Appendix 2

REPORT FROM POLLUTION 2000+: PHASE I

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1. INTRODUCTION

The IWC-POLLUTION 2000+ programme was initiated to investigate pollutant cause-effect relationships in cetaceans.

It arose from the major workshop on chemical pollution and cetaceans held in Bergen in 1995 (Reijnders *et al.*, 1999b) 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' (IWC, 1994, p.35)

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 presented by Aguilar *et al*. (1998) and this was developed further at a special workshop held in Barcelona in 1999 (Aguilar *et al.*, 1999) where the POLLUTION 2000+ programme was born. 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. 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.

It is important to note that in all stages of the development of the programme, the Commission was warned that the programme would be extremely expensive compared to 'normal' IWC projects, given the high costs of collecting and analysing samples. Despite this, the Commission expressed its wish for the development to continue. It should be noted that although considerable funds were allocated by the IWC by its standards, funding problems remained throughout the project.

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

(a) 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;
(b) to validate/calibrate sampling and analytical techniques to address such questions for cetaceans, specifically
    (i) determination of changes in concentrations of variables with post-mortem times;
    (ii) 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 1 of what necessarily would have to be a long-term programme. The results from Phase 1 would be used to determine what might be achieved under Phase 2.

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 documents on specific studies within the project have been regularly submitted to the Scientific Committee in the following: Aguilar and Borrell (2001); Reijnders *et al*. (2002b); Reijnders *et al*. (2003a); Tornero *et al*. (2005b); Tornero *et al*. (2005a); Hall *et al*. (2004); Wilson *et al*. (2004); Wells *et al*. (2005); Reijnders *et al*. (2004) and Reijnders *et al*. (2005).

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* (Reijnders *et al*., 2002a).

This present final report summarises the work carried out under Phase I for each of the two subprojects: bottlenose dolphins and harbour porpoises. It examines the field work, analyses and the results in terms of what was expected to be achieved in the context of the objectives. In doing so, it tries to present a critical review and where appropriate, identifies what might be considered in any Phase II of the project. A more detailed discussion of the potential for a Phase II is given in a separate document. After considering the individual sub-projects, the report goes on to consider more general lessons learned from the conduct of Phase I. Finally it summarises Phase I from the perspective of the IWC and beyond.

2. BOTTLENOSE DOLPHIN SUBPROJECT

2.1 Introduction

This part of the project was directed in particular at objectives (a) and (b)(ii) above. Live bottlenose dolphins, *Tursiops truncatus*, were sampled to evaluate potential biomarkers of organochlorine contaminants (OCs) exposure and effects. The approach involved sampling dolphins expected to reflect a large range of environmental contaminant conditions ('dose'), and then investigating variation in potential biomarkers ('response') relative to these contaminant situations ('dose-response').

2.2 Sampling

The original plans called for obtaining blubber samples from three sites ('pristine', intermediate and heavily polluted) to provide a range of PCB exposures. A full analysis to understand the relationship of PCBs with biomarkers requires considerable knowledge of a number of potential 'confounding variables' e.g. age, sex, reproductive condition, nutritive condition, etc. for at least one sampling site. With such information, it would allow for the investigation of certain biomarkers that might be present in skin (outer portion is epidermis inner portion blubber), and *inter alia* allow an investigation of whether the information from biopsy sampling alone (which provides only a limited amount of associated biological information) could be sufficient to make inferences.

The initial choice for the heavily polluted area was the Mediterranean Sea near the Balearic Islands. However, after a pilot project in 2001 when only five samples were obtained, it was discovered that the behaviour of the animals meant that it would be extremely difficult and expensive to obtain the requisite number of samples (Aguilar and Borrell, 2001). Funding and logistical problems meant that it was not possible to identify a suitable alternative heavily polluted site within Phase I of the programme.

Similarly, the initially chosen pristine site (the Bahamas) for the study proved problematic. Initial sampling in 1998-1999 resulted in 11 samples being collected (Reijnders *et al.*, 2003b). Analysis of the samples confirmed low levels of PCB pollutants (Reijnders *et al.*, 2003b) but for a number of logistical and other reasons it was not possible to continue sampling there. As a possible alternative, exploratory biopsy darting took place in Charlotte Harbor, Florida, a less-developed estuary south of Sarasota Bay. A total of 11 samples were obtained in 2001 but the PCB levels, although slightly lower than those found for Sarasota Bay, were not suitable for a 'pristine' environment. Once again, funding and logistical problems meant that it was not possible to identify a suitable alternative low polluted site within Phase I of the programme. A summary of the information for the three study sites on the biopsy samples and associated pollutant level analysis is given in Annex I.

The choice of an intermediate site was simple. The bottlenose dolphins in Sarasota Bay, Florida represent one of (if not the) best known cetacean populations. The multi-generational resident community numbers around 150 known individuals. Extensive background information (e.g. age, sex, maternal lineage, ranging patterns, health records) is available for most of the individuals from photographic identification, focal animal behavioural observations and capture-release for life history data collection and health assessment studies carried out since 1970 (Scott *et al.*, 1990; Wells, 1991; 2003; Wells *et al.*, 2004). The capture-release programme enables a variety of samples (including epidermis, blubber and blood) to be obtained.

Fortunately, the sampling work carried out at Sarasota Bay was considerably more successful than in the other areas. The capture-release operations provided detailed information on health and body condition, as well as the larger, full-thickness blubber samples and samples of blood and urine needed for 'ground-truthing' potential biomarkers. In total, matched blubber, blood, and urine samples were collected from 47 dolphins (22 males and 25 females) during June 2000 and June 2001. A brief summary of the sampled animals is given in Adjunct 1. Details for each animal, including information on PCB levels and other analyses carried out are given in Adjunct 2a, and the number of samples collected for specific analyses are presented in

Adjunct 2b. In addition, 'co-inspired by' the POLLUTION 2000+ programme (although not funded by it) sampling efforts have continued subsequent to the 2001 field work. This is discussed briefly below but will allow future analyses to be undertaken with a considerably larger sample size.

Table 1

Summary of bottlenose dolphins sampled during 2000-2001 and formally considered part of the POLLUTION 2000+ programme.

	Females	Males	Not known
Biopsy dart sampling total (total=27)			
Bahamas	4	7	-
Charlotte Harbour	2	9	-
Mediterranean (Balearic Islands)	-	-	5
Capture-release total (total=47) (surgical biopsy)			
Sarasota Bay	25	22	-
0-10 years	11	10	-
11-20 years	5	5	-
21-30 years	1	2	-
31-40 years	4	3	-
41-50 years	4	2	-

2.3 Analyses

Samples were distributed for analysis to selected laboratories in Europe and the USA (see Adjunct 3) following agreed standard protocols. The list of primary analyses undertaken included:

(1) blubber contaminant levels (total PCBs and total DDT, as well as values for individual congeners);
(2) immune response parameters in blood (measured as mitogen induced proliferation or interleukin 6 levels);
(3) CYP1A1 expression in integument samples; and
(4) retinoids levels in blubber.

2.4 Summary of results

The primary objectives of the Bottlenose Dolphin Subproject were to:

(1) try to select and examine a number of biomarkers of exposure to and/or effect of PCBs and try to determine whether a predictive and quantifiable relationship with PCB levels in certain tissues exists;
(2) examine the relationships between concentrations of variables obtained by biopsy sampling with those of concentrations in other tissues that can only be obtained from fresh carcases.

The research design called for collection of blubber samples from bottlenose dolphins inhabiting three areas providing distinctly different levels of exposure to PCBs, and for the collection of additional data on associated health parameters from the intermediate site, the only site where it was possible to capture, examine, sample, and release bottlenose dolphins. If a potential biomarker was identified through the detailed sampling and health assessment, then its potential could be investigated through comparisons across sites covering a range of contaminant concentrations.

As discussed under Item 2.2 above, the biopsy darting component of the project encountered several practical difficulties that precluded its successful completion. Initial fieldwork at the Bahamas site was successful in that it showed that it was possible to collect biopsy samples from the animals found there. Although the PCB analysis of the eleven samples obtained suggested this to be a suitable 'pristine' area (Reijnders *et al.*, 2003b), a variety of logistical and funding problems unfortunately rendered it impossible to return there to collect the necessary larger sample sizes.

In view of this, the Steering Group tried to identify other potential 'pristine' sites that could be sampled within the limited budget available and with a reasonable chance of obtaining the necessary sample size. Within those limitations, Charlotte Harbor, Florida, was selected as a potential alternative site. Although it proved possible to collect eleven samples, the analysis of the PCB levels showed that the levels were only marginally lower that the intermediate site and certainly not suitable for consideration as a 'pristine environment'. Funding and timing issues unfortunately meant that it was not possible to carry out the necessary investigative fieldwork to examine further potential sites.

Unfortunately, unforeseen practical difficulties also thwarted the attempts to collect sufficient samples from a heavily polluted area. An area off the Balearic Islands in the Mediterranean Sea had been identified as the potential site. However, due to the elusive behaviour of the animals found there (probably as result of interactions with fishermen), the first season of fieldwork resulted in only five samples being collected despite considerable effort. Although analysis of PCB levels showed that the site could be considered as heavily polluted, calculation of the amount of effort required to collect a suitable sample size meant that the costs of doing so were prohibitive given the available funding (Aguilar and Borrell, 2001). Funding problems also precluded sampling at another identified potential heavily polluted site at Tampa Bay in Florida.

Despite this, the biopsy sampling attempts did highlight some problems with this approach to investigating pollution cause-effect relationships in cetaceans:

(1) blubber is not homogeneous across its thickness but has a stratified structure in tissue matrix, biochemical composition and function. As a consequence, organochlorines and probably some associated biomarkers do not distribute homogeneously across blubber thickness (Aguilar and Borrell, 1991). The biopsy darts probably did not sample the entire thickness of the blubber, complicating interpretation of the data derived from the samples.
(2) the relatively small sample masses obtained from biopsy darting did not permit sufficient testing for the full suite of potential biomarkers.

There was considerably more success with the programme of work at Sarasota Bay (the intermediate site) via the capture-release project. The original plans for the analyses from this project were very ambitious and heavily dependent on funding becoming available. In the event, largely for financial but partly for technical reasons, the Steering Group agreed to limit the analyses to be undertaken. In particular, it was agreed that the analyses of luciferase, porphyrins, and co-planar PCBs would not be conducted because availability of tissue from biopsies was insufficient for all analyses and economical cost to attempt it was high, particularly in the case of the co-planar PCBs.

A major feature of the analyses undertaken was that it highlighted the complex relationships between organochlorine concentrations and potential biomarkers due to the variation in patterns of accumulation and depuration relative to age, sex, nutritive status and reproductive condition (Tornero *et al.*, 2005a; Tornero *et al.*, 2004; Wells *et al.*, 2005). The POLLUTION 2000+ studies confirmed

for cetaceans what had been suspected from other species. For example, male bottlenose dolphins in Sarasota Bay, Florida, accumulate high concentrations of contaminants throughout their life, while concentrations in females, originally similar to males of the same age, decrease dramatically after calving and lactation, with a concomitant increase in concentrations in particularly the first calf. The implications of these findings (e.g. with respect to first calf survival rates) are important inputs to modelling exercises to try to examine potential effects at the population level. An additional complicating factor is that males showed age-related differences in nutritive status (decreased blubber lipid concentration with age). Because of this, observed decreases in retinoid concentrations (a potential biomarker) with age could not be dissociated from the observed negative correlation between levels of organochlorines and retinoid blubber concentration. At present, therefore, it is not clear whether high organochlorine burdens lowered retinoid concentrations or whether depleted lipid reserves were responsible for both the observed high organochlorine concentrations and the low retinoid levels in the blubber (Tornero *et al.*, 2005a; Tornero *et al.*, 2004). This is a question that could be addressed in a Phase II of POLLUTION 2000+.

Similar issues arose when looking at other potential biomarkers such as dermal CYP1A1 expression (Wilson *et al.*, 2004) and several immune assays (SC/58/E39). In the former case, it seems that CYP1A1 expression is not (unlike PCB concentrations) linked to sex and age and that contaminant concentrations, not life-history parameters, may be the determinants of CYP1A1 expression in cetacean integument. In the latter case, it appears that if there is an effect of PCB concentrations (at the levels found in the Sarasota Bay animals) on the immune response system, it is small. However, in both cases, work with larger sample sizes is required.

An innovative approach was used to examine the potential of using an integrated set of biomarkers (immune assays, retinol, the reproductive hormones oestradiol and progesterone, dermal biotransformation enzyme levels measured by specific antibody binding assay) to examine the relationship with PCB concentrations (Hall *et al.*, 2004). However, once again the approach was hampered by small sample sizes and it was found not to be possible to integrate these biomarkers and further explore the relationships between combined biomarker responses and organochlorine variables.

Finally, an important part of the programme was to develop a modelling framework for examining the possible effects of pollutants at the population level. An important step forward in this regard is the work to examine the potential effects of PCBs on reproduction (Hall *et al.*, 2005). Although preliminary and based on small sample size (n=47), for the first time an individual based model was constructed that simulated the accumulation of PCBs in the population and allowed modification of first year calf survival based on maternal blubber PCB levels. The initial results suggested that the current estimated PCB accumulation rate might be depressing the potential annual growth rate of the population by some 3.6% compared to a zero accumulation rate. This preliminary analysis also suggested that higher accumulation rates might produce a maximum depression of about 5% in potential growth rate. Median effective concentration, i.e. concentration at which 50% of the population is affected by PCBs, was estimated at about 10mg/kg (lw). There is clearly considerable uncertainty in the results at present but the work has shown the potential for this approach to look at population level effects. Continuation of this work is an important consideration for Phase II.

In conclusion, with respect to the investigated biomarkers, although their potential value remains a full evaluation of their utility is confounded by the effect of biological variables. The detected patterns of variation require considerable stratification of data relative to life history parameters. The resultant small numbers of samples in any given age/sex/reproductive class/nutritive condition clearly decreases the resolution ability and power of statistical tests. Given the lack of conclusive evidence in support of any potential biomarkers from the samples obtained through capture and release, the across-site comparisons turned out to not be a feasible component of the project.

Having said that, the bottlenose dolphin sub-project has produced extremely valuable baseline results, and in particular has confirmed a number of hypotheses with respect to the influence of life history and nutritive state on PCB levels and the need to account for these when attempting to investigate cause-effect relationships. This groundwork provides a solid framework for future research (either within or outside the POLLUTION 2000+ programme) and highlights the need for (a) larger sample sizes to be obtained; (b) the value of incorporating pollutant studies with long-term capture-release projects; (c) the value of developing a suitable modelling framework for consideration of results at the population level; and (d) the need to consider further the most appropriate way to obtain biopsy samples.

As had been warned from the outset, it was not possible to obtain enough samples to adequately investigate potential biomarkers for POLLUTION 2000+ through IWC support alone. In fact, IWC funding was insufficient to obtain all of the samples analysed for the project; half of the support for sample collection from Sarasota Bay bottlenose dolphins had to be obtained from other sources, including the National Marine Fisheries Service. Continuing research in Sarasota Bay and elsewhere in the USA with support from private and governmental sources has resulted in four years of additional samples (2002-2005), which are currently undergoing analyses (Schwacke *et al.*, 2004). The larger sample sizes provided by combining the POLLUTION 2000+ samples with those from subsequent research should provide opportunities for more refined and complete analyses, perhaps as part of Phase II.

2.5 Key reports and publications (a full list is given in Adjunct 4)

Wells, R.S., V. Tornero, A. Borrell, A. Aguilar, T.K. Rowles, H.L. Rhinehart, S. Hofmann, W.M. Jarman, A.A. Hohn, and J.C. Sweeney. 2005. Integrating life history and reproductive success data to examine potential relationships with organochlorine compounds for bottlenose dolphins (*Tursiops truncatus*) in Sarasota Bay, Florida (Originally SC/56/E19). *Science of the Total Environment* 349:106-119.

Research initiated in 1970 has identified a long-term, year-round resident community of about 150 bottlenose dolphins (*Tursiops truncatus*) in Sarasota Bay, Florida, providing unparalleled opportunities to investigate relationships between organochlorine contaminant residues and life history, health and reproductive parameters. Many individual dolphins are identifiable, and of known age, sex, and maternal lineage (< 5 generations). Observational monitoring provides data on dolphin spatial and temporal occurrence, births and fates of calves, and birth order. Capture-release operations conducted for veterinary examinations provide biological data and

samples for life history and genetic analyses, health assessment, and contaminant residue measurement. Organochlorine concentrations in blubber, milk, and blood (plasma) can be examined relative to age, sex, body condition, lipid content, birth order, and health parameters. Reproductive success is evaluated through tracking of individual female lifetime calving success.

For the current study, 47 blubber samples collected during 2000 and 2001 for the IWC POLLUTION 2000+ Programme were analyzed for PCB concentrations relative to life history factors and reproductive success. Prior to sexual maturity, males and females exhibited similar concentrations. Classical patterns of accumulation with age were identified in males, but not in females. Subsequently, males accumulated higher concentrations of PCBs through their lives, whereas females begin to depurate with their first calf, reaching a balance between contaminant intake and lactational loss. In primiparous females, PCB concentrations in blubber and plasma and the rates of first-born calf mortality were both high. While high PCB concentrations may decrease the fitness of calves, absence or early interruption of lactation may also hamper transfer of pollutants, making it difficult to clarify cause and effect. First-born calves had higher concentrations than subsequent calves of similar age. Concentrations in adult but young females were lower than in older females, reflecting a progressive decrease in PCB transfer rates to offspring as a consequence of an age-related increase in calving intervals. Maternal burdens were lower early in lactation, and increased as calves approached nutritional independence.

Empirical data were generally consistent with a published theoretical risk assessment, and supported the need for incorporation of threats from indirect anthropogenic impacts such as environmental pollutants into species management plans. Long-term observational monitoring and periodic biological sampling provide a powerful, non-lethal approach to understanding the correlations of organochlorine concentrations and health or reproductive parameters for coastal dolphins.

Tornero, V., A. Borrell, A. Aguilar, R.S. Wells, J. Forcada, T.K. Rowles, and P.J.H. Reijnders. 2005. Effect of organochlorine pollutants and individual biological traits on blubber retinoid concentrations in bottlenose dolphins (*Tursiops truncatus*). (Originally SC/56/E16). *Journal of Environmental Monitoring* 7(2):109-114.

This study investigates whether sex, age, lipid content and organochlorine concentrations induced variation on retinoid status and its deposition in the blubber of bottlenose dolphins in order to assess the utility of retinoids as biomarkers of contaminant exposure. Blubber samples were collected from 47 individuals of known age and gender from Sarasota Bay in June of 2000 and 2001. The sample included a representative cross-section of the resident dolphin community, with ages ranging from 2 to 50 years. Organochlorine levels showed the age- and sex-related variation commonly observed in other species, with concentrations increasing in youngsters of both sexes and in adult males, and decreasing in adult females after the onset of maturity. Blubber lipid content was found to be low in the overall population and to significantly decrease with age in adult males. Retinoid blubber concentrations were found to be at levels comparable to other odontocete species previously studied, and to be strongly determined by lipid content. Potentially as a consequence of the latter, retinoid concentration was observed to decrease with age in adult males. This effect could not be statistically dissociated from the also observed negative correlation between levels of organochlorines and retinoid blubber concentration. As a consequence, it could not be clarified whether in this population high organochlorine burdens lowered retinoid concentrations or, conversely, depleted lipid reserves were indeed responsible for both the observed high organochlorine concentrations and the low retinoid levels in the blubber. With current knowledge, both options should be considered and investigated.

Hall, A.H.; McConnell, B.J.; Rowles, T.K.; Aguilar, A.; Borrell, A.; Schwacke, L.; Reijnders, P. J. H. and Wells, R. S. 2006. An individual based model framework to assess population consequences of polychlorinated biphenyl exposure in bottlenose dolphins. *Environmental Health Perspectives* 114 (suppl. 114): 60-64.

Based on the papers above described, this study examines the effect of different polychlorinated biphenyl (PCB) accumulation scenarios on potential population growth rates in bottlenose dolphins from Sarasota Bay, Florida. To achieve this an individual based model framework that simulates the accumulation of PCBs in the population and modifies first year calf survival based on maternal blubber PCB levels is developed. Results suggest that the current estimated PCB accumulation rate for the Sarasota dolphin population might be depressing the potential annual growth rate by 3.6% compared to a zero accumulation rate. Higher accumulation rates would produce a maximum depression of about 5% in potential growth rate. Median effective concentration, this is, concentration at which 50% of the population is affected by PCBs, was estimated at about 10mg/Kg. However our predictions are limited by both model naivety and parameter uncertainty. We emphasise the need for more data collection on the relationship between maternal PCB levels and calf survivorship, the annual accumulation of PCBs by females and the transfer of PCBs to the calf through lactation. Such data require continued efforts directed towards long term studies of known individuals in wild and semi-wild populations.

Wilson, J.Y. Wells, R., Aguilar, A., Borrell, A., Tornero, V., Reijnders, P., Moore, M. and Stegeman. Cytochrome P450 1A1 (CYP1A1) expression in integument biopsies from a coastal bottlenose dolphin population related to blubber ΣPCB concentration. International Whaling Commission paper SC/58/E49.

Integument biopsy is a non-destructive way to collect samples from free-ranging cetacean populations that ideally can allow for the determination of both contaminant concentrations and biomarker responses in protected species such as marine mammals. Cytochrome P450 1A1 (CYP1A1) expression is induced by polycyclic aromatic hydrocarbons (PAHs) and planar halogenated aromatic hydrocarbons (PHAHs) such as the non- and mono-ortho PCBs, and has been used extensively as a biomarker of PCB exposure and effects in other species. Contaminant concentrations and CYP1A1 expression were measured in integument biopsies collected from bottlenose dolphins resident in Sarasota Bay, FL. This population of dolphins has been the subject of a long-term population and health assessment project that facilitates the rare evaluation of the influence of life-history parameters such as age and sex with CYP1A1 expression. CYP1A1 expression was seen in vascular endothelial cells, vascular smooth muscle, and nerve cells in the dermis but not in epithelial cells, connective tissue, or adipocytes; similar to what has been documented in other cetacean species. Regional variation of endothelial CYP1A1 expression in the dermis was seen in this study and indicates that lipid dynamics, lipid or fatty acid content, or nutritional status may be important factors for endothelial CYP1A1 expression in integument. Athough age and sex are important determinants for PCB concentrations, CYP1A1 expression appeared not to be strongly related to neither age or sex. Total PCBs, but not Σmono-ortho PCB or TEQ concentrations, were positively correlated with endothelial CYP1A1 expression. Thus, contaminant concentrations, but not life-history parameters, may be the stronger determinants of CYP1A1 expression in cetacean integument.

Hall, A.J., Stott, J., Blanchard, M., Rowles, T.K., Wells, R.S., Borrell, A., Aguilar, A. and Reijnders P.J.H. The relationship between immune measures and blubber organochlorine concentrations in bottlenose dolphins (*Tursiops truncatus*) from Sarasota Bay, Florida. International Whaling Commission paper SC/58/E39.

As part of the IWC Pollution 2000+ project (Reijnders, Aguilar & Donovan, 1999) to determine the impact of pollutants on cetacean populations, a study examining the effect of PCBs on immunity in free-living bottlenose dolphins (Tursiops truncatus) was implemented. In conjunction with the long term study of bottlenose dolphins in Sarasota Bay, Florida, tissues (blubber and blood) from 47 animals sampled in 2000 and 2001 were assessed for OC contaminant exposure and immune response. The relationship between these two variables was explored, taking into account factors such as age, sex and handling time.

For most measures of immune response considered, no significant correlations with PCB levels in blubber were found (immune response measured using in vitro leukocyte subpopulations, mitogen induced proliferation assays or interleukin 6 (IL6) levels). However, two leukocyte subpopulation ratios did show a relationship with blubber PCB concentrations: naïve to memory (N to M) cell ratios; and T to B cell ratios. The difference between the N to M cell ratios in the pre (immediately after capture) and post (after handling) samples from 2000 indicated a significant negative relationship with blubber PCBs. However, given the potential effects of sex and age it was impossible to discriminate whether this relationship was due directly to PCB exposure or, because all the highly exposed animals

were old males, due to age. With respect to T to B cell ratios, a significant relationship with the length of the animal was also found. Length might be more closely associated to immunity than age as the development of both will reach a plateau in adulthood. The ratio of T to B cells was higher in the older animals, which again were also the individuals with the higher PCBs. Thus, the relatively small sample size and the significant relationships between age and immunity, and between age and PCBs, prevented reaching definitive conclusions about the impact of PCBs on immune function in this species.

Hall, A.J., Wells, R.S., Aguilar, A., Borrell, A., Rowles, T., Stott, J., Wilson, J.Y., O'Hara, T., Siebert, U., Bjørge A., Tornero, V., and Reijnders, P. 2004. Biomarkers of contaminant exposure and relationships with blubber contaminant levels in bottlenose dolphins *Tursiops truncatus*. International Whaling Commission paper SC/56/E15.

As part of the Pollution 2000+ project this study investigates the potential of using a *combination* of a wide range of biomarkers for exposure/response assessment to organochlorine (OC) contaminants in bottlenose dolphins (*Tursiops truncatus*) from Sarasota Bay, Florida. This analysis endeavours to *integrate the biomarkers* and investigate their relationship with exposure, as assessed by blubber OC concentrations. A considerable number of individual OC contaminants were analysed for in the blubber samples (28) and the individual biomarkers (including the effect of covariates of exposure such as sex, age and nutritive condition) comprised a further 14 variables. Such a large datas*et al*lowed us to investigate any relationships between the individual parameters using principal component analysis (PCA) for data reduction followed by least squares linear and polynomial models for inference. In this overall analysis we were addressing the following questions:

(1) Is there a significant relationship between the set of independent biomarker variables (immune assays, retinol (vitamin A), reproductive hormones (oestradiol and progesterone) and dermal biotransformation enzyme levels (as measured by specific antibody binding assay) and the blubber contaminant levels, accounting for the effects of sex, age and nutritive condition?
(2) How predictive is this relationship?
(3) Can we combine a set or subset of these measures to determine exposure and response effects in bottlenose dolphins?

A very high proportion (c.80%) of the variance in OC contaminants in the blubber was explained by sex, age (and blubber lipid since the OC data were lipid normalised). Adult males have significantly higher concentrations of total PCBs (and therefore of all OC contaminants because of the high degree of correlation between them) than adult females, although there is no difference between the sexes when the animals are immature. All the blubber OC contaminant concentrations were positively linearly correlated (correlation coefficients ranged from 0.7 to 0.95) indicating that data reduction methods could be used to capture a very high proportion of the variance. The first two PCA components captured almost 95% of the variance.

There was no correlation between the different biomarker responses (immune assays, retinol (vitamin A), reproductive hormones (oestradiol and progesterone) and dermal biotransformation enzyme levels (as measured by specific antibody binding assay), except between the female reproductive hormones. A PCA could not reduce the variance in the data sufficiently for an integrated factor to be used in any further analysis. A multivariate analysis of variance model was also tested, with the independent variables reduced to categories and multiple dependent variables but there were an insufficient number of observations (n=47) to produce estimable effects. It was therefore not possible to integrate these biomarkers using these methods and therefore relationships between combined biomarkers responses and OC variables were not explored further.

Schwacke, L., Hall, A., Wells, R., Bossart, G., Hohn, A., Fair, P., Kucklick, J., Rosel, P., and Rowles, T. 2004. A 5-year plan for health and risk assessment for bottlenose dolphin populations along the southeast U.S. coast. International Whaling Commission paper SC/56/E20.

Several independent bottlenose dolphin health assessment projects, some of them within the framework of the Pollution 2000+ project, have been conducted over the past decade or more along the southeast U.S. coast. Individually, these projects have contributed a wealth of information on disease prevalence and health parameters, as well as on contaminant concentrations in dolphin tissues and their relationship with age, gender and reproductive status. Collectively, the data obtained from the various projects could be of even greater consequence, providing the basis for documenting broad-scale trends in health status and chemical and biological toxicant exposure, and for correlating such exposures with deleterious health effects. Combining information from the multiple studies increases effective sample sizes and broadens gradients of exposure levels, thereby increasing statistical power for testing hypothesized correlations between environmental variables and health status. Additionally, it will allow for more generalisable inferences about reference ranges for wild dolphin populations for the suite of measured health parameters. In this paper, results that have been obtained to date are summarized and plans for standardization of protocols, epidemiological analyses and expansion of health assessment sites are presented. Our ultimate goal is to provide the knowledge and information required for risk assessment efforts, which will identify potential hazards and eventually quantify the likely impacts of anthropogenic actions and environmental factors for bottlenose dolphin populations along the U.S. coast.

3. HARBOUR PORPOISE SUBPROJECT

3.1 Introduction

Changes in levels of contaminants and indicators of exposure to and/or effect of contaminants are known to occur after death due to the inevitable changes in physiological status and breakdown of tissue. Bycatches in fishing operations are a potentially important source of abundant, high-quality specimens for pollution studies. However, because in most cases sampling can only be conducted some (usually unknown) hours after death, it is essential to quantify any *post mortem* related changes to be able to correctly interpret levels of variables (pollutant levels or biomarkers) with respect to levels in the living animal. Such fundamental research has never been carried out. While such studies will allow us to calibrate the accuracy of such material for later studies under the POLLUTION 2000+ project, they have potentially important significance for other studies that have not taken such possible changes into account. Harbour porpoises are often subject to bycatches throughout the North Atlantic area (Donovan and Bjørge, 1995) and are one of the POLLUTION 2000+ project target cetaceans; it was therefore decided to conduct the post-mortem calibration study in that species (Aguilar *et al.*, 1999; Reijnders *et al.*, 1999a).

3.2 Sampling

It was recognised from the outset of the study that the collection of appropriate (i.e. very recently dead) and sufficient number of samples would be difficult outside direct harvests. After substantive discussion and extensive consultation with other experts in order to optimise sampling, even taking into account the high numbers of bycaught animals, it became apparent that the prospects for meeting the initial goal of 30 known time-to-death animals of various age/sex/reproductive classes was poor. Through the much-appreciated initiative of Andy Read (Duke University), a source of samples was found in the Bay of Fundy region. Harbour porpoises are [irregularly] bycaught in weir nets of fishermen from Grand Manan. The expectation was that it would be possible to sample perhaps as many as 10 animals of known and short times-to-death in that fishery.

A Field Protocol for conducting such sampling was developed and published (Reijnders *et al.*, 2002a) and field personnel were instructed on how to handle the specimens and collect and preserve the tissue samples.

Thanks to the efforts of Andy Read, Heather Koopman and Tara Cox, and financial support from Duke University and various sources, it was possible to retrieve six freshly dead bycaught porpoises from the Grand Manan fishery in the summer of 2001. A summary of the information for the porpoises caught is given in Table 2.

After the porpoises were retrieved from the nets, the experimental protocol was followed in which the animals were kept in water and sampled at pre-set intervals to mimic to the extent possible the likely situation in a true fishery. Details of the procedure are given in the protocol and the papers summarised below. The number of samples collected and the analyses performed are summarised in Table 3.

3.3 Analyses conducted

Samples were distributed for analyses to selected laboratories around the world, (see Adjunct 3), following agreed standard protocols.

Table 2

Summary of Bay of Fundy harbour porpoises sampled during summer 2001 for the post-mortem calibration study.

Code	Sex	Length (cm)	Estimated age-group/ reproductive status
GM-01-69	Male	139	Sexually mature
GM-01-84	Male	119	Sexually immature
GM-01-85	Male	126	Sexually immature
GM-01-184	Male	109	<1 year
GM-01-191	Male	129	Sexually immature
GM-01-199	Female	150	Pregnant

Table 3

Types, number and usage of samples collected from the Bay of Fundy harbour porpoises in summer 2001.

		No. of samples collected at each time point				
Sample	Analysis	Immediate	3 hr	9 hr	24 hr	48 hr
Blubber	PCB	5	6	5	6	6
	Immunohistochem	5	6	6	6	6
	Enzyme induction	5	6	6	6	6
	Luciferase	5	6	6	6	6
	Retinol	5	6	6	6	6
Blood	Misc. analyses*	5	0	0	0	0
Liver	Enzyme induction	1	0	0	0	6
	Porphyrins	1	0	0	0	6
	Retinol	1	0	0	0	6
Lymphoid organs	Histopathology	0	0	0	0	6

*Chemistry, thyroid hormones, PCB, porphyrin, retinol, luciferase.

During the analyses on enzyme induction, very low CYP1A1 expression was found. It was therefore decided by the Steering Group that it would not be worthwhile from either a scientific or economic perspective to continue with further EROD and western blot analyses. As a consequence, efforts concentrated on immunohistochemistry analyses.

Originally it was also planned to include measurements of porphyrins in urine and PCBs, retinol, and immune function in blood. However, due to the difficulty of obtaining urine samples and the small quantities of blood sampled, the Steering Group decided to prioritise and focus on the following analyses:

(a) Blubber contaminant levels (PCBs, DDT)
(b) Retinoids levels in blubber
(c) Cyp1A1 expression in liver and integument
(d) Histology on lymphoid organs
(e) Thyroid hormones in blood (serum)
(f) Luciferase in blubber
(g) Porphyrins in blood (plasma)

3.4 Summary of results

The primary objective of the Harbour Porpoise Subproject was to determine changes in concentrations of selected variables with post-mortem times.

The goal of the subproject of investigating post-mortem changes of biomarkers and organochlorine concentrations in harbour porpoises proved feasible, though not without difficulties (including of course, sample size). The original experimental design called for the collection of freshly dead bycaught harbour porpoises and the subsequent collection (at pre-set intervals from zero to 48 hours after death) of tissues and blood for later analyses on PCBs and a set of associated biomarkers. As noted above, the collection of fresh porpoise carcases was difficult. After examination, it was found that for almost all of the initially identified possible candidate sites, the collected animals were usually dead for an unknown period (in some case perhaps two days or more), rendering them unsuitable for the objectives of the experiment. Even after extensive and dedicated work, ultimately only six animals could be collected and subsequently sampled at different time intervals; even there it proved impossible to collect suitable samples for all of the targeted tissues.

Despite the limitations in sample size, some important (and hitherto uninvestigated) results were obtained. For example, with respect to post-mortem changes in pollutant levels (total PCBs and total DDT), the results of the subproject revealed that no time trends were observed (Tornero *et al.*, 2005b) in the sampled animals. This important finding shows that blubber can be regarded as a reliable tissue for the assessment of the examined organochlorines in specimens preserved in 'natural' (at least in the case of bycaught specimens given normal soak times) conditions of up to 48 hours. With respect to potential biomarkers of exposure and effect, the results on post-mortem stability of retinoids demonstrated that neither significant differences nor trends were detected in the concentration of retinoids over the study period of 48 hours, i.e there was no significant degradation in tissue retinoids (Tornero *et al.*, 2005b).

An additional major feature of this subproject was the discovery that that concentrations of retinoids in liver and in blubber were highly correlated. Since blubber can easily be sampled from live animals using biopsy techniques, it can thus be regarded as an appropriate and reliable tissue for the assessment of these variables in specimens preserved in natural conditions up to 48 hours (Tornero *et al.*, 2005b).

The performed luciferase analyses in blubber also yielded interesting results with practical implications (SC/58/E28). Analyses of luciferase (a biomarker for exposure to dioxine-like compounds (DLCs) such as dioxins, benzofurans, nonsubstituted and mono-ortho-substituted PCBs), revealed no significant changes over time in the estimated concentrations of DLCs over the 48 hour study period. Thus, as had been shown for tPCBs and tDDT, it can be concluded that blubber is a reliable tissue to assess luciferase (and hence DLCs) in unpreserved specimens kept under 'normal' conditions up to 48 hours post-mortem.

Studies on snap-frozen pre-scapular lymph nodes and formalin-fixed, paraffin-embedded lymphoid organs collected 48 hours *post mortem* showed that histological examination of the snap-frozen material was reliable except

for a detailed slide description, that was impaired by distinct autolytic changes and freezing artifacts of the samples. Conversely, histological examination of formalin-fixed, paraffin-embedded lymphoid organs revealed no significant findings and appeared not to be affected by autolysis. Serum samples of four individuals were also analysed for thyroid hormones (triiodothyronine (T3), thyroxin (T4) and free thyroxin (fT4)), and values obtained were considered to be within 'normality'. However, one serum sample coagulated and thus precluded analyses. It is unclear whether such coagulation reflected the blood quality at the sampling time or because of the long freezing time prior to analysis (SC/58/E43). Ultimately, however, given the provisos concerning sample size, it can be concluded that bycaught porpoises with soak times of up to 48 hours, are with some exceptions, generally reliable for assessment of lymphoid organs.

The results for other potential biomarkers were inconclusive. Opening the visceral cavity produced an unrealistically sudden fall in body internal temperature; given this the practice was discontinued after the first attempt. As a consequence, the liver tissue samples necessary for the study on stability of porphyrines could not be collected at appropriate time intervals. While some analyses were performed to check porphirine levels in the few tissues collected, and results showed that concentrations were above analytical detection limits, the prespecified post-mortem test could not be carried out.

The results of the study on the expression of cytochrome P450 1A1 (CYP1A1) using immunohistochemistry, enzymatic assays and western blots was also inconclusive although for other reasons. The EROD activity assay and the immunohistochemical analyses showed that CYP1A1 levels were very low both in the integument and in the liver of the harbour porpoises; only 3 out of 33 samples were above the detection limit. It appears, therefore, that from this perspective, harbour porpoises and white-sided dolphins might be relatively insensitive to the enzyme inducing effects of organochlorines such as PCBs. Indeed, harbour porpoises appeared to be less sensitive to organochlorine pollution or, at least, to require higher pollutant concentrations to induce CYP1A1 than other cetacean species. Whatever the reason for this insensitivity, the low levels of expression found made it impossible to determine effects of time of sampling on CYP1A1 levels (SC/58/E42). This aspect may be further investigated under Phase II of POLLUTION 2000+.

3.5 Key reports and publications (a full list is given in Adjunct 4)

Tornero, V., Borrell, A., Pubill, E., Koopman, H., Read, A., Reijnders, P.J.H. and Aguilar, A. 2005b. Post-mortem stability of blubber retinoids in by-caught harbour porpoises (*Phocoena phocoena*): implications for biomarker design studies (Originally SC/56/E4). *Journal of Cetacean Research and Management*, 7(2): 147-152.

The effect of post-mortem time (0-48 hours) on retinoid concentrations in the blubber and liver of harbour porpoises under natural conditions was investigated to assess the reliability of samples collected from animals after they died. Organochlorine compounds and lipid content were also determined to test whether they affected retinoid status. Organochlorine concentrations remained low throughout the post-mortem period and were considered unlikely to influence retinoid body dynamics. Retinoid concentrations in liver were 5-6 times higher than those in blubber and both were highly correlated. As opposed to liver, blubber can be easily sampled from live individuals using non-destructive biopsy techniques and is therefore considered an alternative tissue to assess retinoid status in marine mammals. Neither significant differences nor trends were detected in the concentration of retinoids over the studied period, indicating that degradation agents (ultraviolet rays, oxygen exposure and heat) did not affect tissue retinoids. Blubber can thus be regarded as a reliable tissue for the assessment of the retinoid status of unpreserved specimens kept up to 48 hours in conditions similar to those of the study.

Borrell, A., A. Aguilar, S. Zeljkovic, A. Brouwer, H. T. Besselink and P.J. H. Reijnders. 2006. Post-mortem stability of blubber DLCs, PCB and tDDT in by-caught harbour porpoises (*Phocoena phocoena*). International Whaling Commission paper SC/58/E28.

With the aim of assessing the reliability of samples collected from animals after they died, the effect of post-mortem time (0-48 hours) on dioxin-like compounds or DLCs (such as 2,3,7,8-substituted dioxins and benzofurans as well as nonsubstituted and mono-ortho-substituted PCBs), tPCB (total polychlorinated biphenyls) and tDDT (dichloro diphenyl trichloro etane and family) was investigated in the blubber of harbour porpoises that were left after death under natural conditions. Neither significant differences nor time trends were detected in the concentration of DLCs over the study period, indicating that degradation agents (ultraviolet rays, oxygen exposure and temperature) did not affect them. tDDT and PCBs were calculated in 2 timepoints and again no trends were observed. Blubber can thus be regarded as a reliable tissue for the assessment of the organochlorine compounds (DLCs, PCB, DDT and others) of unpreserved specimens kept up to 48 hours in conditions similar to those of the study.

Wilson, J.Y., E., Koopman, H., Read, A., Reijnders, P.J.H. 2006. CYP1A1 Expression in Bay of Fundy Harbour Porpoise liver and integument. International Whaling Commission paper SC/58/E42.

Samples of liver and integument (epidermis and underlying blubber) were collected from by-caught harbour porpoise up to 48 hours post-mortem. The expression of cytochrome P450 1A1 (CYP1A1) was determined using immunohistochemistry, enzymatic assays, and western blots to determine if time from death causes a significant decrease in the expression level within the tissues.

CYP1A1 levels, using EROD activity assay, proved to be very low in the integument. Only 3 out of 33 samples were above the detection limit. This low expression was supported by immunohistochemical analyses. No sample had any cell expressing CYP1A1 levels ≥4 (on a scale of 0-15), and more than 75% of the samples had none of the three cell types that stain for CYP1A1 in cetacean integument. Only bottlenose whales of those odontocetes studied in the NW-Atlantic, have lower CYP1A1 expression. Like integument, EROD activity assay showed that CYP1A1 expression was low in liver of these animals. Immunohistochemical data in liver of these animals also showed low levels of CYP1A1 expression. The low levels of CYP1A1 expression made it impossible to determine if there was any effect of time of sampling on the CYP1A1 levels measured.

Low expression of CYP1A1, particularly in liver, could indicate that either the animals are unexposed to contaminants or that harbour porpoises are relatively insensitive to OC compounds. Given the fact that levels of PCBs are similar in white-sided dolphins which show higher expression of CYP1A1, and that in pilot whales with PCB levels 4-5 times lower show much higher CYP1A1 than in harbour porpoises in this study, it is concluded that porpoises and white-sided dolphins are relatively insensitive to the enzyme inducing effects of OCs such as PCBs. At least they appear to require higher concentrations to induce CYP1A1 than other cetacean species.

Siebert, U., Beineke, A., Koopman, H., Read, A., Reijnders, P.J.H. 2006. Note on investigations on lymphoid organs and thyroid hormone levels in serum of harbour porpoises from the Bay of Fundy. International Whaling Commission paper SC/58/E43.

Lymphoid organs

Six snap-frozen pre-scapular lymph nodes from different animal and 9 formalin-fixed, paraffin-embedded lymphoid organs were submitted for investigations. All samples were collected 48 h after death of the animals. As far as interpretable, histological examination of the snap-frozen material revealed no significant findings in the submitted lymph nodes. However, a detailed slide description was impaired by distinct autolytic changes and freezing arctifacts of the samples.

Histological examination of formalin-fixed, paraffin-embedded lymphoid organs revealed no significant findings or only mild to moderate lymphoid hyperplasia of most investigated lymphoid organs. Lymphoid hyperplasia most likely represents the result of an immunologic reaction after antigenic stimulation and can be interpreted as a physiologic host defence mechanism. The reason fort he mild thymic depletion (GM-01-13) remains unknown. In general, thymic depletion can be the result of physiologic thymic involution in adult individuals. However, thymic atrophy can also be associated with different diseases or debilitating conditions. Therefore, the animals' age and health status should be considered in the interpretation of this finding. Focal hemorrhages in the lymph node of animal Gm-01-69 can possibly be due to a traumatic impact. Histological examination was not affected by significant autolytic changes contrary to the snap-frozen material.

Thyroid hormones

Serum samples of five harbour porpoises were submitted for analyses of thyroid hormones. One serum sample (GM-01-69) was coagulated so that thyroid hormones could not be measured. Thyroid hormones measured were triiodothyronine (T3), thyroxin (T4) and free thyroxin (fT4). For one animal (GM-01-184) there was not enough serum to analyse all thyroid hormones. Values for T3 were varying between 120.58 and 199.99 ng/dl, T4 between 8.00 and 12.40 µg/dl and fT4 between 19.8 and 28.8 ng/dl. It remains unclear if one serum sample coagulated because of the blood quality at the sampling time or because of the long freezing time.

4. ONGOING RESEARCH

As discussed above, the results of the research on retinoids, both within the POLLUTION 2000+ project and elsewhere, have shown that:

(a) this biomarker may be of utility in gauging cause-effect relationships of organochlorine pollutants in cetaceans (Borrell *et al.*, 1999; Borrell *et al.*, 2002; Tornero *et al.*, 2005a; Tornero *et al.*, 2004); and
(b) that it can be reliably assessed in a reasonably fresh by-caught cetacean (Tornero *et al.*, 2005b).

Further steps required in the calibration of this biomarker include the determination as to whether it remains stable in long-term preserved tissues and a further understanding of possible cause-effect relationships.

In several countries, harbour porpoise samples from different stranding networks are stored. However, for one stranding network – the UK strandings network – a large (n>400) and extremely well documented sample harbour porpoise tissue collection exists. To take advantage of this within the frame of the POLLUTION 2000+ project, a research initiative was developed aiming to:

(a) Examine the effect of long-term freezing on retinoid stability.
(b) Examine the effect of PCB exposure on blubber retinoid concentrations.

With respect to the former, a set of blubber tissue samples (n=50) from harbour porpoises of same sex and similar age and PCB concentrations, but that had been preserved for varying time periods, is to be analysed for retinoids. Since, in this way the effect of gender, age and PCB levels is standardised, it is expected that preservation-related effects can be identified.

With respect to the latter, a previous study on UK stranded harbour porpoises (Jepson *et al.*, 1999; Jepson *et al.*, 2005) showed that individuals that died because of an infectious disease had significantly higher PCB levels than those that died due to physical trauma but that otherwise were considered to be healthy. After taking into account confounding variables, this result was taken as a potential indication of the immunosupressive effect of PCBs. Retinoids are known to mediate in the impact of organochlorines on the immune system (Borrell *et al.*, 1999), so the Jepson *et al*. (1999; Jepson *et al.*, 2005) studies clearly offer optimal grounds for investigating the cause-effect relationships sought by the POLLUTION 2000+ project. In this context, two sample sets (each n=80) comprising harbour porpoises whose cause of death has been trauma and disease, respectively, will be analysed in search of differences in retinoid status. To avoid gender or age-related effects, the individuals object of the research will be standardised as much as possible in relation to these variables.

Given that this study was not initiated until after the initial steps of the POLLUTION 2000+ project had been completed, it is currently ongoing.

5. CONTRIBUTION TO THE WORK OF THE COMMISSION AND OTHERS

This programme has been undertaken by the IWC Scientific Committee on request of the Commission to:

'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' (IWC, 1994).

The POLLUTION 2000+ programme is part of the Commission's consideration of the broad issue of the effects of environmental change on cetaceans, including global warming, ozone depletion, pollution (chemical and noise), direct and indirect effects of fisheries, coastal development and tourism. Given the broad nature of the subject, it was agreed to focus initially on chemical pollution and climate change. The POLLUTION 2000+ project was developed after considerable effort including the holding of a major international workshop and several subsequent smaller workshops. Throughout the project, the Commission was informed that such work was considerably more expensive than work it usually funded. Whilst the Commission did provide more funds than usual, it must be said that funding issues did slow down progress considerably, despite the considerable in-kind funding obtained (*ca* £200,000) and the dedication of many researchers.

There are a number of important products of the programme. One obvious example includes the publication of the book *Chemical Pollutants and Cetaceans* (Reijnders *et al.*, 1999b). In addition, there is series of published/submitted peer reviewed publications in scientific journals and presentations at international workshops and conferences outside the IWC. The design of the programme

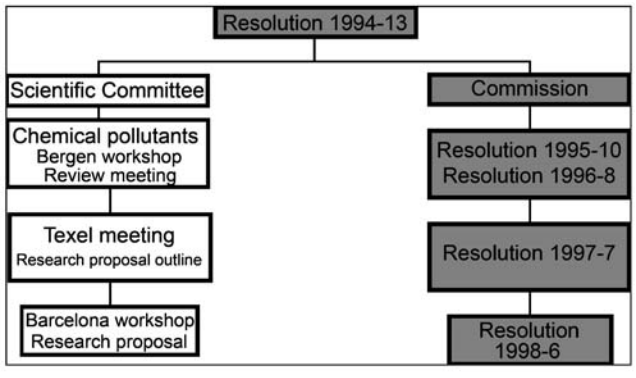

Fig. 1. The Scientific Committee and Commission paths to POLLUTION 2000+ Phase I.

itself, and the associated Field Protocol (Reijnders *et al.*, 2002a) have themselves provided a valuable scientific framework for these types of ecotoxicological studies and have become widely used as a standard within the scientific community, with endorsement by a number of international bodies including ASCOBANS, ACCOBAMS and ICES.

Despite funding and logistical problems, the scientific output has certainly contributed towards the Commission's request to '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'.

6. PERSPECTIVES FOR CONTINUATION INTO PHASE II

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 odontocetes 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 that this applies to other species 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 (Aguilar and Borrell, 1994; Jepson *et al.*, 1999; Jepson *et al.*, 2005) and SC/58/E42. 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, 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.

We thus **recommend** that, before any decisions are taken with respect to a possible Phase II, an interdisciplinary specialist workshop, similar to the Barcelona workshop, is held to develop a full proposal including the specification of short-term objectives, case-study populations and procedures for the programme continuation.

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Adjunct 1

Summary information on PCB concentrations in bottlenose dolphin blubber from three study sites: Bahama Islands, Charlotte Harbor (Florida), and Sarasota Bay (Florida)

	Total PCBs (ppm, lipid)	
	Mean	*n*
Males		
Bahama Islands	9.5	7
Charlotte Harbor	10.1	9
Sarasota Bay: all males	102.0	22
Sarasota Bay: males >5 years old	142.4	14
Females		
Bahama Islands	5.1	4
Charlotte Harbor	7.6	2
Sarasota Bay: all females	13.5	25
Sarasota Bay: females >5 years old	7.8	18

Adjunct 2a

Details of 47 sampled bottlenose dolphins from Sarasota Bay, Florida, 2000-2001

Sample year	Dolphin	Sex	Age (years)	tPCB - mg/Kg (lipid)
2000	123	Female	2	26.50
2000	125	Female	2	46.13
2001	133	Female	2	14.67
2000	109	Female	5	39.22
2000	117	Female	5	29.55
2000	127	Female	5	24.37
2001	113	Female	5	16.20
2000	119	Female	9	8.67
2000	101	Female	10	5.94
2000	155	Female	10	23.13
2001	175	Female	10	24.85
2001	3	Female	12	4.03
2001	131	Female	13	2.60
2000	11	Female	16	6.78
2001	9	Female	17	1.86
2000	33	Female	18	3.33
2000	25	Female	22	5.99
2001	163	Female	30	2.22
2001	90	Female	31	2.82
2000	43	Female	34	12.90
2001	5	Female	38	9.99
2000	13	Female	41	4.64
2001	84	Female	43	12.72
2001	63	Female	47	3.87
2000	15	Female	50	3.63
2000	196	Male	2	38.73
2001	212	Male	2	27.81
2001	214	Male	2	19.65
2001	216	Male	3	32.14
2000	146	Male	4	52.33
2000	148	Male	4	13.22
2000	198	Male	4	21.30
2000	178	Male	5	44.38
2001	118	Male	9	32.44
2001	138	Male	9	37.49
2001	92	Male	13	49.28
2001	182	Male	14	46.30
2001	6	Male	17	45.42
2000	32	Male	20	43.45
2001	106	Male	20	151.44
2001	66	Male	25	139.98
2000	14	Male	27	64.33
2000	28	Male	35	84.76
2000	174	Male	36	116.21
2000	58	Male	36	215.45
2000	48	Male	41	98.93
2000	26	Male	43	868.41

Adjunct 2b

Summary of samples collected from Sarasota Bay bottlenose dolphins 2000-2001, and corresponding planned analyses

Tissue	Analysis	Total	Females	Males
Blubber	PCB concentration	47	25	22
	Immunohistochemistry	47	25	22
	Enzyme induction: activity	21	11	10
	Luciferase	16	9	7
Blood	Chemistry, hematology	47	25	22
	Reproductive hormones	47	25	22
	Thyroid hormones	47	25	22
	Immune response	47	25	22
	Total PCBs	21	11	10
	Coplanar PCBs	21	11	10
	Porphyrins	47	25	22
	Luciferase	47	25	22
	Vitamin A	47	25	22
Urine	Porphyrins	38	16	22

Adjunct 3

List of laboratories involved in the analyses for POLLUTION 2000+

Analysis	Laboratory involved	Principal investigator
PCBs	University of Barcelona	Alex Aguilar
Enzyme induction	Woods Hole Oceanographic Institution	Joanna Wilson
Immunohistochemistry	Woods Hole Oceanographic Institution	Joanna Wilson
Porphyrins	University of Siena	Christina Fossi
Luciferase	BioDetection Systems, Amsterdam	Bram Brouwer
Thyroid hormones	University of Kiel (FTZ)	Ursula Siebert
Histopathology	University of Kiel (FTZ)	Ursula Siebert
Immunology	University of California (Davis)	Jeff Stott
Retinoids	University of Barcelona	Assumpcio Borrell

Adjunct 4

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