Report of the IWC POLLUTION 2000+ Phase II Workshop

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1. INTRODUCTORY ITEMS

The Workshop was held at the Marine Mammal Center, Sausalito, CA, USA from 22–24 February 2010.

1.1 Welcoming remarks

Jeff Boehm, Executive Director of the Marine Mammal Center, thanked the participants for coming and offered the services of the Center for the Workshop. Ylitalo (Convenor) welcomed the participants.

1.2 Introduction of participants

The Workshop participants introduced themselves and their areas of expertise with regard to pollutants and cetaceans. A list of Workshop participants and Steering Committee members are shown in Annex A.

1.3 Election of Chair

Ylitalo was elected Chair.

1.4 Appointment of rapporteur

Bolton was appointed rapporteur. All participants assisted in the preparation of the report.

1.5 Adoption of Agenda

The adopted Agenda is given in Annex B.

1.6 Available documents

Fossi *et al.* (2008), Fossi *et al.* (in press), Godard *et al.* (2004), Hall *et al.* (2006), Hall *et al.* (2005), Marsili *et al.* (2008), Muir and Howard (2006), Pierce *et al.* (2008), Spinsanti *et al.* (2006). Documents distributed during the meeting: IWC (2008), Pauly *et al.* (1998), Dorneles *et al.* (2007), Dorneles *et al.* (2008a), Dorneles *et al.* (2008b), Lailson-Brito *et al.* (2010), Dorneles *et al.* (2010).

2. BACKGROUND AND GOALS OF POLLUTION 2000+ PROGRAMME

2.1 POLLUTION 2000 Phase I

Rowles gave an overview presentation that summarised the origins, goals and findings of previous IWC-POLLUTION 2000+ workshops, namely the 1995 Bergen Workshop and the 2007 Barcelona Workshop. The IWC-POLLUTION 2000+ Programme was initiated to investigate pollutant cause-effect relationships in cetaceans. It arose from a major Workshop on chemical pollution and cetaceans held in Bergen in 1995 as part of the IWC's instruction to the Scientific Committee that it should: 'give priority to research on the effects of environmental changes on cetaceans in order to provide the best scientific advice for the Commission to determine appropriate response strategies to these new challenges'. Based on the findings of the Bergen Workshop there was the recommendation to move forward with planning a research strategy for evaluating impacts of pollutants on cetaceans. That plan was developed and finalised through several workshops: Texel 1997 developed a proposal for the follow up research programme, Barcelona 1999 further developed the programme and Texel 2000 finalised the specific protocols and biomarkers for the study. Interim progress reports as well as working documents on specific studies within the project have been regularly submitted to the IWC Scientific Committee. A fundamental concept behind the Phase I study was to attempt to examine a pollution 'gradient' for populations of the same species (i.e. a 'clean', moderately exposed and heavily exposed population). In an ideal world the objective would be to determine a predictive model linking tissue pollutant levels in individuals with effects at the population level. Even though this was clearly not a realistic short-term goal, it was proposed to be a potential long-term goal. Given the variety of factors influencing population dynamics, it was noted that eventually some level of probability of certain effects occurring at the population level could be assigned, given certain levels of specific pollutants in individuals. Polychlorinated biphenyls (PCBs) were identified as the chemicals of interest for this programme due to their widespread global distribution and the extensive information on the levels and effects of these compounds for a variety of mammals. One of the first important tasks (and indeed achievements) of the programme was to develop an integrated protocol for sampling, storage and shipping procedures for cetacean samples to ensure that tissue samples to be collected were adequate and would reach the designated laboratories in suitable condition for the analyses. This was developed at the Texel meeting in November 2000. It included protocols for collecting samples for pollutant analysis, indicators and biological variables and is published in the *Journal of Cetacean Research and Management* (Reijnders *et al.*, 2007).

In 2000, Phase 1 of the POLLUTION 2000+ was begun with two short-term objectives:

(1) to select and examine a number of biomarkers of exposure to and/or effects of PCBs and try to determine whether a predictive and quantifiable relationship with PCB levels in certain tissues exists with a focus on bottlenose dolphins; and
(2) to validate/calibrate sampling and analytical techniques utilising harbor porpoises to address such questions for cetaceans, specifically
    (a) determination of changes in concentrations of variables with post-mortem times;
    (b) examination of relationships between concentrations of variables obtained by biopsy sampling with those of concentrations in other tissues that can only be obtained from fresh carcasses.

The examination of these two objectives was considered to be Phase I of what necessarily would have to be a long-term programme. The results from Phase I would be used to determine what might be achieved under Phase II. The results of the two Phase I subprojects were as follows.

Bottlenose dolphin sub-project

(a) Blubber retinol levels were negatively correlated with tissue lipid content and PCB concentrations, however, it could not be ascertained which of the variables were responsible for the decrease in retinol.
(b) There was a positive correlation between dermal CYP1A1 expression and both total PCBs and toxic equivalent quotient concentrations. These concentrations appeared to be stronger determinants of dermal CYP1A1 expression than sex, reproductive status or age.
(c) While immune assays (*in vitro* leukocyte subpopulations, mitogen induced proliferation assays and interleukin 6

levels) were dependent on body length, they showed no correlation with PCB concentrations.
(d) PCB concentrations were not correlated to reproductive hormone (oestradiol and progesterone) levels.
(e) An approach using an integrated set of biomarkers to examine the relationship with PCBs failed.

In all cases: sample size (*n*) was insufficient to allow conclusive results, because potential cause-effect relationships, if existing, were weak.

In addition, an individual-based model was developed to set a framework for examining population level effects. That approach demonstrated how a potential link beween PCB levels and first calf survival could affect annual population growth rate, using the Sarasota bottlenose dolphin population data as an example. However, the framework also showed how sensitive this framework would be to the shape and uncertainty around the dose-response relationship used in the model.

Harbour porpoise subproject
In this post-mortem calibration project it was found that with a post-mortem period of up to 48 hours, and animals kept under 'natural' conditions there was no effect on:

(a) total PCB concentrations;
(b) total DDT concentrations;
(c) retinol levels;
(d) luciferase measures (indicator for dioxin-like exposure);
(e) histology of formalin-fixed, paraffin-embedded lymphoid organs; and
(f) levels of thyroid hormones (T3, T4 and fT4) in serum.

The histology results of snap-frozen pre-scapular lymph nodes were inconclusive as a result of autolytic changes. Tests for CYP1A1 expression using immunohistochemistry, enzymatic assays and western blots were also inconclusive.

2.2 Phase II – goals and objectives
A Phase II Planning Workshop was held in Barcelona in 2007. The Workshop recommended to the Scientific Committee that Phase II of POLLUTION 2000+ should focus initially on the following:

(1) developing a modelling framework;
(2) evaluating model populations that may be more promising for studies for Phase II. It is proposed that initial evaluation focuses on:
    (a) bottlenose dolphins, due to the large body of ecotoxicological information obtained during Phase I;
    (b) humpback whales, because of the significantly large number of biopsy samples from populations whose demography is well known; and
(3) developing a protocol for validating in model species the use of biopsy samples for the specific analyses needed in Phase II.

In subsequent meetings the Scientific Committee recommended that a new Steering Committee be formed and move forward to host an intersessional workshop with the following goals.

(1) Develop integrated modeling approaches and a risk assessment framework for evaluating the cause and effect relationships between pollutant exposure and cetacean populations:
    (a) further refine the conceptual model developed at the Workshop in Barcelona;
    (b) develop the draft models and risk assessment framework;
    (c) review and assess modelling approaches to meet the framework;
    (d) evaluate existing models that could be tested and develop a plan for testing these models with available datasets;
    (e) assess the model characteristics needed and a plan for developing new models if needed;
(2) Develop a prioritisation hazard identification framework to evaluate the broad number of environmental pollutants; and
(3) Identify data needs and available datasets or case studies that would be appropriate for the models that are exposure driven, source driven or effects driven.

The Sausalito Workshop discussed some of the species that were given for consideration at the Barcelona Workshop. Although a number of tissue samples from bowhead whales are available from subsistence activities, at this time it appears that there is an insufficient contaminant gradient among the samples to develop dose-response relationships. Limited chemical contaminant data are available for Southern right whales for dose-response assessments. For minke whales, while there is a gradient in contaminant exposure, it was noted that population dynamics data are lacking in order to elucidate model parameters. Humpback whales afforded the greatest opportunity to represent a mysticete species as many biopsy samples are available for humpback populations that are exposed to different pollutant levels, and for a few populations there are good demographic data (e.g. Gulf of Maine) and photo-identification catalogs so that individual whales could be biopsy sampled over time. Sufficient contaminant and biological data are available for bottlenose dolphins including the Sarasota Bay population, other well-studied populations in US coastal waters, as well as animals in the Navy dolphin program in San Diego, California. Harbor porpoise were also considered as they are a coastal species with site fidelity; thus they could be used to evaluate the population effects of certain classes of chemical contaminants near 'hot spots' or point sources. Similarly, other small coastal cetacean species, such as South American small cetaceans (*Sotalia guianensis* and *Pontoporia blainvillei*), could be used for these types of studies. Some populations are widely distributed off the coast of Brazil with a general chemical contaminant gradient increasing from North to South, with the highest concentrations being found in the southeast region (Rio de Janeiro and São Paulo states). In this latter region, there are some photo-identification catalog data, but demographic and exposure data tend to be somewhat spotty. However, these cetaceans have a high degree of site fidelity, and many of the urban embayments in the southeast region have unique contaminant signatures, which again could be used to elucidate effects from different chemicals or mixtures. In species for which there are both nearshore and offshore populations, it could be useful to compare these populations, as nearshore animals would likely have higher levels of many contaminants whereas offshore/oceanic populations may have higher levels of mercury and cadmium due potentially to enrichment of these compounds in upwelling regions of marine waters and other environmental and physiological factors (Dorneles *et al.*, 2007). Similarly, other cetacean species, such as Mediterranean striped dolphin (*Stenella coeruleoalba*) and bottlenose dolphin (*Tursiops truncatus*), could be used for these types of studies. Some populations are widely

distributed off the coast of Italy and Spain with a chemical contaminant gradient increasing from South to North in the Mediterranean Sea region. High levels of POPs and responses of biomarkers were detected in specimens of striped dolphin of the Pelagos Sanctuary (Ligurian Sea) in comparison with other Mediterranean areas (Fossi *et al.*, 2008; Marsili *et al.*, 2008; Spinsanti *et al.*, 2006).

3. RISK ASSESSMENT FRAMEWORK

3.1 Overview of Risk Assessment Paradigm

Schwacke and Hall presented information on how a risk assessment process for cetaceans could be carried out using a tiered approach. At the end of each phase or tier, risk characterisation results will be evaluated to determine whether there is sufficient concern and/or uncertainty to justify continuation of additional assessment tiers. If the results from the current analysis indicate that estimated exposures do not likely exceed a threshold for effects, then the process is considered complete. Alternatively, if the results indicate a significant risk with an appropriate level of confidence to warrant management action then the risk assessment is considered complete and will then be used to inform a risk management plan. If a potential risk exists but further research or data collections are required to achieve an appropriate level of confidence to be practical for management planning, then the risk assessment process should be continued, advancing to the next tier. The results from initial tiers will inform plans for subsequent tiers, identifying research priorities and data collection needs. A generalised framework for risk assessment tiers is elaborated below (Fig. 1). It should be emphasised that the identified tiers, information sources and research approaches are only general suggestions and may not be feasible or appropriate for some cetacean species and/or contaminants of concern. For cetaceans the largest data gap is in the effects category.

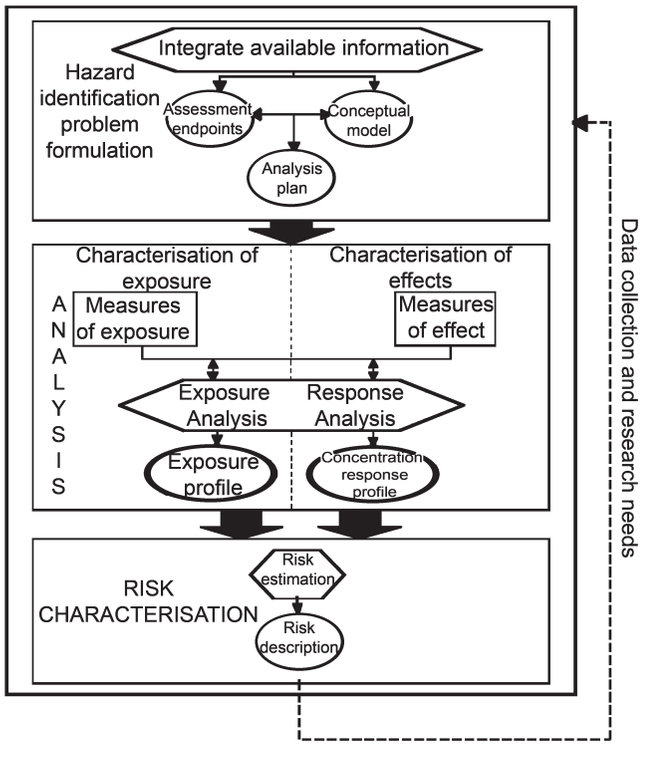


Fig. 1. Proposed risk assessment framework – adapted from US Environmental Protection Agency Guidelines for Ecological Risk Assessment (1998). Risk assessment would be an iterative process following a tiered approach.

3.2 Tiered risk assessment approaches

Tier 1

HAZARD IDENTIFICATION/PROBLEM FORMULATION

The first tier of the risk assessment should be a timely analysis utilising existing environmental characterisations and/or information on status and trends of marine ecosystems (e.g. Mussel Watch) to identify priority hazards. In many cases, evidence of die-offs (cetaceans or other wildlife) may inform the hazard identification process or even be the impetus for a risk assessment. From these data, conceptual models of potential exposure and/or effects pathways can be constructed to aid in the definition of specific assessment endpoints and problem formulation.

EXPOSURE CHARACTERISATION

Existing measurements (or analysis of archived samples) for contaminant concentrations from biomonitoring efforts could support Tier 1 exposure characterisation. Alternatively, concentrations in prey species in combination with models such as pharmacokinetic or bioenergetic models, or even simple bioaccumulation factors could be used to estimate cetacean tissue concentrations. Most of the available contaminant data will come from marine mammal tissue levels as levels for prey are scarce. The use of contemporary environmentally relevant contaminant data is best as organic contaminant levels change with time.

EFFECTS CHARACTERISATION

Effects characterisations for Tier 1 could be based on laboratory or epidemiological studies using surrogate species. The aim would be to identify a threshold level for minimal effects from the existing literature.

RISK CHARACTERISATION

To characterise risks as part of Tier 1, simple threshold models could be employed, potentially as part of a probabilistic analysis. If probabilistic analyses are not feasible, uncertainty factors could be applied for conservative risk characterisation.

Tier 2

HAZARD IDENTIFICATION/PROBLEM FORMULATION

For Tier 2 and subsequent tiers, hazard identification and problem formulation should be based on risk characterisation from the prior tier. In addition, sampling and analysis for nonspecific biomarkers may be pursued to aid in identification of hazards from a general class of compounds (e.g. CYP1A expression as a marker for Ah-receptor agonists).

EXPOSURE CHARACTERISATION

In Tier 2, minimally invasive sampling techniques such as remote dart biopsy may be employed to gather information (*i.e.*, exposure distributions) on a specific population's exposures. Dart biopsy samples of blubber and skin can be analysed for a variety of persistent organochlorine contaminants (blubber) as well as mercury or other metals (skin). In addition to the remote tissue sampling, monitoring surveys such as photo-identification studies can be initiated or intensified to better understand the population's distribution, movements and demographics to aid in the characterisation of exposures.

EFFECTS CHARACTERISATION

Tier 2 effects characterisation may also be based on existing data from laboratory studies but additional complexity in analyses may be pursued. For example, rather than estimating single point threshold for effects, raw data from published studies may be integrated to define a continuous concentration-response function. In addition, laboratory studies such as in-vitro studies (fibroblast cell colture, skin slices) to elucidate toxic mechanisms or species sensitivities could also contribute and help to refine effects characterisation. Experimental in-vivo studies using surrogate species could also be pursued.

RISK CHARACTERISATION

Concentration-response functions derived from the effects characterisation can be integrated with exposure distributions for probabilistic analyses and/or incorporated into simple population models. A key component of this preliminary modeling exercise should be sensitivity and uncertainty analyses which will help to identify data needs.

Tier 3

HAZARD IDENTIFICATION/PROBLEM FORMULATION

See Tier 2.

EXPOSURE CHARACTERISATION

Exposure characterisation may be refined by conducting additional sampling, possibly to include capture-release techniques (for small cetaceans) or sampling of subsistence hunt animals. The purpose of the expanded sampling would be to elucidate important covariates for exposure (e.g. age, sex and reproductive state) in order to more accurately describe the population's exposures. Additional chemical analyses such as analysis for PCB hydroxylated metabolites could also help to refine exposure characterisation.

EFFECTS CHARACTERISATION

For the final tiers of the risk assessment, epidemiological studies could be designed based on findings of previous tiers and conducted to refine effects characterisation. Many types of studies, including correlational, case-control, or longitudinal studies may be feasible depending on the species and population under study. Correlational studies (comparing effects among populations) would be particularly useful if baseline populations with lower exposures could be identified.

In some cases, it may also be appropriate to examine effect biomarkers such as retinol or functional immune indicators that could help to refine derived concentration-response functions (if the marker provides direct measure of reproductions or survival) or even expand the effects characterisation to include multi-stage models. Multi-stage models would be appropriate for biomarkers of indirect effects – e.g. retinol → immune function → susceptibility to infectious agents → survival.

RISK CHARACTERISATION

More complex population models, such as Individual Based Models (IBM), Monte Carlo simulations, spatially-explicit models or other stochastic population models which would allow for the inclusion of exposure distributions and concentration-response functions along with associated uncertainties, would be appropriate for Tier 3 assessment.

4. PRIORITISATION SCHEMA FOR CHEMICAL HAZARDS FOR CETACEANS

4.1 Overview of contaminants of emerging concern in marine ecosystems

Information on contaminants of emerging concern in marine ecosystems was presented by Collier. Many new chemicals are appearing in marine and coastal waters as a result of human activities, and some are being found in a diverse array of biota as well. Collectively, these are now referred to as chemicals of emerging concern (CECs). Examples include halogenated flame retardants, perfluorinated compounds, current use pesticides, hormones, pharmaceuticals and nanomaterials. NOAA's Status and Trends Program is developing a pilot program in the State of California to assess whether CECs are being found in coastal environments, and the Arctic Monitoring and Assessment Programme is also analysing for many CECs in both biotic (marine mammals) and abiotic (air, water and sediment) matrices. For example, a 2009 NOAA Mussel Watch Program report identified several polybrominated diphenyl ether (PBDE) hotspots in the US, such as Southern California. In addition, international 'mussel watch' programs have been established in the U.K. and other parts of the world (e.g., East Asia). Data obtained from regional, national and international monitoring programs such as NOAA Mussel Watch could help identify CECs that may pose the greatest risk for cetaceans as well as the geographical regions where these compounds occur. However, programs like Mussel Watch that rely on measured tissue concentrations need to be supplemented with other approaches that would capture risks posed by non-accumulative CECs. Very recently, the California State Water Board, together with the David and Lucile Packard Foundation, have empaneled a group of experts to provide an overall assessment of the risks posed by CECs to the coastal ecosystems of California.

The Workshop discussed factors that could affect contaminant exposure in cetaceans. For example, sewage treatment processes can vary from place to place even within 'developed' countries. Certain contaminants may not be eliminated from the sewage waste stream even during secondary treatment. In addition, minimal oxygen zones are known to inhibit metabolism of many compounds including hormones and other endocrine disruptors, which could lengthen the potential time frame for exposure. Comparing contaminant levels in nearshore to offshore cetacean populations may also be useful in determining populations that are at higher risk to exposure effects.

4.2 Summary of exposure and effects in cetaceans

Kucklick presented a summary on pollutant exposure and effects in cetaceans, focusing on findings in bottlenose dolphins. Contaminant exposure in marine mammals is assessed through samples obtained through strandings, dart biopsies, samples collected from health assessments, and through modelling. Of these, dart biopsies have been used extensively for assessing lipophilic pollutant in marine mammal blubber. However, the reliability of this technique has been called into question due to the potential for stratification of pollutants in blubber. A study was conducted examining the stratification of lipophilic contaminants in stranded bottlenose dolphins (J. Kucklick, pers. comm.). Concentrations of lipophilic pollutants were not significantly different among the three layers for the animals studied in agreement with a field study finding no significant

differences between surgical versus dart biopsies; however, dart biopsy results appeared to provide more variable concentrations than surgically collected biopsies. Tissue distributions of lipophilic pollutants can vary mainly based on lipid distribution, however there are some differences in overall pollutant profiles among tissues. For instance, blood and blubber levels of total lipophilic pollutants have been shown to be highly correlated, however individual contaminant distributions may vary among tissues based on the physical property of the compound being studied. Proteophilic compounds include hydroxylated PCB metabolites, organomercury, organotin and perfluorinated compounds. These compounds are best measured in blood, however mercury can be determined in skin samples. In the US, lipophilic pollutants, mercury, and perfluorinated contaminants vary with location. For lipophilic pollutants, the highest concentrations are observed in bottlenose dolphins living near large urban centers and near known sources of point-source pollution. Levels of perfluorinated compounds also vary in bottlenose dolphin blood samples based on location as do concentrations of mercury in skin biopsy samples.

Exposure assessment in marine mammals should be mindful of temporal trends of contaminants. For example, many legacy pollutants appear to be declining or are stable in most locations whereas some current use flame retardants are increasing in concentration in marine mammal blubber. There have been a number of effect studies done on marine mammals and these fall into several categories including correlative, in vitro work, and modeling at the individual and population levels. Correlative studies have mainly been done on endocrine and immune endpoints. In vitro work has been focused primarily on immune function. Overall the number of studies on toxicity are fewer than on exposure; however there have been correlative effects observed primarily in immune function. Several promising new approaches are currently under development including cDNA microarrays for bottlenose and striped dolphins as well as new cell lines (fibroblast cell culture) and organotypic cultures for in vitro studies.

4.3 Prioritisation protocol for chemical hazard identification

An objective of the Workshop was to develop a prioritisation hazard identification framework to evaluate the broad number of environmental pollutants of concern to cetaceans. The Workshop agreed upon an international prioritisation survey of subject matter experts in marine mammals and/or toxicology. To develop the survey, the general approach was to establish cetacean, geographical, and contaminant categories; assess existing information on contaminant exposures and biological effects (negative impacts on reproduction and health); determine where information was strong enough to prioritise contaminants; develop international survey format; and identify and query subject matter experts.

4.3.1 Classification methods

To develop a survey, two work groups were formed to establish cetacean life history and contaminant categories (see Items 4.3.1.1 and 4.3.1.2 below). Once the categories were agreed upon by the Workshop, the Workshop assessed existing information on contaminant exposures and biological effects (negative impacts on reproduction and health); determined where information was strong enough to prioritise contaminants and worked towards developing an international survey format. The Workshop then worked towards identifying subject matter experts from various countries to participate in the survey.

4.3.1.1 CETACEANS BY LIFE HISTORY

The cetacean life history work group categorised the cetaceans using the diet composition and trophic level data presented in Pauly *et al*. (1998) (see below).

Cetacean Category 1: Trophic Level 3.2–3.3	
Northern right whale	Pygmy right whale
Southern right whale	Blue whale
Bowhead whale	Gray whale
Cetacean Category 2: Trophic Level 3.4–3.9	
Fin whale	Humpback whale
Common minke whale	Antarctic minke whale
Sei whale	Bryde's whale
Commerson's dolphin	
Cetacean Category 3: Trophic Level 4.0–4.2	
Arnoux's beaked whale	Pacific white-sided dolphin
Baird's beaked whale	Fraser's dolphin
Southern bottlenose whale	Common bottlenose dolphin
Northern bottlenose whale	Striped dolphin
Narwhal	Long-beaked common dolphin
White whale (beluga)	Hector's dolphin
Rough toothed dolphin	Harbor porpoise
Tucuxi	Vaquita
Franciscana dolphin	Burmeister's porpoise
Indo-Pacific hump-backed dolphin	Dall's porpoise
Atlantic hump-backed dolphin	Finless porpoise
Irrawaddy dolphin	Short-beaked common dolphin
White beaked dolphin	
Atlantic white-sided dolphin	
Dusky dolphin	
Peale's dolphin	
Indo-Pacific bottlenose dolphin	
Yangtze dolphin (Baiji) (possibly extinct)	
Ganges dolphin	
Amazon dolphin (Boto)	
Indus dolphin	
Cetacean Category 4: Trophic Level 4.3–4.5	
Other beaked whales	Risso's dolphin
Strap-toothed whale	Spinner dolphin
Sperm whale	Pantropical spotted dolphin
Pygmy sperm whale	Atlantic spotted dolphin
Dwarf sperm whale	Guiana dolphin
Melon-headed whale	Clymene dolphin
Pygmy killer whale	Southern right whale dolphin
Killer whale	Northern right whale dolphin
Long finned pilot whale	Heaviside's dolphin
Short finned pilot whale	Chilean dolphin

4.3.1.2 CHEMICALS BY FATE AND BEHAVIOR

The chemical contaminants work group classified the chemicals based chemical property (e.g., lipophilic, proteophilic) and by bioaccumulation and exposure potential (see below).

Lipophilic chemicals
Legacy organochlorines
PCBs
OC pesticides
Sulfone metabolites of PCBs and DDTs
Chlorinated paraffins
Polybrominated diphenyl ethers (PBDEs)
Polychlorinated dibenzo dioxins/furans
New persistent organic pollutants
- – New/replacement brominated flame retardants/flame retardants
- – Musks
- – Methoxychlor, endosulfan

Proteophilic chemicals
Perfluorinated compounds
Mercury, cadmium, other heavy metals
Organotins
Phenolic metabolites (e.g. hydroxylated PCBs and PBDEs)

Low bioaccumulative/high exposure chemicals
Current use pesticides (CUPs)
- – Picloram
- – Pyrethroids
- – Carbamates
- – Diquat

Pharmaceuticals and personal care products (PPCPs)
- – Surfactants
- – Triclosan
- – Phthalates
- – Chlorophenols
- – Bisphenol A
- – Pharmaceuticals that have been measured in prey (e.g. statins, diazepam)

Other chemicals
Polycyclic aromatic hydrocarbons (PAHs) and their metabolites
PBDE 209
Nanomaterials
Non-PAH chemicals associated with discharges from oil and natural gas production

4.3.1.3 GEOGRAPHIC REGIONS

After some discussion, the Workshop agreed to use the 18 geographical regions of the IUCN ecosytem-based regional framework used by the World Commission of Protected Areas – Marine Regions (Kelleher *et al.*, 1995). The regions include the following: Antarctic, Arctic, Mediterranean, Northwest Atlantic, Northeast Atlantic, Baltic, Wider Caribbean, West Africa, South Atlantic, Central Indian Ocean, Arabian Sea, East Africa, East Asian Sea, South Pacific, North East Pacific, North West Pacific, South East Pacific and Australia/New Zealand.

4.4 Chemical hazard survey design and outcomes

Several approaches were discussed with regard to a chemical hazard survey design. The Workshop agreed that the prioritisation survey should be quick and easy to fill out, in order to maximise response rates. It should also be designed so that the maximum amount of information on prioritisation of chemicals of concern, species at risk and identification of potential hot spots can be obtained from a single survey.

Desired outcomes from the needs assessment survey are the following:

(1) prioritisation of chemicals of concern;
(2) prioritisation of species at risk; and
(3) identification of potential hot spots.

It was agreed that each Workshop participant would send the survey to 2–3 subject matter experts, with a cover letter from the Steering Committee. The selection criteria for subject matter experts to query should be some combination of expertise in marine mammals, toxicology or analytical chemistry. The Workshop proposed that the survey would be finalised in spring 2010, and would then be sent to subject matter experts and compiled during 2010. A final report on the prioritisation survey results would be presented at the 2011 IWC Scientific Meeting.

5. IDENTIFICATION OF BIOMARKERS FOR POPULATION MODELLING APPROACHES

The Workshop purposefully selected biomarkers that have been validated in cetaceans and would most likely provide relevant information for the assessment of effects at the population level (see Table 1). It was recognised that there are many additional biomarkers of effects providing very valuable information at the molecular and cellular levels (including drug metabolising enzyme expression, genotoxicity endpoints, oxidative stress, etc.) and that there are promising research efforts currently focused on identifying and validating links between effects at these lower levels of biological organisation and effects at the individual or population levels. The use of toxicopanomics (genomics, transcriptomics, proteomics, metabolomics, etc), *in vitro* (cell culture), and *ex vivo* (organ slice culture) tools are deemed of particular interest in this endeavor.

Table 1
List of biomarkers of effects most likely to provide information for population-level effects in cetaceans.

Hazard identification (validated in cetaceans)
Cytochrome P450 1 enzymes (particularly CYP1As)
PAH-DNA adducts
Metallothioneins

Biomarkers of exposure (validated in cetaceans)
Cytochrome P450 1 enzymes (particularly CYP1As)
PAH-DNA adducts

Biomarkers of effects with potential for population-level assessment
Retinol
Immune assays
Reproductive hormones
Thyroid hormones

Although there is increased variability using dart biopsy samples with respect to contaminants and biomarkers of exposure and effects, these samples can still provide useful information, particularly if all the biopsies for a program are collected the same way, facilitating comparisons between samples in the same data set, even across geographical regions, species, sexes and age classes. Currently, development of *in vivo*, *ex vivo*, and *in vitro* biomarkers is ongoing (see Annex C for selected biomarkers and their descriptions) but information is lacking on how most of these biomarkers can be linked to population-level effects, such as fecundity and survivorship. Biomarkers of stress or resilience, which could reflect on the overall general health and reproduction of cetaceans, would be useful at a population level, but current biomarkers such as cortisol levels are not specific to contaminant exposures. Biomarkers can help pinpoint which populations are most in need of in-depth study of population effects. Some biomarkers, such as hormone levels, may have some direct relevance to fecundity. It was noted that categorisation of biomarkers by known relevance to population level effects would be useful, particularly those associated with reproduction.

Another Workshop discussion point included selecting the appropriate whale populations, as well as tissues needed to help develop and validate biomarkers of effects in cetaceans. Samples collected during subsistence activities could be used to conduct biomarker studies on certain Alaskan beluga whale populations as demographic data, exposure level gradients and temporal trends are available. As noted in Phase I, the Workshop also recognised that harbor porpoise might also be useful for biomarker development and validation, particularly well-studied populations (e.g. United Kingdom). Dolphins in the US Navy Dolphin Program could provide a unique opportunity for study, as the population is a known quantity demographically and samples are collected routinely as part of their health assessment. In addition to full thickness biopsies, sloughed skin, blowhole, blow, urine and fecal samples could also be used for biomarker development. Rosa noted recent data on subsistence-harvested bowhead whales

indicate that the baleen may provide a record of fecundity (calving intervals) based on its trace element content. The Workshop noted that surrogate species could also be used to examine links between biomarkers and survival because cetaceans cannot be studied directly. In some surrogate species, LC50 values, in addition to other effect threshold values, have been established for certain contaminants.

Development of new technologies for measuring biomarkers of effects is currently under way. For example, new techniques are being developed to link skin biomarker results with effects in other organs, such as liver or gonad, and therefore skin samples may have the potential to give additional information about the overall health of an animal. Validation of these techniques could be conducted on animals that are freshly stranded or collected during subsistence activities. Although it is unclear how biomarkers are related to survivorship, as the state of knowledge improves, these relationships can be characterised in surrogate species, as well as in cetaceans. Longitudinal studies on individuals using gene probes, and linking gene expressions to exposures and outcomes (such as survivability) could also prove to be useful.

6. MODELLING APPROACHES

6.1 Overview of Phase I model

Hall described a risk assessment model that examined the effect of different polychlorinated biphenyl (PCB) accumulation scenarios on potential population growth rates using, as an example, data obtained for the population of bottlenose dolphins from Sarasota Bay, Florida. To achieve this goal, an individual-based model framework was developed that simulates the accumulation of PCBs in the population and modifies first-year calf survival based on maternal blubber PCB levels. In this example, the current estimated annual PCB accumulation rate for the Sarasota Bay dolphin population may be depressing the potential population growth rate. However, these predictions are limited both by model naivety and parameter uncertainty. More data on the relationship between maternal blubber PCB levels and calf survivorship, the annual accumulation of PCBs in the blubber of females, and the transfer of PCBs to the calf through the placenta and during lactation are needed. Such data require continued efforts directed toward long-term studies of known individuals in wild and semi-wild populations. During discussion, it was noted that contaminant data are available for some prey species of Sarasota Bay dolphins but, because they are opportunistic feeders, analysing all their potential prey for contaminants is prohibitively expensive. As a result, examination of effects related to tissue burdens rather than oral dose would be useful to incorporate into the model. In addition, the model could be refined to examine sublethal effects in cetaceans, as well as temporal trends in legacy contaminant exposure. The Workshop also recognised that case study models are useful in that they can help answer the 'what if' questions, for example, would reducing fisheries bycatch by half or reducing environmental levels of a specific contaminant by half have a larger beneficial effect on survival of a population? The information obtained could be helpful in making resource management decisions.

6.2 Dose response approaches

Human risk assessments routinely rely on using surrogate (laboratory model) species concentration (or, if given orally, dose) response data. It is now well accepted that this is the best available strategy to use, despite all the drawbacks and caveats but given that directed studies using human subjects are not possible there is little alternative. This situation is directly comparable to that in the cetaceans and it should be emphasised that in the absence of robust concentration-response data from cetaceans, surrogate species data (including pinnipeds and vertebrate laboratory animal models) should be used. Surrogate data already used in cetacean risk assessments include studies in mink (Kihlström *et al.*, 1992; Restum *et al.*, 1998) and monkeys (Barsotti *et al.*, 1976) because the studies reported tissue levels in relation to a reproductive outcome (offspring survival) of direct relevance to understanding perturbations in population dynamics and growth rate.

These standard toxicological studies are carried out on a wide variety of species but it should be possible to combine the data from different species (e.g. using a Bayesian approach) to improve the reliability of the concentration response curve, particularly if the LC50's for the different species are generally of the same order of magnitude (e.g. mink and monkey LC50's in relation to offspring survival are both around 30 mg/kg lipid weight in adipose). Additional recent studies on, for example, sled dogs fed contaminated whale blubber might yield data with population-relevant endpoints and the data from these should be investigated (Sonne *et al.*, 2008a; 2006; 2008b). A wider literature search should also be carried out to see what other recent studies have been published (see reference list below for some promising recent studies that report concentration – response information). There are many caveats in using these surrogate data and where possible reproductive strategy matching would be preferable (e.g. using data from primates might be better than other model species as they give birth to single offspring rather than litters). Older pinniped datasets also exist (e.g. Reijnders, 1986) and if the raw data could be obtained (the published results are in summary form not amenable to concentration-response modelling) these would be a very valuable addition to the existing datasets. Other surrogate species concentration response data that could be incorporated in future models include those recently estimated using a mark-recapture study with contaminants as covariates of survival. For example, a study by Hall *et al.* (2009) reported the concentration-response relationship between first year survival probability and PBDE and PCB uptake during lactation in grey seals. In addition there may be more recent cetacean studies (e.g. Pierce *et al.*, 2008) that report useful reproductive endpoints.

For some species in some situations it might be possible to use physiologically-based pharmokinetic (PBPK) model (e.g. Hickie *et al.*, 1999) to determine blubber concentrations from ingested fish and vice versa. That would then allow the integration of dose-response data from studies for which only ingested doses have been reported. Many of the vertebrate laboratory model species studies do not measure tissue concentrations at the end of the study, only reporting ingested concentrations or doses. A variety of toxicological, contaminant feeding studies are carried out to determine a range of endpoints and if collaborations were set up it might be possible to obtain tissue levels for concentration response studies at the conclusion of the research when the laboratory animals are usually sacrificed. For example, current research is focussing on the effects of POPs and emerging contaminants on neurological and neurophysiological endpoints. Additional added value for marine mammalogists could therefore be gained by encouraging integration and collaboration between marine mammal and other toxicologists.

Effects of relevance to population level impact can be grouped into direct and indirect effects. The direct effects can

be readily incorporated into a population effects model by including a concentration response function. Endpoints include effects on reproduction particularly fecundity, neonatal survival, juvenile survival and adult survival. Where age-specific survival rates in relation to changes in exposure are available these could also be embedded into a model.

Indirect effects include impacts on growth (possibly through thyroid mediated effects) and immune suppression which could result in higher juvenile mortality rates or higher mortality rates following exposure to infectious disease, respectively. Although more difficult to model, additional steps in the model process could be included and strandings data could be utilised to estimate age-specific survival in relation to cause of death. For example, using bottlenose dolphin strandings data available from a wide geographical area and infectious disease as the cause of death, age-specific survival probabilities for different geographical groups could be generated using for example maximum likelihood and Bayesian uncertainty models (Joly *et al.*, 2009; Moore and Read, 2008). If these groups have different exposures (comparing cleaner regions with regions of higher exposure), relative age-specific survivals from infectious disease mortality could be compared. Another approach, if individual tissue concentrations were available, would be to split the data into animals that have less than or greater than some independent estimated toxic threshold, e.g. using the Kannan *et al* estimated threshold for effects of 17mg/kg (Kannan *et al.*, 2000), and compare infectious disease mortality rates. For example, it would be possible to use the large body of harbour porpoise strandings data from the UK and Europe where blubber PCBs and other POP contaminants have been measured in over 500 individuals (Deaville and Jepson, 2008) in an age-specific infectious disease survival model by using these blubber levels as covariates.

6.3 Individual-based model approach

There is an individual-based model (IBM) framework (Hall *et al.*, 2006) available to the community for use and development and this will continue to be refined with the ultimate objective of making it available as open source software via the web. However, it is recognised that other population dynamics models for cetaceans could also be modified to specifically include the direct effects of exposure due to contaminants e.g. matrix population models, state space models, PVA models etc.

There are also a variety of population model outputs and some may be of more interest in the context of the risk assessment framework than others. For example are we concerned about the decline in overall abundance and if so, over what timescale? Or is a decline or depression in population growth rate (i.e. lambda as used by Hall *et al.*, 2006) more important? Other parameters include quasi-extinction probability (defined as the probability of a population falling below a critical density) which has been used in relation to the impact of infectious disease mortality in marine mammals.

6.4 Sensitivity analysis

Sensitivity analysis is an important part of the rationale for using a model to determine the impact of contaminants on populations to investigate where the uncertainties in the data lie. For example a variety of model simulations can be run to see which parameters and relationships have the largest impact on the outcome of interest (population growth rate or abundance etc.) This then helps to prioritise and focus research on parameters that most affect the critical outcome of interest. Studies carried out to date suggest uncertainties around the concentration response relationship can have a large impact.

It is also recognised that other stressors (e.g. climate change, ocean noise, habitat degradation, exposure to contaminant mixtures, etc.) could also affect cetacean population dynamics. It is envisaged that in the future some of these additional stressors should be incorporated into the risk assessment framework and, using sensitivity analyses, the relative impact of various combinations could be determined.

6.5 Example of risk assessment modelling approach in bottlenose dolphins

The following is an example of how the population level risk assessment approach would be carried out. Long term studies into the ecology and health of bottlenose dolphins along the east coast of the US have been ongoing (Schwacke *et al.*, 2004) and have generated an excellent body of relevant data that can be used to answer the question: is PCB exposure and uptake likely to result in a reduction in the potential population growth rate? This illustrates an approach that is possible at Tier III because intensive studies, including live capture/release have been conducted on bottlenose dolphin. A stochastic individual based model framework that has been developed (Hall *et al.*, 2006) and is currently being refined, will be used in this example approach (recognising that this may not be applicable at all Tiers or for all identified case study species).

(1) Hazard identification/problem formulation

The first stage of the process involves identifying a possible hazard. For example, the possibility that PCBs may pose a risk to the health and population of bottlenose dolphins along the east coast of the US was raised based on information about high PCBs in the coastal food web and in lower level marine biota.

Conceptual model:

PCBs in sediments and fish → biomagnification through food web → bottlenose dolphins

(2) Exposure characterisation

The second stage is to determine exposure in the species of interest. In this example PCBs were measured in blubber (as the most appropriate target tissue for these compounds, listed in chronological order):

(a) blubber samples from stranded animals on a lipid weight basis (together with information on confounding factors such as sex, and age class (length));
(b) blubber from biopsy dart samples from live animals; and
(c) blubber from biopsy wedges from live capture release studies.

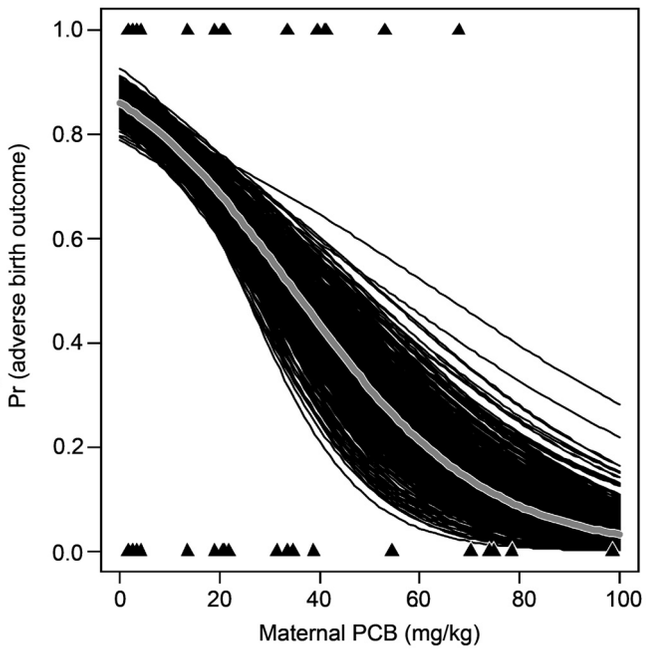
(3) Effect characterisation

There is a wealth of published toxicological data (going back to the early 1970s) indicating that exposure to PCBs affects the health of vertebrate laboratory model species. Direct effects of PCBs on fecundity and offspring survival have been reported in many different species, including marine mammals. This evidence suggests that higher exposure in bottlenose dolphins could have effects at the population level since fecundity and survival are key parameters in determining the population dynamics in general and potential

population growth rate in particular. However, to reach the final goal of risk characterisation a concentration-response relationship specifically linking contaminant blubber levels to fecundity or survival is required.

(a) Bottlenose dolphin concentration-response data. One dataset has been published linking maternal PCB blubber concentration with offspring survival probability in captive bottlenose dolphins (Reddy *et al.*, 2001). However the uncertainty around this relationship is very large and the EC50 is much lower when compared with the results of toxicological studies in other species.

(b) Surrogate species concentration-response data. Other published datasets from surrogate species include two studies in mink (Kihlström *et al.*, 1992; Restum *et al.*, 1998) and monkeys (one study, Barsotti *et al.*, 1976). There are disadvantages and caveats associated with using these data but when the relationships were compared there was no evidence of a statistically significant difference between the two models. This enabled the generation of a generic concentration response curve combining these data (weighted by number of offspring in each concentration category). The resulting relationship is shown in Fig. 2a, with associated uncertainty estimated by resampling with replacement 500 times from the original data and recalculating the regression equation. A Bayesian approach was also taken (Fig. 2b) to determine the most appropriate concentration-response curve. This resulted in an EC50 of 29.5mg/kg. This combined concentration-response was then embedded into the model at the next stage.



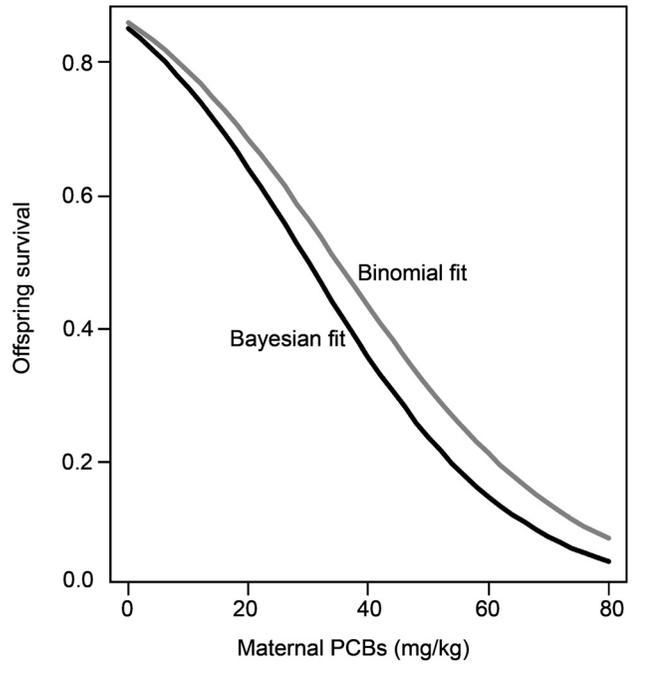


Fig. 2. Example of combined surrogate species (combined results from published mink and monkey studies) dose response relationship relating maternal PCB fat concentration to the probability of (a) adverse birth outcome (black lines show 500 predictions from resampling the data) and (b) a Bayesian fit to the data compared to the binomial fit.

(3) Risk characterisation

This approach is based on the framework outlined in Hall *et al.* (2005) (see paper for details of the model parameters). The effect of PCB exposure on potential population growth rate was assessed (through maternal PCB exposure affecting first calf survival). A simplified flow diagram of how the model functions is shown in Fig. 3.

The model was run for 100 years (Fig. 4 shows 25 simulations for clarity). The black line connects the mean of the population size for each year, and blue lines connect the 95% CI of the population size for each year). In summary the mean potential population growth rate over the final 40yr of the model runs was 1.0007 (95% CI 0.9934-1.0051). In this scenario, there was no evidence that potential population growth rate was affected. However, it should be recognised that PCBs may have additional effects on fecundity or survival not incorporated in this version of the model.

7. IDENTIFICATION OF DATA GAPS AND RESEARCH NEEDS

The Workshop prioritised the research needs with respect to the amount of time and effort needed as follows:

A = can be conducted with existing information and efforts.
B = can be conducted if existing efforts were bolstered.
C = new effort required.

Short term (within 18 months)

- Develop a standardised sampling protocol, including blood, blubber, skin, and fecal samples (A).
- Through modeling, investigate how contaminants that impact individual health can then affect population dynamics (A).
- Investigate how to measure proteophilic contaminants in cetaceans (B).
- Determine framework for a global cetacean sample inventory that is not attached to other animal data (B).
- Determine dose-response levels of contaminants in cetaceans, including linking with toxicologists to determine tissue residue levels in vertebrates; accessing raw dose-response data related to pinnipeds; and targeting ideal surrogate species by contaminant class (B).
- Address the issue of mixed contaminant exposure as it relates to biological effects among cetaceans (B).

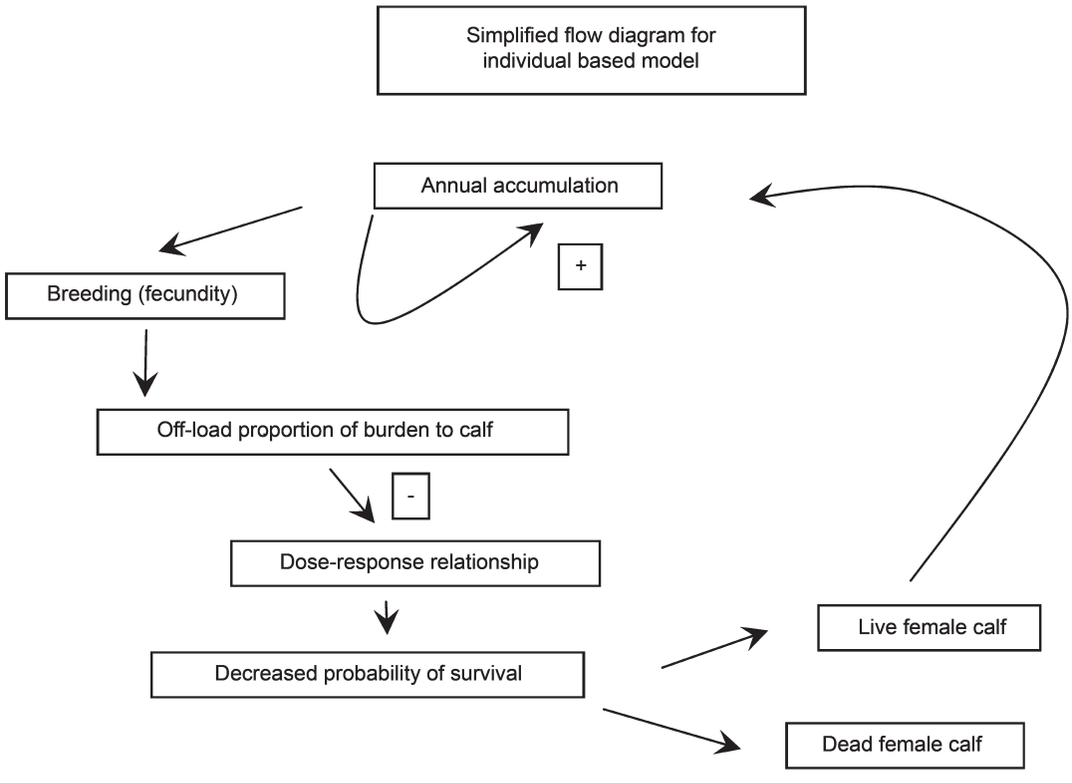


Fig. 3. Simplified flow diagram of bottlenose dolphin PCB exposure model.

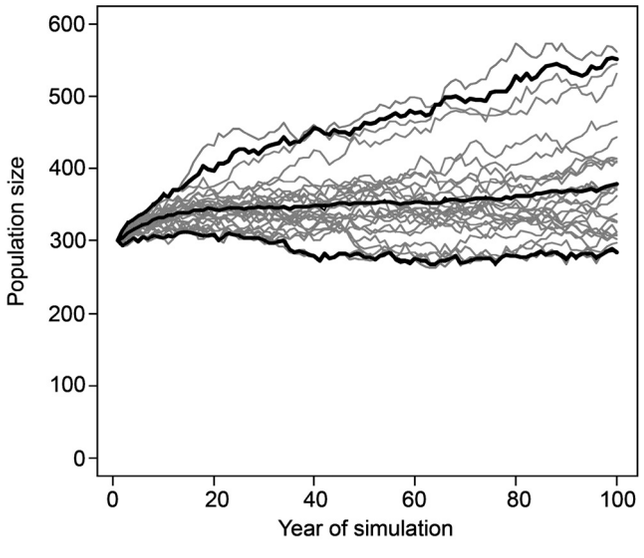


Fig. 4. Simulations to project the population of bottlenose dolphins for 100 years. The figure shows results for 25 simulations.

Examples include review of existing mixed contaminant research; in vitro studies (fibroblast cell culture, skin slices); surrogate species; and SCID mice with cetacean immune systems (B).
- Develop blubber sampling technique involving larger sample mass (C).
- Maximise use of captive cetacean populations by sharing a prioritised list of research, surveillance, and data needs (C)

Moderate term (>18 months–3 years)
- Intensify epidemiology studies. May include efforts to integrate existing databases and standardising definitions (B).

Long term (3–5 years)
- Better understand measurements of reproduction and nutritive state to be used as measures of biological effect (B).
- Validate contaminant load in biopsies by comparing results with internal organs or body burden (B).
- Develop and validate biomarkers and chemical metabolites as measures of effects (impacts on reproduction, survival, and health) (B).
- Develop rapid, inexpensive screening assays for contaminants (C).
- Develop and evaluate measurements of contaminant metabolites for evidence of exposure. May include technique and tool development and pharmacokinetics (C).

8. RECOMMENDATIONS

The Workshop **recommended** the following:

(1) Improve existing concentration-response (CR) function for PCB-related reproductive effects. Re-initiate efforts to derive a CR function based on surrogate species for reproductive effects in relation to PCB exposure. This can build upon prior efforts by Hall *et al.* (2006) that resulted in a CR component for an individual-based model based on data from captive bottlenose dolphin, mink, and monkeys. The CR component could be improved by conducting a literature search and integrating data from more recent studies.

(2) Derive additional CR functions to address other endpoints (i.e. survival) in relation to PCB exposure. This may be accomplished through a multi-stage modeling approach, e.g. a series of functions that provide a connection from PCB exposure → functional immune endpoints → increased pathogen susceptibility → increased likelihood of mortality. Additional CR

functions could be derived using data from surrogate species (e.g. experimental studies and/or wildlife and human epidemiological studies) as well as through synthesis of recently acquired information from small cetaceans (European harbor porpoise strandings and US bottlenose dolphin capture-release health assessments).

(3) Integrate improved concentration-response components into a population risk model (e.g. individual-based model) for one or more case study species (e.g. bottlenose dolphin and/or humpback whale).

(4) Develop new biomarkers and improve the linkages between lower and higher levels of organisation (molecular → individual → population). The highest priority for biomarker development should include those with direct relevance to population-level endpoints such as reproduction and survival.

9. ADOPTION OF REPORT

The report was adopted on 24 February 2010.

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Annex A

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Annex B

Agenda

1. Introductory items
   - 1.1 Welcoming remarks
   - 1.2 Introduction of participants
   - 1.3 Election of Chair
   - 1.4 Appointment of Rapporteur
   - 1.5 Adoption of Agenda
   - 1.6 Available documents

2. Background and goals of Pollution 2000+
   - 2.1 Pollution 2000 Phase I
   - 2.2 Phase II goals and objectives

3. Risk assessment framework
   - 3.1 Overview of risk assessment paradigm
   - 3.2 Tiered risk assessment approaches

4. Prioritisation schema for chemical hazards for cetaceans
   - 4.1 Overview of contaminants of emerging concern in marine ecosystems
   - 4.2 Summary of exposure and effects in cetaceans
   - 4.3 Prioritisation protocol for chemical hazard identification
     - 4.3.1 Classification methods
       - 4.3.1.1 Cetaceans by life history
       - 4.3.1.2 Chemicals by fate and behavior
       - 4.3.1.3 Geographic regions
   - 4.4 Chemical hazard survey design and outcomes

5. Identification of biomarkers for population modelling approaches

6. Modelling approaches
   - 6.1 Overview of Phase I model
   - 6.2 Dose response approaches
   - 6.3 Individual-based model approach
   - 6.4 Sensitivity analyses

7. Identification of data gaps and research needs

8. Recommendations

9. Adoption of Report

10. References

Annex C

Other Suggested Biomarkers to be Validated in Cetacean Biopsy Samples

The **E2F transcription factor** is a member of the E2F family (E2F1-6), which is important in regulating the cell cycle and has a dual role: controlling some genes that regulate the progression of DNA synthesis or being involved in apoptotic processes (Attwooll *et al.*, 2004; La Thangue, 2003). Overexpression of E2F-1 promotes upregulation of several genes involved in the activation of apoptosis and appears to interact with and be modulated by the aryl hydrocarbon receptor. DNA damage in general seems to be responsible for induction of the apoptotic pathway by E2F-1 (Stevens and La Thangue, 2004). Stress signals (such as UV exposure or hypoxia) can induce expression of E2F-1 (O'Connor and Lu, 2000), supporting the use of this gene as a putative biomarker for response to ecotoxicological stress.

The **heat shock protein 70 (HSP70)** is a stress-related protein belonging to a multigene family. HSPs are stress-related proteins induced by a variety of agents and conditions that either directly damage proteins or indirectly act by causing production of abnormal proteins in cells (Nollen and Morimoto, 2002). HSPs are induced as a first response, their main role being to protect cells exposed to stress. Among all HSP families, HSP70 is often used as an early biomarker for environmental stress assessment in a wide variety of organisms. Nevertheless, most studies that use HSP70 as a biomarker are carried out in invertebrate species such as terrestrial arthropods (e.g. *Chilopoda* and *Diptera*) (Pyza *et al.*, 1997) or marine invertebrates and vertebrates (Cruz-Rodriguez and Chu, 2002; Porte *et al.*, 2001). Studies on vertebrates in the wild are very limited, with most studies focusing on fishes (Boone and Vijayan, 2002; Deane *et al.*, 2004) and information about marine mammals is lacking so far.

Oestrogen receptors (ERs) are members of the nuclear receptor superfamily and are ligand-inducible transcription factors. Two isoforms of ERs are known, ERα and ERβ, which have differing tissue distributions and physiological roles (Muller and Korach, 2002) and are encoded by different genes located on different chromosomes. Ligand-induced signalling is due to binding of oestrogen (or a structurally similar compound such as organochlorines or PBDEs) and a specific transcriptional response is subsequently activated. The affinity of chemicals with oestrogenic or antioestrogenic activity is due to the ability of these compounds to interact with ERs (Mueller 2004). Because of the central role of ERs in cell differentiation and proliferation, abnormalities in ER signalling pathways can interfere with sexual development and the endocrine system, both in wildlife and humans. The exposure to exogenous compounds (such as EDCs) with high affinity for ER may therefore cause impairment of endocrine functions. To date, most studies on ERs and their interactions with xenobiotic compounds have been carried out *in vitro* to understand better the toxic effects they can have on living organisms (Tiemann, 2008). Compounds like PCBs and PBDEs that have dioxin-like property can bind oestrogen receptors and interfere with signalling pathways, having an agonist potency measured *in vitro* more preferential for ERα than ERβ. The binding of xeno-oestrogens or xeno-antioestrogens to ER, indeed, can enhance responses of endogenous oestrogens or agonistically bind the receptor to inhibit the physiological action of those oestrogens (Carpenter *et al.*, 2002).

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