]. Moreover, the total Cr levels in the whales were found to be high and considering that Cr(III) is poorly absorbed by mammals [6], for the whales to accumulate these levels of total Cr, the original exposure to Cr would almost certainly have been to Cr(VI).

We treated whale cells with both a water soluble (sodium chromate) and a water-insoluble particulate (lead chromate) to determine its ability to induce DNA damage. The concentration units for the two chemicals are different (uM for sodium chromate and ug/cm^2 for lead chromate) because sodium chromate dissolves fully in water and thus is a solution, while lead chromate does not and instead forms a slurry of particles in water. The cells were treated with this slurry of intact particles. Since all the lead chromate is not dissolved to express it in units of molarity would overestimate the dose. Thus, its units are weight per surface area.

2.2.2. Cytotoxicity assay

Cells were seeded into a 6 well plate and allowed to grow for 48 h. The cells were treated for 24 h with 1-25 uM of sodium chromate or 0.05-10 ug/cm^2 lead chromate. After 24 h we harvested the cells using a standard protocol [7]. Briefly, cells were harvested, counted and re-plated into 100 mm dishes at colony forming density, allowed to grow approximately 2 weeks, then stained and counted. Treated dishes were compared to the untreated control.

2.2.3. Chromosome damage assay

Cells were seeded into 100 mm dishes and allowed to grow for 48 h and treated as they were in the cytotoxicity assay described above. Cells were harvested and analyzed using published protocols [7]. Briefly, cells were harvested, treated with potassium chloride to

swell the cells, fixed and dropped onto slides. Slides were stained with Giemsa, coverslipped and 100 metaphases were analyzed per treatment concentration.

3. Results

3.1. Determining if exposure has occurred

Once a skin biopsy was collected from a free ranging whale the skin was used to measure the levels of chromium present. The different valence states of chromium cannot accurately be determined, so we measured the total level of chromium in the tissue (Figure 1). For North Atlantic right whales in the Bay of Fundy, 7 biopsies were collected. The total chromium levels in right whale skin (wet weight) ranged from 4.9 to 10 ug Cr/g tissue with an average of 7.0 ug Cr/g tissue. (Figure 1A). For sperm whales, 331 biopsies were collected from 17 different regions around the globe. In sperm whales, the total chromium ranged from 0.9 to 122.6 ug Cr/g tissue with an average of 9.3 ug/g w.w (Figure 1B). The highest mean levels by region were found in the Bahamas with an average of 81.9 ug/g w.w for 2 animals. The average was slightly higher in sperm whale tissues than North Atlantic right whale tissues (Figure 1C).

3.2. Determining if Cr is cytotoxic and genotoxic to cultured whale cells

3.2.1. Cytotoxicity

Both soluble and particulate forms of chromium are cytotoxic to sperm whale and Northern right whale skin cells. The data show that 1, 2.5, 5, 10 and 25 uM sodium chromate (the soluble form of chromate) induced 78, 60, 49, 11, 1 percent survival, respectively, in right whale cells; and 80, 51, 13, 2 and 0 percent survival, respectively, in sperm whale cells (Figure 2A). Doses of 0.1, 0.5, 1, 5 and 10 ug/cm^2 lead chromate induced 126, 64, 49, 11 and 1 percent cell survival in right whale cells and doses of 0.05, 0.1, 0.5, 1, 5 and 10 ug/cm^2 lead chromate induced 100, 91, 68, 63, 36 and 7 percent cell survival, respectively (Figure 2B).

3.2.2. Genotoxicity

Both soluble and particulate forms of chromium induced chromosome damage in sperm whale and North Atlantic right whale skin cells. We measured genotoxicity by induced chromosome damage in two ways: Percent of metaphases with damage and total damaged chromosomes in 100 metaphases. In sperm whale cells 1, 2.5, 5, 10 and 25 uM sodium chromate damaged 7, 17, 20 and 31 percent of metaphases, respectively, and induced 7, 16, 24 and 40 total aberrations, respectively. At the highest dose cell cycle arrest occurred and no metaphases were seen. In right whale cells 1, 2.5, 5 and 10 uM sodium chromate damaged 7, 15, 23 and 33 percent of metaphases, respectively, and induced 8, 18, 30 and 48 total aberrations, respectively (Figure 3A and 3B). For lead chromate, 0.5, 1, 3 and 5 ug/cm^2 lead chromate damaged 6, 12, 15 and 27 percent of metaphases and induced 7, 13, 24 and 28 total aberrations, respectively in sperm whale cells. 0.5, 1, 2, 4 and 5 ug/cm^2 lead chromate damaged 16, 19, 23, 32 and 26 percent of metaphases and induced 17, 22, 28, 40 and 30 total aberrations, respectively (Figure 3C and 3D).

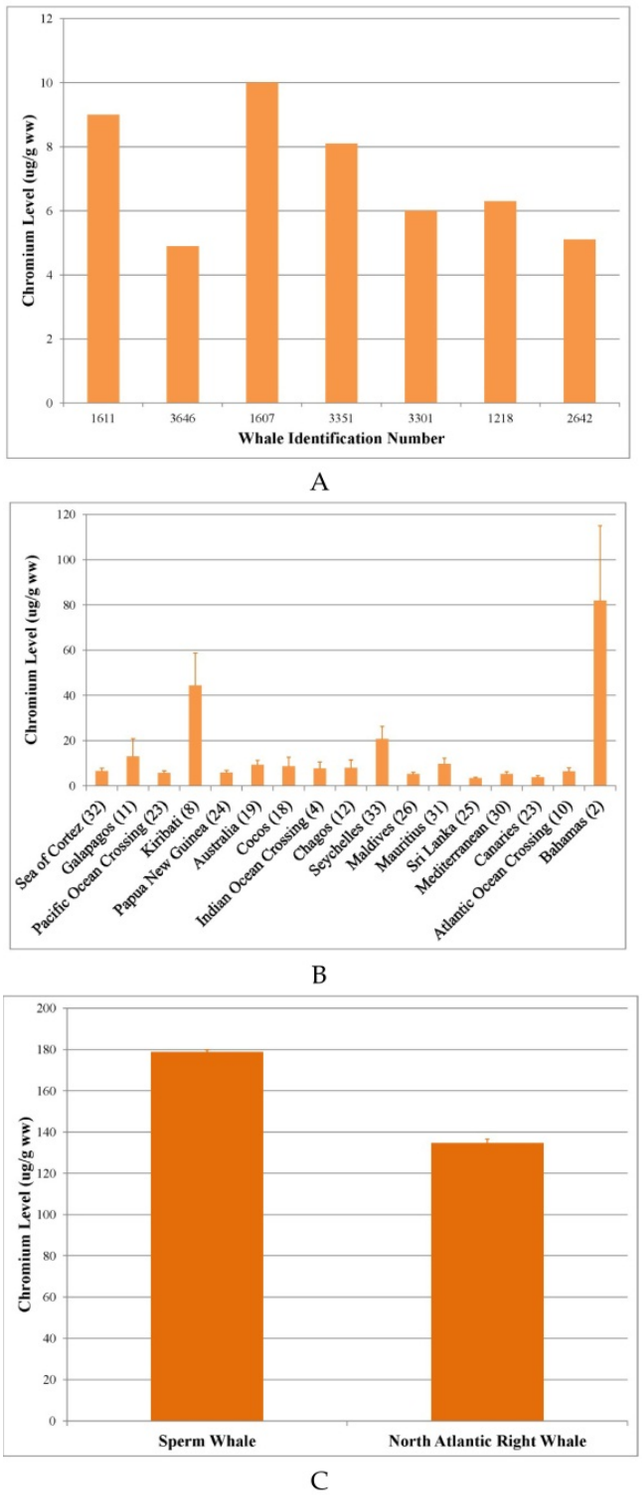
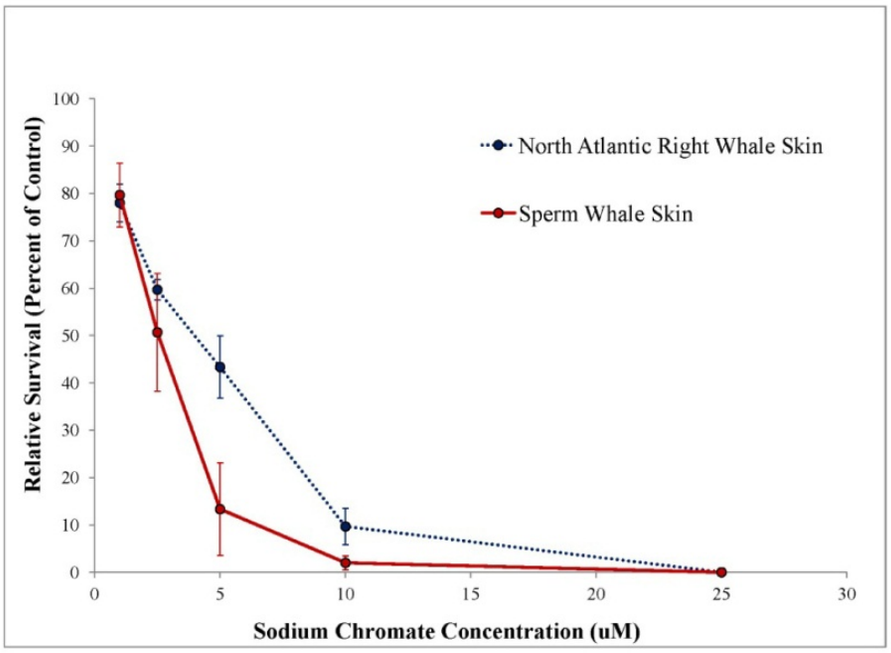
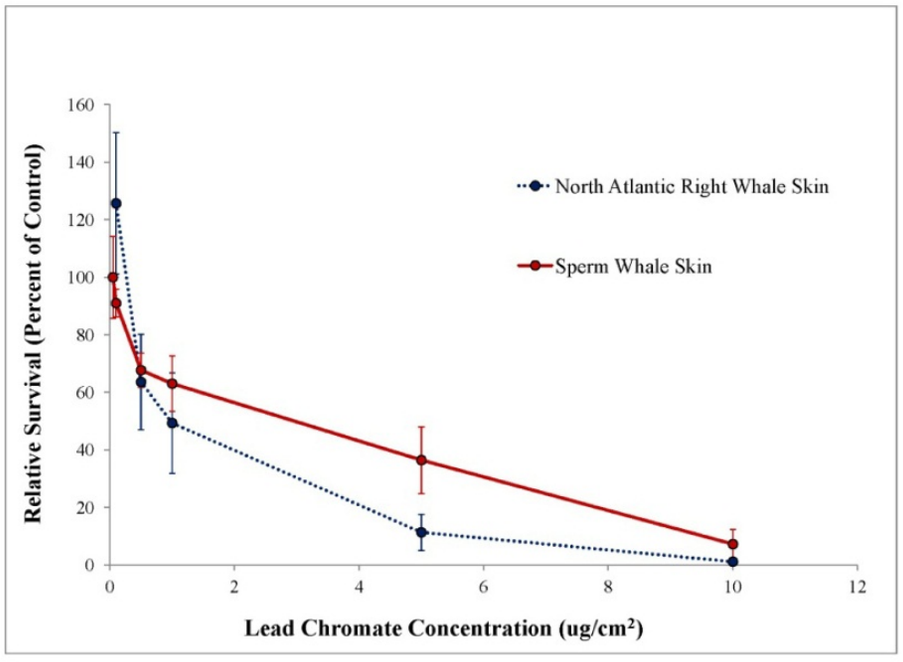


Figure 1. Chromium Levels in Whale Skin. This figure shows levels of Cr found in whale skin biopsies. A) This panel shows individual levels of Cr detected in each of seven North Atlantic right whales; B) This panel shows the mean levels of Cr in sperm whales grouped by region +/- the standard error. Number in parentheses on the x-axis indicate the number of whales sampled in that region; C) This panel shows the average level of Cr found in North Atlantic right whale and sperm whale skin. The North Atlantic right whale average is based on 7 animals biopsied in the Bay of Fundy. The sperm whale average is based on 331 animals biopsied worldwide.

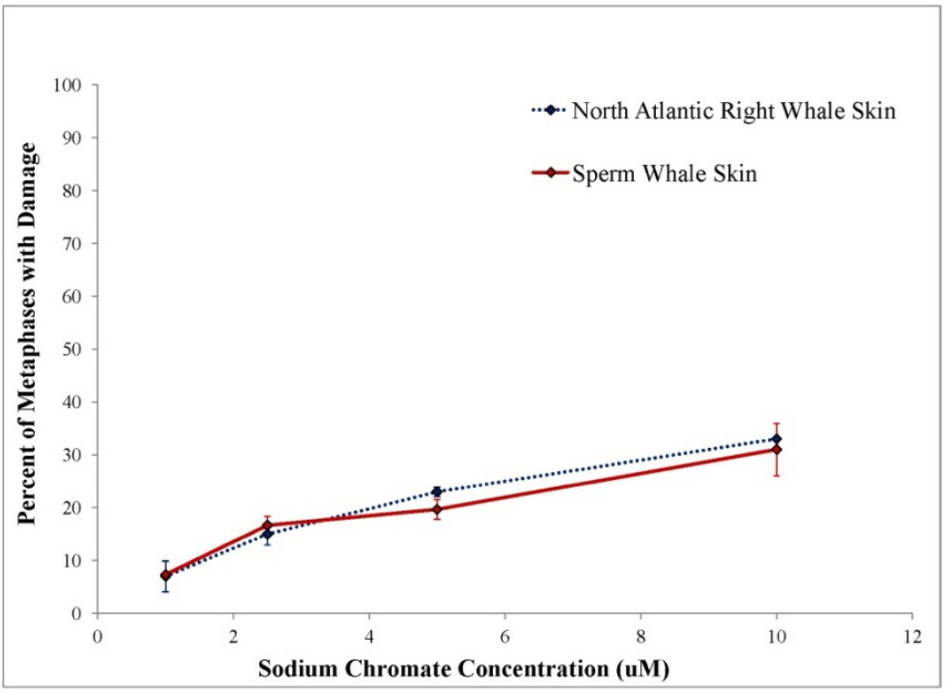


A

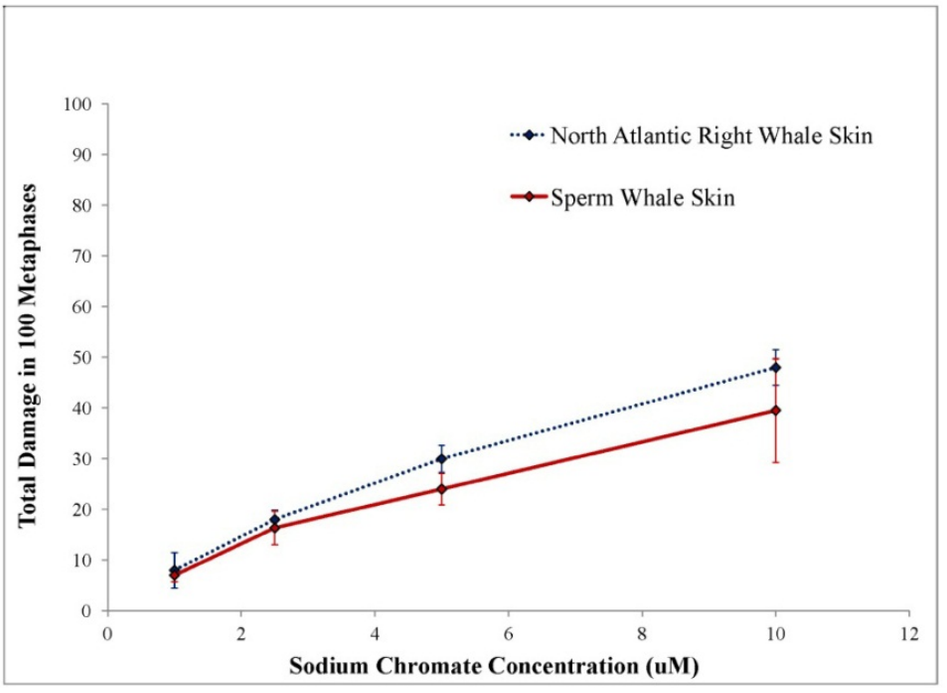


B

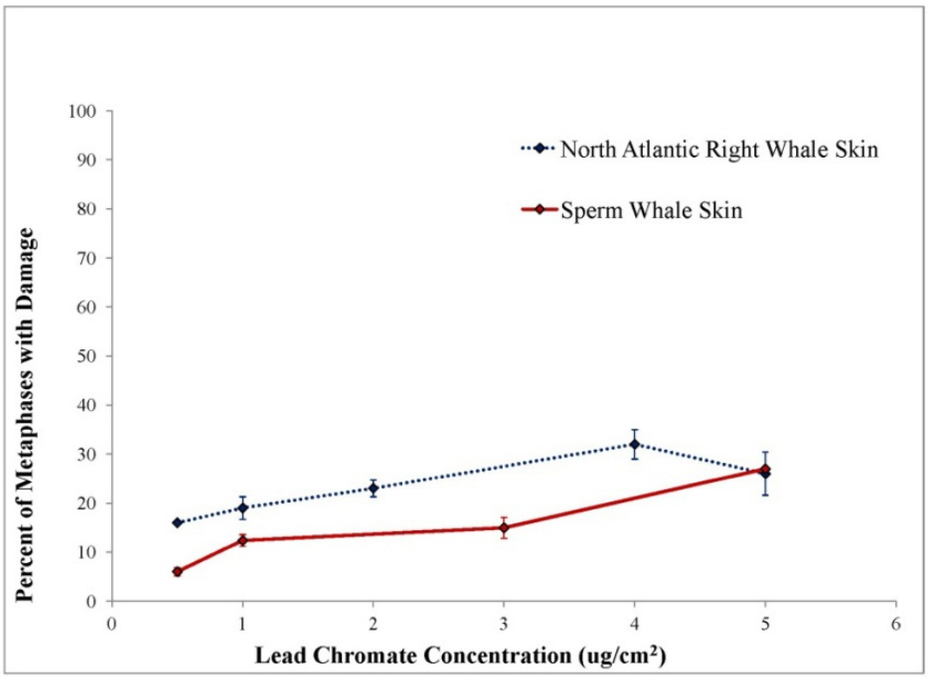
Figure 2. Cytotoxicity of Chromium in North Atlantic Right Whale and Sperm Whale Skin Cells. This figure shows the relative survival of cells treated for 24 h with chromium. There is a concentration-dependent decrease in the number of surviving cells. Error bars represent the standard error of the mean from three independent experiments. A) Cells treated with sodium chromate; B) Cells treated with lead chromate.


North Atlantic Right Whale Skin
Sperm Whale Skin
Percent of Metaphases with Damage
Sodium Chromate Concentration (uM)
0
10
20
30
40
50
60
70
80
90
100
0
2
4
6
8
10
12

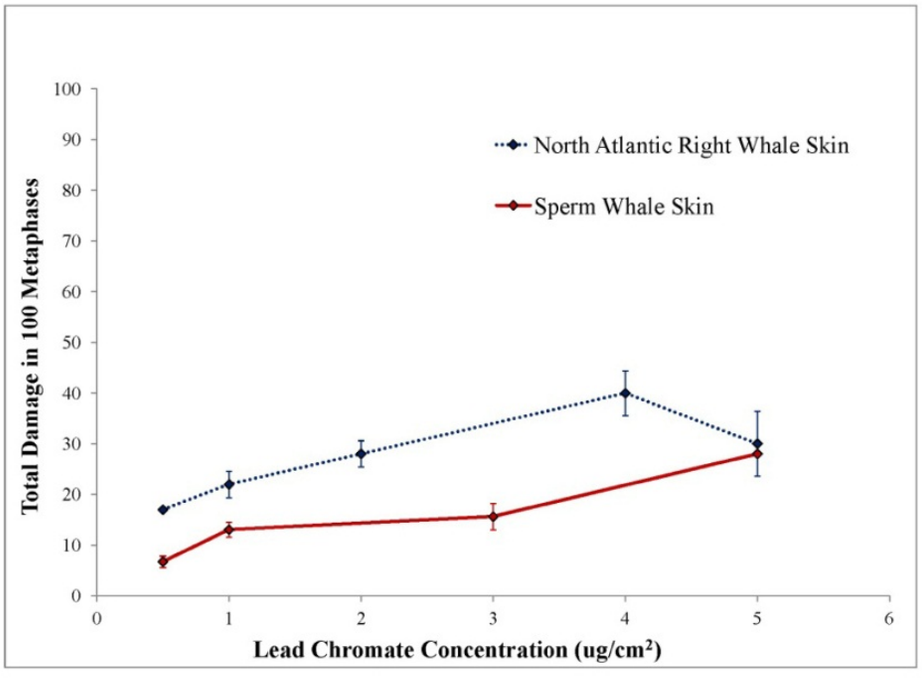
A


North Atlantic Right Whale Skin
Sperm Whale Skin
Total Damage in 100 Metaphases
Sodium Chromate Concentration (uM)
0
10
20
30
40
50
60
70
80
90
100
0
2
4
6
8
10
12

B



C



D

Figure 3. Genotoxicity of Chromium in North Atlantic Right Whale and Sperm Whale Skin Cells. This figure shows the genotoxicity of a 24 h chromium treatment in whale cells. There is a concentration-dependent increase in the amount of damage. Error bars represent the standard error of the mean from three independent experiments. A) Percent of metaphases with damage in cells treated with sodium chromate; B) Total aberrations in 100 metaphases in cells treated with sodium chromate; C) Percent of metaphases with damage in cells treated with lead chromate; D) Total aberrations in 100 metaphases in cells treated with lead chromate.

3.3. Toxic context

The next challenge is to compare the tissue culture doses to the levels in the whales. Comparisons can be made to gain toxicological context; however, one must always bear in mind that cells grown on a dish are in a different environmental context than cells in a tissue and so the comparisons will not be precise. To contextualize the chromium toxicity data, we have converted our treatment concentrations to parts per million and converted the levels observed in the biopsies to molarity. This way we can determine if the levels we are using in the cell cultures are environmentally relevant concentrations.

Our sodium chromate treatments convert to a range of 0.052-1.3 ppm and our lead chromate treatments convert to a range of 0.34-6.8 ppm (Table 1). Considering that average tissue levels for sperm whales and right whales were 9.3 and 7.0 ppm, respectively, our treatment concentrations are well below the average levels measured in whale skin (Tables 1 and 2). In fact, if we were to treat the cell cultures with the lowest sperm whale regional average measured in Sri Lankan waters (63.8 uM) we would kill all of the cells because our highest treatment concentration of 25 uM was highly cytotoxic (Figure 2) as measured by our cytotoxicity assay. Even our lowest detectable level in the sperm whale, 0.9 ug/g, is equivalent to treating cells with 6.38 uM and would induce cytotoxicity in approximately 65% of North Atlantic right whale cells and 90% of sperm whale cells. This level of treatment would also induce DNA damage in approximately 20% of both sperm whale and right whale metaphases. Given that our experimental doses are so much lower than the levels found in whales; this outcome raises concern about the impact of chromium pollution on whales regardless of the difference in environmental context between cells in a dish and cells in a tissue.

Sodium Chromate (uM)	Total Chromium(ppm)
1	0.05
2	0.10
2.5	0.13
3	0.16
5	0.26
10	0.52
25	1.3
Lead Chromate (ug/cm^2)	**Total Chromium (ppm)**
0.5	0.34
1	0.68
2	1.4
3	2.0
4	2.7
5	3.4
10	6.8

Table 1. Chromate Treatment Conversions.

Right Whales (individual levels)		
Whale ID Number	**Cr Tissue Level (ppm)**	**Total Cr (uM)**
3646	4.9	94
2642	5.1	98
3301	6	115
1218	6.3	121
3351	8.1	156
1611	9	173
1607	10	192
Mean all right whales	7.1	135
Sperm Whales (average by region)		
Location	**Cr Tissue Level (ppm)**	**Total Cr (uM)**
Sri Lanka	3.32	63
Canaries	3.69	70
Maldives	5.18	99
Mediterranean	5.21	100
Pacific Crossing	5.55	106
Papua New Guinea	5.72	109
Atlantic Ocean Crossing	6.28	120
Sea of Cortez	6.51	125
Indian Ocean Crossing	7.65	147
Chagos	7.95	152
Cocos	8.63	166
Australia	9.19	176
Mauritius	9.59	184
Galapagos	12.9	248
Seychelles	20.6	396
Kiribati	44.3	852
Bahamas	81.9	1575
Mean all sperm whales	9.3	179

Table 2. Tissue Level Conversions

4. Conclusions

Ocean pollution is emerging as a global priority. No longer can the world's oceans be considered an easy collective dumping site because even the most remote areas of the ocean are accumulating high levels of waste and pollutants. We now understand that any kind of pollution eventually ends up in the ocean. It may be carried by the wind, freshwater rivers

and/or streams, coastal erosion, or watercraft of any sort. We have even found the most remote places on earth to be impacted. However, the true extent and the impacts of our wastes are poorly understood. One reason for this lack of understanding is due to the difficulties the ocean environment poses. Many places are geographically difficult to access; these areas require a large financial, technical, personnel and personal burden to study. In the rare cases that these obstacles are overcome, there remain the additional challenges of contending with environmental challenges (e.g. weather) and the simple fact that marine life is difficult to see, and can only be observed for a short period of time. Finally, the vast majority of marine animals cannot be used in laboratory experiments, and we do not currently have a useful model species to use in their place. Yet, despite these difficulties there are ways to study the impacts of pollution on marine life without sacrificing a large number of organisms and threatening their populations.

Thus, marine mammals provide the best model for studying marine pollution. They are found near-shore (e.g. sea otters, seals, sea lions, sirenians, and some cetaceans) and off-shore (e.g. most cetaceans, some seals, and some sea lions), and their diets range the entirety of the food chain from plankton and krill to giant squid and other marine mammals. A handful of marine mammals have cosmopolitan or near-cosmopolitan distribution, which enables for a more controlled comparison of marine pollution in different areas; these include sperm whales, bottlenose dolphins, orcas, and humpback whales. All marine mammals attract ecotourism, thus there are many watercraft that seek them out for observation. Along with these watercraft come the exhaust from burning fossil fuels, noise pollution that can interfere with the animals' communication and any trash that may be accidently lost overboard.

The health of all marine mammals depends on clean air and water; they all live in the water exclusively or near-exclusively, and since they are mammals they need air to breathe. Not all are directly affected by pollution in the sediment, but may still be indirectly affected through their prey. Marine mammal prey is highly varied from species to species. Studying a variety of them, we can determine if the effect of pollution is specific to animals that are higher on the food chain (likely caused by biomagnification), or if the effect is conserved throughout the food chain (likely caused by bioaccumulation).

Furthermore, marine mammals are suitable organisms to study because they capture the attention and support of the mass media and general public. These animals are one of the major bases for marine ecotourism because of their charismatic behaviors and their similarities with humans; they breathe air, are warm-blooded, nurse their young, and communicate with songs and chatter. Their similarities also enable them to be studied as a model species for humans [8]. The ocean is a finite resource, and has a finite capacity for pollution intake. Marine mammals can serve as a model species for humans to demonstrate how we will be affected by marine pollution in the future if it is not monitored and regulated better. They spend either all or nearly all their lives in the ocean, and they will exhibit impacts by marine pollution sooner than humans will.

Skin biopsies can provide a wealth of information about marine mammal pollution. Here we have shown how chromium, a little studied chemical in the marine environment, has contaminated whales in even the remotest regions. Using the levels of chromium we reported in the biopsies we have determined that the treatment concentrations that induced DNA damage in our cell culture toxicological analysis were orders of magnitude lower. To add further supporting context, human workers exposed occupationally to Cr(VI) had levels of chromium in lung tissue of 20.4 ug/g [9] while levels of skin were reported to be 0.05 ug/g [10]. These workers died of lung cancer. Considering that the average skin levels found in sperm whales and North Atlantic right whales of 9.3 ug/g and 7 ug/g, these levels appear to be extremely high compared to occupationally exposed humans and are of concern.

By measuring levels of metals and other pollutants we can inform our in vitro toxicological experiments by using more environmentally relevant treatment concentrations. In addition, we have used the biopsies to develop cell lines to test potential environmental pollutants like chromium in a species specific model rather than use human or mouse models to inform on pollutants specifically being exposed to a marine mammal population. These results demonstrate that environmental pollutants found in the ocean are accumulating in the whales and are likely to have important health repercussions to the animals living in the oceans.

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