

POLLUTION 2000+ Phase II Workshop¹

1. INTRODUCTORY ITEMS

The Workshop was held at the Centre de Cultura Contemporània de Barcelona (CCCB), Barcelona, Spain from 11-12 April 2007.

1.1 Convenor's opening remarks

The Convenor, Reijnders, welcomed the participants and thanked the FDS Foundation for overall support in the organisation of the meeting. Participants are listed in Annex A.

1.2 Election of Chair

Aguilar was elected Chair

1.3 Appointment of rapporteurs

Aguilar, Reijnders and Donovan acted as rapporteurs.

1.4 Adoption of the Agenda

The adopted Agenda is given in Annex B.

1.5 Available documents

The documents available for the Workshop were IWC (2007) and Reijnders *et al.* (2007), as well as published documents as needed.

2. BACKGROUND

2.1 The origins of POLLUTION 2000+

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'.

At the Bergen Workshop, the main conclusions were that:

- (1) there are sufficient data on the adverse effects of pollutants on other marine mammals and terrestrial species to warrant concern for cetaceans;
- (2) a considerable amount of fundamental research is needed to adequately address the question of pollutants on all cetaceans; and
- (3) if any progress is to be made within a reasonable timeframe, a multidisciplinary, multinational focussed programme of research is required that concentrates on those species where there is the most chance of success.

Based on the Workshop report, an outline proposal for a follow-up research programme was drafted at the Texel Meeting in 1997. This was developed further at a special Workshop held in Barcelona in 1999 where the

POLLUTION 2000+ programme was born. The POLLUTION 2000+ programme could be said to have started from late 2000 with the holding of the first Steering Group meeting in Texel (The Netherlands) in November 2000. 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 proposal was to try 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 with effects at the population level. This is clearly not a realistic short-term goal but it might be achievable in the long-term. Given the variety of factors influencing population dynamics, then one might eventually be able to assign some level of probability of certain effects occurring at the population level, given certain levels of specific pollutants in the body. Polychlorinated biphenyls (PCBs) were identified as the chemicals of interest for this programme because of their widespread global distribution and the extensive information on the 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 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.

2.1.1 Objectives of POLLUTION 2000+ Phase I

Two short-term objectives were identified at the Barcelona Workshop:

- (1) to try 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; and
- (2) to validate/calibrate sampling and analytical techniques 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.

¹ Presented to the meeting as SC/59/Rep6.

2.1.2 Summary of results of POLLUTION 2000+ Phase I BOTTLENOSE DOLPHIN SUBPROJECT

- (a) Retinol levels are 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) Dermal CYP1A1 expression is not linked to sex or age, and is determined by PCB concentrations.
- (c) Immune assays (in vitro leukocyte subpopulations, mitogen induced proliferation assays and interleukin 6 levels) dependent on body length, showed no correlation with PCB concentrations.
- (d) Non-significant results were found with reproductive hormones (oestradiol and progesterone).
- (e) An approach using an integrated set of biomarkers to examine the relationship with PCBs failed.

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

An individual based model was developed to set the framework for examining population level effects. That showed a potential to link PCB levels with annual growth of the Sarasota bottlenose dolphin population, and enabled estimation of the concentration at which 50 of the population was affected by PCBs.

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 results on histology 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.

PERSPECTIVES FOR CONTINUATION INTO PHASE II

As detailed in Reijnders *et al.* (2007), the protocols for sample collection and preservation, the post-mortem calibration study and the investigation on the long-term stability of retinoids have all been completed within the framework of Phase I. In addition, substantial advances have been made towards the elucidation of cause-effect relationships, particularly with regards to the potential effects of PCBs on reproduction, retinoid levels and some immune system-related variables. However, as was anticipated from Phase I of the programme, in these latter aspects findings are still weakly conclusive, *viz.* correlative, and mostly refer to only one odontocete species, the bottlenose dolphin.

In this context, the natural continuation of the project into Phase II would consist of:

- (a) further investigation into linking pollutant concentrations with biological effects; and
- (b) extending the above findings to other species, including mysticetes.

The development of Phase II into appropriate case-studies and specific research activities is complex and requires further discussion. The design of a research project to

understand the precise causal mechanisms involved in, for example, the higher mortalities of first born bottlenose dolphin calves, the appropriateness or otherwise of assumptions found in one species applies to others and the implications at the population level requires discussion and contributions from specialists in a variety of fields (*i.e.* reproductive biology, population modelling, ecotoxicology) – as was the case for the development of Phase I. Similarly, research conducted under the POLLUTION 2000+ project and other programmes strongly suggests moderate to severe effects of PCBs on the immune system of some small odontocetes, possibly mediated through a depression of retinoid levels. Making further progress in this line of research requires again interdisciplinary discussion on promising variables and techniques as well as elucidation of populations from which suitable sample sizes can be obtained.

It is important to point out that the results of the POLLUTION 2000+ study on harbour porpoises have suggested that at least some biomarkers applied to the assessment of cause-effect relationships may be species-specific. This conclusion may reasonably be extended to biological effects, such as PCB-related reproductive impairment. For pragmatic reasons detailed in the original proposal, Phase I focused only on two small cetacean species. The species-specificity of effects suggests that simple extension of the findings to other species is problematic. A Phase II project should therefore also consider the inclusion of other species, including mysticetes, as well as following up remaining issues on bottlenose dolphins and harbour porpoises.

As mentioned, most of the objectives of Phase I had been achieved and the involved research has resulted in a number of already published articles, though a small part of that referring to the potential effect of PCBs on the immune system as well as the analysis of retinoids in the harbour porpoise blubber samples from the UK stranding network are now in the final stages of the research or in the review process for publication (Reijnders *et al.*, 2007).

However, Phase I was not able to expand, as planned, the in-depth studies that were carried out on the Sarasota Bay bottlenose dolphin population to other populations of the same species but subject to a different, gradient-distributed, exposure to PCBs. Thus, sampling the initially targeted populations (Bahamas as a 'clean' population and Balearic Islands as a 'polluted' population) was not possible because of logistic difficulties (Bahamas) or by elusive behaviour of the dolphins and subsequent low success in obtaining biopsies (Balearic Islands). Partially because of this, but also by dedication of funding to other research activities, the initially planned validation of the techniques for biopsy sampling by examining relationships between concentrations of variables obtained in biopsies with those obtained in other tissues that can only be obtained from fresh carcasses could not be made and is thus a priority action under Phase II.

2.2 Recent developments in pollutant studies relevant to a possible Phase II

Four presentations were given on new developments in this area of research.

Fossi presented the development of new tools to investigate toxicological hazard due to endocrine disrupting organochlorines and emerging contaminants in skin biopsies of free ranging Mediterranean cetaceans.

As a new 'prognostic' tool she explored interspecies and gender susceptibility to OC-EDCs and PBDEs using qualitative and semi-quantitative evaluation of target proteins, such as CYP1A1 and CYP2B in cultured cetacean fibroblasts of different cetacean species, by western blot, immunofluorescence technique and quantitative real time polymerase chain reaction (qRT-PCR). The need to develop a new powerful 'diagnostic' tool to detect exposure and effects of old and emerging contaminants in Mediterranean cetaceans, led her to develop a suite of sensitive non-lethal investigation tool (Western Blot [WB], RT-PCR and fibroblast cell culture) using skin biopsies of free-ranging animals. As a second new diagnostic tool she proposed the qRT-PCR studies in skin biopsies as a powerful molecular technique to investigate ecotoxicological hazard. Ten commonly used housekeeping genes (Hogs) were partially sequenced in the *Stenella coeruleoalba* (skin biopsy) and, for each gene, PCR primer pairs were specifically designed and tested in qRT-PCR assays. In conclusion, the information obtained from these different research projects will be the basis for further applications and validation of *in vivo* (skin biopsies) and *in vitro* (fibroblasts) methodologies (WB, immunofluorescence, (qRT-PCR) to study the ecotoxicological hazard of marine mammals to classical and emerging contaminants.

Piña gave a presentation on the use of two vital (implying no requirement to kill or damage the specimen) biomarkers in fish: CYP1A1 expression in scales of laboratory zebra fish as well as natural populations of carp, and the level of micronuclei in blood of carp and catfish fish, and the greater purple heron and white heron (micronucleus (MN) index). An increase in CYP1A1 was found in the more polluted population, and the MN index was significantly elevated in animals from the higher polluted areas. Cumulative biomarkers seem to describe better the chemical impact than dynamic ones, like CYP1A1 expression. The development of this kind of biomarker in marine mammals was also discussed.

De Guise presented recent data on the *in vitro* immunotoxicity of PCBs, individually and in mixtures, on different species of marine mammals. It was noted that the immunotoxicity of PCBs differed markedly between species, with no clear relationship to phylogeny, and the mouse model's poor performance at predicting effects in other species. Several lines of evidence suggest that the effects on immune functions were mostly related to the non-coplanar PCB congeners, the mechanisms of action of which are relatively poorly documented, rather than to the better understood dioxin-like coplanar PCBs. It is therefore not surprising that the toxic equivalent (TEQ) approach did not predict the immunotoxic effects of PCBs. Current studies are quantifying the dose-response relationships for approximately 20 individual PCB congeners in different marine mammal species. Given the strong relationship between immunotoxicity and susceptibility to infectious diseases in laboratory animals, the coupling of such dose-response relationship with appropriate exposure assessment could lead to species-specific risk assessment at the cell level, based on cause and effect relationships, to assess potential health effects at the individual and potentially population level.

Krahn reported on the development of a simple, multi-linear equation model-derived from the combination of two specific fatty acid ratios-that enabled the ages of individual killer whales to be predicted with good precision ($\sigma = \pm 3.8$ years). The model was applied to several less well-studied

resident and transient killer whale populations to predict their age distributions from their blubber fatty acid compositions. These results provided evidence for the first time that adult male transient killer whales appear to have a lower life expectancy than their resident counterparts.

3. POLLUTION 2000+ PHASE II

3.1 Objectives

The Workshop **agreed** that within the time available there was insufficient time to fully specify Phase II of the programme. However, sufficient progress was made to **recommend** that Phase II would be valuable in fulfilling the mandate given to the Committee by the Commission and that the initial components of that Phase should include the following elements:

- (1) further validation of the use of biopsy sampling techniques to address issues related to pollution, as well as to extend the work to include other pollutants and biomarkers;
- (2) the work should be extended to new species, particularly baleen whales, to the extent feasible; and
- (3) further work to develop an integrated modelling framework to assess cause effect-relationships between pollutants and cetaceans at the population level, building on the progress made during Phase I and on recent research. This general framework will be of relevance to studies involving all species and compounds.

3.1.1 New analytical techniques for determination of novel pollutants and biomarkers

With respect to the suggestions made by the Scientific Committee, the Workshop spent considerable time evaluating the state of knowledge of present techniques to investigate new compounds and biomarkers of affect and/or exposure. This information is summarised in Annex C. In the light of this, the Workshop **agreed** that it would be premature at this stage to focus in the early part of Phase II on new compounds and biomarkers but **recommends** that developments on these should be evaluated by the Steering Committee for possible later inclusion in the programme.

3.1.2 Validation of biopsy sampling techniques

RATIONALE

In many cetacean species blubber is very thick and its structure is heterogeneous, both histologically and biochemically. It may also vary in structure and composition in different body locations. With the current technology to collect biopsies, the samples excised typically reach depths of only a few centimetres. Depending on the species this may represent only a small fraction of the blubber. In addition, biopsying is not an exact science, therefore samples may be obtained from different body locations. These limitations pose questions about the consistency or representativeness of the samples collected. In addition there are indications that the structure of the blubber is different among species, even if taxonomically related (e.g. white and killer whale).

Therefore, at least in the context of pollution studies, the biopsy technique requires to be validated in any species to be included in Phase II to determine how representative of the whole blubber thickness the biopsy samples are and to assess replicability and potential variation between body

locations. Validation can be conducted by comparing in a set of fresh dead individuals of various categories (sex, reproductive class, age and body condition):

- (a) subsamples obtained from different depths of a blubber thickness containing together all blubber strata with a biopsy from the same body location obtained using standard techniques; and
- (b) in the same set of individuals, biopsy samples collected from different body locations in the body region susceptible to be sampled when using this technique in the field (i.e. posterior and lateral-dorsal part of the trunk).

Blubber thickness may vary seasonally (especially in migratory baleen whales), so consideration must be given to the season during which the individuals used in the validation exercise are sampled.

Given the potential importance of biopsy sampling to pollutant and other studies, the Workshop **recommended** that the conduct of appropriate validation studies be an important component of Phase II. To this end, it notes that the development of a robust and properly designed protocol for such studies is essential. This protocol will be of general value and can be applied in research projects that may not formally be part of Phase II.

There was insufficient time to develop such a proposal at the Workshop and the Workshop **recommended** that the Scientific Committee establishes a mechanism for such a protocol to be developed and for it to identify priority species/populations for this work as a sub-project under Phase II. In order to assist in this process an initial draft of such a protocol is given as Annex D.

WORK ON A MODELLING FRAMEWORK

The ultimate goal of the POLLUTION 2000+ programme is to be able to predict population level effects of pollutants on cetaceans should these occur. As was stated at the outset of the process, this is an extremely ambitious and difficult task and, if possible, will only be accomplished over a long time period with a concerted multidisciplinary effort. There are a number of approaches to such questions which can vary from 'bottom-up' i.e. look at the cellular level, through organs to individuals to population parameters and populations, to 'top-down' i.e. observing population level effects and trying to identify whether the cause is pollutant-related and, if so how and whether predictive 'safe' or 'harmful' levels of pollutants in particular tissues/organs can be identified. Despite some progress on the broad issue made as a result of initial work incorporated into Phase I, the problem remains complex, not only for cetaceans but for most wild animal species. Our information/technique gaps occur at both the data acquisition and the modelling levels. If reasonably estimated quantifiable effects on population parameters can be obtained through the programme, the actual population modelling itself is relatively trivial as many such models are available and are used by the IWC Scientific Committee.

Given this complexity, the Workshop believed that it is essential that work begins now on developing an appropriate integrated modelling framework that will: (1) be relevant to studies of all species and all compounds as information/techniques become available; (2) incorporate a number of potential modelling and both bottom-up and top-down approaches (and the many options in between); and (3) will focus on the types of data and models that are already available or appear likely to be available in the near future. Such an approach is similar to that suggested by the

habitat degradation workshop (reference) and the disease workshop (reference). This approach will also help to set priorities for appropriate key species/populations and data needs. One very simple schematic diagram incorporating some preliminary ideas for an outline of the type of modelling framework that could be developed is shown in Fig. 1.

The present Workshop therefore **recommended** that the Scientific Committee establishes a Steering Group to carry out the necessary careful planning to hold a workshop to further the development of this modelling framework. The modelling workshop will need to incorporate a broad range of expertise including modellers from a range of related disciplines (and taxonomic interests), toxicologists, epidemiologists as well as cetologists familiar with the data available from various potential focal species and populations (see Item 3.2).

3.2 Extension of work to other species

It is clear from discussions at the Workshop that there is sufficient evidence to warrant caution in extrapolating from one species to another, even species that are taxonomically close. While it is clearly not feasible to cover all cetacean species, the Workshop **agreed** that it was important to look at additional species to the bottlenose dolphins and harbour porpoises that were the focus of Phase I. It **agreed** that there were a number of important factors to consider when determining the final 'focal' species, most relating to the likelihood of being able to meet particular objectives set (e.g. validation of biopsy sampling). These include (in no specific order):

- (1) feasibility (availability, cost etc.) of acquiring the requisite samples and appropriate associated information;
- (2) level of knowledge already available (e.g. at the known individual level, c.f. the bottlenose dolphin sub-project from Phase I) or likely to become available; and
- (3) availability of species occurring over a 'gradient' of pollutant levels.

In identifying the methods and case-study populations on which Phase II should focus, the Workshop agreed that the following elements were relevant:

- (i) to complete issues pending from Phase I, namely, the validation of biopsy sampling;
- (ii) to concentrate on the biomarkers that showed more potential for indicating the effect of pollutants;
- (iii) to extend findings of Phase I to other species with populations subject to a pollutant gradient, including mysticetes; and;
- (iv) to prioritise pollution studies in populations from which good background demographic information is available, as is the case in the Sarasota bottlenose dolphin population.

Taking this into account, the following potential case-study populations were identified, although this is not intended to be an exhaustive list.

- (1) Humpback whales from the Atlantic and the Pacific Oceans, from which both biopsy samples and demographic information are already available through the JONAH and SPLASH projects. Preliminary analyses indicate that the two populations are subject to different levels of exposure and the demographic information available would permit examining potential differences in biomarkers.

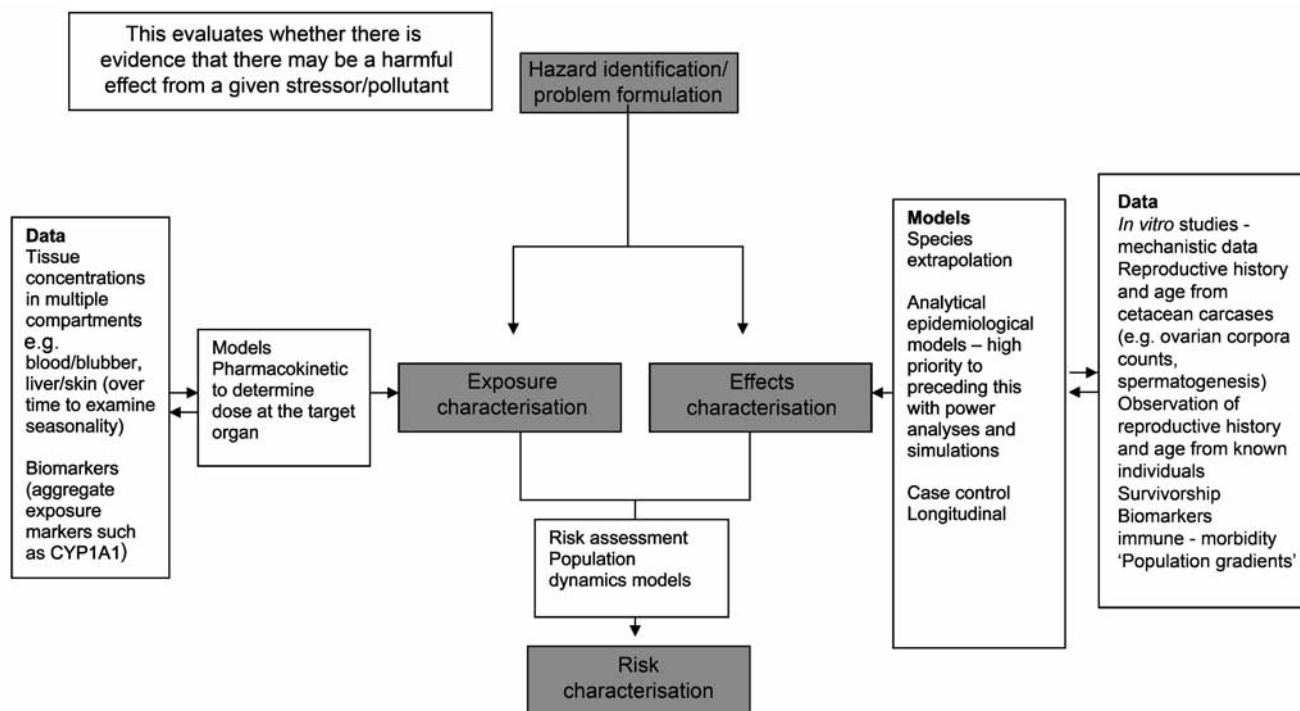


Fig. 1 Simple schematic diagram of an outline that may be useful in designing an integrated modelling framework to examine cause-effect relationships between chemical pollutants and cetaceans, and ultimately effects at the population level. Note the interaction between models and data – data inform modelling exercises and modelling exercises inform data needs.

- (2) Bowhead whales from the Pacific and Atlantic Oceans. Because of extensive work done on the species, tissue samples can be obtained from the Aboriginal Whaling Scheme in the first case and through dedicated biopsy sampling in the second. A feasibility study should be conducted to determine levels of pollutant exposure and, if existing, examine potential differences in biomarkers.
- (3) Minke whales from the Greenland and Norway. Tissue samples can be obtained from the Aboriginal Whaling Scheme in the first case and from the commercial catch in the second. Samples of ovaries and reproductive rates can be obtained from both populations. A feasibility study should be conducted to determine differences in pollutant exposure and, if existing, examine potential differences in biomarkers as well as association of pollutant levels with reproductive output.
- (4) Southern right whales can be sampled through biopsies and stranded animals in Argentina, South Africa, Australia and New Zealand, areas from which some information on individual reproductive rates and overall demographic information are available. A feasibility study should be conducted to determine differences in pollutant exposure and, if existing, examine potential differences in biomarkers as well as association of pollutant levels with reproductive and recovery rates.
- (5) Bottlenose dolphins from the Atlantic Ocean. The Workshop felt that the limitation in the number of individuals sampled from the Sarasota population that precluded some studies of reaching clear conclusions could not be overcome due to the small size of the resident population. However, the long-term demographic knowledge available from it and the continued access to individuals through both captures and biopsies has a strong potential for testing further

biomarkers and for establishing that population as a baseline to which compare others subject to a gradient of pollutants exposure. To this end, populations whose pollutant levels are already known to be significantly higher than those of Sarasota were: those from the Gibraltar Straits, the Alborán Sea, the Black Sea and in some parts of the US. A population subject to significantly lower levels of pollutants was also identified in Brazilian waters. In all these areas the potential for biopsy sampling is high and for some there is even some information on demography and potential for live captures.

Scientists with knowledge of these and other potential priority species/populations should be present at the proposed modelling framework Workshop.

4. CONCLUSIONS

The Workshop **recommended** to the Scientific Committee that Phase II of POLLUTION 2000+ should begin with initial focus on:

- (1) developing on a modelling framework;
- (2) evaluating model populations that may be more promising for studies for Phase II. These populations will be evaluated to determine if they meet the criteria specified in 3.2. It is proposed that initial evaluation focuses on:
 - (a) bottlenose dolphins, because of the large body of ecotoxicological information obtained from that species 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.

To accomplish this, the Workshop recommends establishing a new Steering Group to take this recommendation forward. The establishment of this group, its composition, Terms of Reference, budget and Work plan should begin at the 2007 Annual Meeting. It should take into account the lessons learned during Phase I as given in the final report of Phase I of the project (IWC, 1999). In making this recommendation, the Workshop **emphasised** the need for an adequate funding mechanism to be put into place, noting that pollutant research is very expensive. The need for working in partnership with other international and national groups is stressed in this regard.

In making this recommendation for Phase II, the Workshop repeats a number of points already made about Phase I:

- (1) the recommendations for future work do not suggest that other work on cetaceans and pollutants is not important, rather this is intended to complement other work and provide a focus for research of general value to the scientific community whilst retaining an IWC focus on conservation and management issues;
- (2) similarly, the lack of focus in the initial part of Phase II on 'novel' compounds does not imply that the Workshop believes that they do not represent a potential threat to

cetacean health, rather that until further developmental work has taken place, it is not feasible for such compounds to figure highly in POLLUTION 2000+ given its limited resources; and

- (3) in general, likely effects of chemical pollutants on cetaceans are at best neutral and there is sufficient evidence already to warrant concern – the fact that establishing quantitative cause-effect relationships and predicting population level effects is complex and time-consuming does not imply that governments should wait to take action to reduce levels of potentially harmful chemicals into the environment.

5. ADOPTION OF REPORT

The report was adopted at 17:00 on 12 April 2007.

REFERENCES

- International Whaling Commission. 1999. Planning Workshop to Develop a Research Programme to Investigate Pollutant Cause-Effect Relationships in Cetaceans – 'POLLUTION 2000+'. *J. Cetacean Res. Manage. (special issue)* 1:55-82.
- International Whaling Commission. 2007. Report of the Scientific Committee. *J. Cetacean Res. Manage. (Suppl.)* 9:1-73.
- Reijnders, P., Wells, R., Aguilar, A., Donovan, G., Bjørge, A., O'Hara, T., Rowles, T. and Siebert, U. 2007. Report of the Scientific Committee. Annex K. Report of the Standing Working Group on Environmental Concerns. Appendix 2. Report from POLLUTION 2000+: Phase I. *J. Cetacean Res. Manage. (Suppl.)* 9: 261-74.

Annex A

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Annex B

Agenda

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Annex C

Pollutants and biomarkers of potential relevance to POLLUTION 2000+

	State of technique	Pros	Cons
Pollutants/biomarkers already in Phase I			
PCBs	Fully developed	<ul style="list-style-type: none"> - Basic reference for measuring pollution exposure - Widespread pollutant - High tissue levels - High toxicity - Very stable in tissues - Moderate cost - Can be measured in biopsies - Indicator of exposure to dioxin-like compounds - Reasonably stable in tissues - Can be measured in biopsies - Possible indicator of immune depression - Demonstrated relationship with PCBs - Good background information - Reasonably stable in tissues - Low cost - Can be measured in biopsies - Does not require extremely fresh tissue - Preliminary studies showed some - though low - association to PCB exposure - Low cost - Can be measured in biopsies - Highly relevant to assess impact of pollutants at the population level; functional assays, CD4,2,11,19, 21 	<ul style="list-style-type: none"> - Does not indicate exposure to other pollutants - Expensive technique - Does not indicate exposure to other pollutants - Expensive technique - Lipophilic, therefore concentrations are also affected by nutritive condition - Unknown stability in tissues - Varies within the tissue at microscopic scale - Little background information - Integration not specific - Preliminary studies showed no association with PCBs in bottlenose dolphins - Unknown stability in tissues - Little background information - Expensive technique - Cannot be measured in biopsies (but immunogenetics can) - Preliminary studies did not show association to PCB exposure - Little background information - Sensitivity to PCB exposure not assessed - Cannot be measured in biopsies - Dubious reliability in non very-fresh tissues - Sensitivity to PCB exposure not assessed - Cannot be measured in biopsies - Requires fresh blood samples. - Sensitivity to PCB exposure not assessed - Little background information - Cannot be measured in biopsies - Unknown stability in tissues - Sensitivity to PCB exposure not assessed - Little background information - Lack skin to liver or blood correlation and validation
Luciferase	Fully developed	<ul style="list-style-type: none"> - Can be measured in biopsies - Indicator of exposure to dioxin-like compounds - Reasonably stable in tissues - Can be measured in biopsies - Possible indicator of immune depression - Demonstrated relationship with PCBs - Good background information - Reasonably stable in tissues - Low cost 	
Retinol	Fully developed	<ul style="list-style-type: none"> - Can be measured in biopsies - Does not require extremely fresh tissue - Preliminary studies showed some - though low - association to PCB exposure - Low cost - Can be measured in biopsies - Highly relevant to assess impact of pollutants at the population level; functional assays, CD4,2,11,19, 21 	
Dermal CYP1A1 expression	Fully developed	<ul style="list-style-type: none"> - Can be measured in biopsies - Highly relevant to assess impact of pollutants at the population level - Low cost - Can be measured in biopsies though technique requires validation - Reasonably stable in tissues - Moderate cost - Moderate cost - Low cost 	
Immune assays	Fully developed	<ul style="list-style-type: none"> - Can be measured in biopsies - Highly relevant to assess impact of pollutants at the population level; functional assays, CD4,2,11,19, 21 - Interleukins, cytokines, some cell markers 	
Reproductive hormones	Fully developed	<ul style="list-style-type: none"> - Highly relevant to assess impact of pollutants at the population level - Low cost - Can be measured in biopsies though technique requires validation - Reasonably stable in tissues - Moderate cost - Moderate cost - Low cost 	
Formalin-fixed lymph nodes	Fully developed	<ul style="list-style-type: none"> - Can be measured in biopsies - Highly relevant to assess impact of pollutants at the population level; functional assays, CD4,2,11,19, 21 - Interleukins, cytokines, some cell markers 	
Snap-frozen lymph nodes	Fully developed	<ul style="list-style-type: none"> - Can be measured in biopsies - Highly relevant to assess impact of pollutants at the population level - Low cost - Can be measured in biopsies though technique requires validation - Reasonably stable in tissues - Moderate cost - Moderate cost - Low cost 	
Thyroid hormones in serum	Fully developed	<ul style="list-style-type: none"> - Can be measured in biopsies - Highly relevant to assess impact of pollutants at the population level; functional assays, CD4,2,11,19, 21 - Interleukins, cytokines, some cell markers 	
Porphyrines	Fully developed for liver; urine and blood	<ul style="list-style-type: none"> - May be measured in biopsies (skin) but technique is not yet developed - Potentially, a specific biomarker of PCBs - Low cost 	

Cont.

State of technique	Pros	Cons
Potential new pollutants/biomarkers		
Emerging contaminants: PBDEs analysis	- Relatively high levels in the marine environment. - Can be measured in biopsies	- Expensive technique
Emerging contaminants: PFOS and PFOA analysis	- Widespread in the environment. - Analytical technique developed for various matrices	- Little information available in biota. - Scarce information about toxicological properties - Difficulties in sample treatment and instrumental analysis - Expensive - Cannot be measured in biopsies because they do not accumulate significantly in blubber - Appear to be decreasing rapidly in the environment - Expensive - Logistically difficult
Fibroblast cell culture	- Suitable to explore susceptibility to OCs and other contaminants. - Suitable 'cell model' to develop new biomarkers - Can be applied to biopsies	
CYP1A1 - CYP2B by Western Blotting	- Demonstrated relationship with OCs and PBDEs treatments in fibroblast cell culture - Good background information - Reasonably stable - Non-expensive	- Semi-quantitative evaluation of induction-exposure - Validation in free-ranging cetaceans is in progress - Specificity
qRT-PCR (gene expression CYP1A1)	- Potential indicator of contaminants exposure of OCs, PAHs, PBDEs and other Ten housekeeping genes (HKGs) already partially sequenced in <i>Stenella coeruleoalba</i> skin - Stable in skin biopsy stored in RNA Later or other preservative - Can be measured in biopsies - Would allow potential development of new biomarkers	- Validation in free-ranging cetaceans is still in progress - Low specificity, it is only an aggregate measure
Proteomic in liver cells	- Potential indicator of health status and contaminants exposure - Stable in blood stored in RNA Later	- There are a lot of unknown proteins - It is only a qualitative method - Cannot be measured in biopsies
qRT-PCR (gene expression of cytokines and acute phase proteins)	- Potential indicator of health status and contaminants exposure - Stable in blood stored in RNA Later	- Validation in free-ranging cetaceans is still in progress - Low specificity, it is only an aggregate measure - Cannot be measured in biopsies - It is only a qualitative method
Proteomic in skin biopsy and Initial stage other tissues and fluids	- Potential indicator of contaminant exposure - Potentially applicable to skin biopsy - Would allow potential development of new biomarkers - Potential for development of microarray for rapid detection of multiple gene expression - <i>Tursiops</i> genome will be completed soon	- Requires gene identification - Requires determining gene expression to quantitative endpoint
Genomics	- High potential for information on pollutants effects - Indicator of EDCs exposure (demonstrated relationship in fibroblast cell culture) - Reasonably stable in skin biopsy - Can be measured in biopsies - Potential indicator of the health status and pollutants body burden	- A new technique, significant evaluation is still needed - Validation in free-ranging cetaceans is required
Metabolomics Biomarkers for EDCs by Western Blotting and RT-PCR		
Glycosylation of (blood) proteins		- Validation in free-ranging cetaceans is required

Annex D

Preliminary draft protocol for the validation of biopsy samples

1. INTRODUCTION

Remote biopsy sampling is a routine technique for obtaining tissue samples from many cetacean species in the wild. Many kinds of analysis have been carried out on such samples ranging from genetic analyses of various kinds to stable isotope and fatty acid analyses. For any such analyses it is essential to compare the results obtained from analysing blubber biopsy samples to those for full-thickness blubber collected by necropsy. Focussing on tissue residue analyses, differences in concentrations of persistent organic pollutants (POPs) by blubber depth appear to be species-specific, as well as sex-specific – i.e. some species or one sex class show pronounced stratification and the other(s) have blubber that is more homogenous. Thus, it has been recommended that blubber samples collected from cetaceans for tissue residue analyses should include all layers in order to be representative of an individual animal's pollutant load. However, collecting all blubber layer via biopsy is possible only for the smaller cetaceans (e.g. bottlenose dolphins). Furthermore, differences in POP concentrations have also been found among biopsy samples taken from different locations on the animal, although these differences were not as pronounced as the depth differences for the particular cetacean species studied (i.e. killer whales and white whales). Finally, because blubber biopsies are also being used to determine biomarkers of exposure, health, disease and reproductive status, it is important to assess whether measurements of these parameters also show stratification. As a result, this draft protocol is designed to ascertain how biopsy samples in selected species compare to full-thickness necropsy samples with respect to the analytical results for a variety of parameters.

This initial draft attempts to identify the key factors that need to be considered in developing a final protocol of similar detail for that developed for Phase I. It of course requires further work.

NOTE: The protocol is designed to be carried out on recently dead carcasses – ‘biopsy’ and where appropriate ‘full’ samples will be taken at a number of standard sites on the animal.

The objective of the validation is to determine whether information obtained from biopsy samples is sufficiently reliable (by species, population, reproductive status, age class) and reflective of animal state.

2. LIFE-HISTORY PARAMETER DATA ON THE CARCASSES TO BE COLLECTED FOR EACH SAMPLE

2.1 Sex

Physical carcass measurements

Length, girth at standard positions, blubber thickness at standard positions.

Reproductive status

Obtained from gross examination and histological analyses.

2.2 Age where possible, if not age class

Obtained from earplugs or growth layer groups (GLGs) in teeth to assign animals to defined age class groups. Otherwise age classes may be inferred from other information (e.g. length, reproductive maturity, physical maturity).

3. POTENTIAL POLLUTANT, BIOMARKERS AND OTHER VARIABLES RELATED TO INTERPRETING THE BIOPSY SAMPLE DATA TO BE EXAMINED

3.1 Persistent organochlorine pesticides (POPs)

3.2 Biomarkers of exposure (e.g. CYP1A; retinol)

3.3 Biomarkers of health/disease (e.g. immune assays, if possible on skin or blubber)

3.4 Lipids/lipid classes in blubber

3.5 Fatty acids

3.6. Nutritional state

3.7. Hormones in blubber

3.8. Stable isotopes

4. SAMPLE SIZES

4.1 Potential categories

A number of potential ‘confounding’ variables need to be examined. These include: species; sex class; reproductive class; age class; time of year; health status; and disease state.

4.2 Number of samples

The number of animals in each category needs to be determined statistically through a power analysis. These numbers will initially represent an ‘intelligent guess’ but can be more accurately determined statistically as initial results become available.

5. STATISTICAL ANALYSES

The sampling plan should be developed in concert with a statistician so that sample number is decided up front. This should include, *inter alia* number of biopsy sites per animal, number of individuals per group, number of groups, etc.