Organochlorine contaminants in cetaceans: how to facilitate interpretation and avoid errors when comparing datasets

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ABSTRACT

This paper reviews current scientific literature to provide information for avoiding errors commonly made in comparing and interpreting datasets from laboratories measuring organochlorine contaminants in cetaceans. Before making comparisons and interpretations using heterogeneous datasets (e.g. those from different laboratories or those from different methods in the same laboratory), it is essential to consider specific information about the animals sampled (e.g. age, sex, reproductive status, body condition and health status), sampling procedures (e.g. necropsy of subsistence, stranded or bycaught individuals; remote or surgical biopsy), methods for measuring and conventions for expressing analytical results for lipids and contaminants (e.g. percent lipid, percent dry weight, contaminant concentration units, totals of contaminant groups such as PCB congeners) and quality assurance performance. Reformatting should be carried out, as necessary, to unify the datasets (e.g. into like units and weight basis) and allow a critical evaluation of the data to be made. As part of the data interpretation, caveats or limits in the comparability of the datasets (based on quality assurance results) should be provided. In addition, the biological relevance of the data must be considered in interpreting the datasets.

KEYWORDS: POLLUTANTS; POLLUTANT BURDEN; ORGANOCHLORINES; BIOPSY SAMPLING

INTRODUCTION

Levels of toxic contaminants in marine mammals at or near the top of the marine food web, as well as in their prey, are needed to provide important information on how contaminants are transferred between trophic levels and how contaminants affect biota at all trophic levels (AMAP, 1998; Reijnders *et al.*, 1999). Some marine mammals may be at risk from contaminants (even when environmental concentrations are relatively low) because of their biology, physiology or ecology. For example, the transfer of PCBs and DDTs from the female to their offspring is widely observed in marine mammals (Muir *et al.*, 1992; Aguilar and Borrell, 1994b; Norstrom and Muir, 1994; Lee *et al.*, 1996; Krahn *et al.*, 1997; Beckmen *et al.*, 1999). This transfer of a significant portion of the organochlorine (OC) contaminant burden from a female to her offspring, particularly during sensitive portions of the foetal and neonatal life cycle phases, could result in serious health problems. The offspring of primiparous females whose body burdens of contaminants are particularly high are especially vulnerable (Aguilar and Borrell, 1994b; Beckmen *et al.*, 1999; Ylitalo *et al.*, 2001). High concentrations of OCs have been associated with reproductive impairment, immunosuppression, alteration in bone development and growth, and increased susceptibility to disease (Brown, 1986; Reijnders, 1986; Olsson *et al.*, 1994; De Guise *et al.*, 1996; 1997; Kamrin and Ringer, 1996; Ross *et al.*, 1996; Zakharov *et al.*, 1997; Beckmen *et al.*, 1999), but direct cause-effect linkages remain unproven.

The assessment of differences and trends among populations and species, geographical regions and time periods is a central issue in evaluating the impact of OCs on marine mammals and is often required for determining the status of particular populations or their environment. This evaluation usually requires combining data from a variety of studies (both intra- and inter-laboratory) involving heterogeneous sample sets and dissimilar methods of analysis and interpretation. There are a number of confounding factors: sampling methods (e.g. necropsy of stranded or harvested individuals compared to biopsy techniques); analytical methods (e.g. use of different analytical methods or absence of quality assurance procedures); and biological factors (e.g. age, sex, reproductive status, nutritive condition) that can profoundly affect the ability to make a meaningful comparison. This review combines biological perspectives with the necessary knowledge of environmental chemistry to allow scientists to more readily and accurately compare data among studies.

CETACEAN SAMPLING METHODS

Necropsy sampling

Necropsy sampling is commonly used to process the carcasses of cetaceans harvested by subsistence hunters or for research, taken as 'bycatch' or found stranded. The systematic examination and collection of tissue samples during necropsy will not be described here, but details of the proper conduct of a necropsy can be found in papers such as Rowles *et al.* (2001). Record keeping is exceptionally important during necropsy and each sample should be identified with a unique sample number, animal identification number, a species code, a site code and the date. In addition, parameters such as length, weight and sex of the animal are recorded. Appropriate tissues should also be collected for ageing each animal (e.g. teeth of odontocetes or earplugs of mysticetes).

Thought should be given when sampling to the requirements (e.g. quality or freshness of the sample) for the analyses to be conducted. When samples originate from stranded or bycaught animals, the animals are most probably necropsied at unknown post-mortem times and this is likely to have an effect on contaminant and lipid levels in tissues (Aguilar *et al.*, 1999; Krahn *et al.*, 2001), as well as on

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physical and biochemical indicators of exposure (e.g. hormones, immune parameters, enzyme induction). For example, if the carcass is exposed to sun or wind, lipid can be lost or decomposed and volatile contaminants can evaporate (Borrell and Aguilar, 1990; Krahn *et al.*, 2001). It is therefore recommended, whenever possible, to sample and analyse only reasonably fresh specimens. Investigation of the effects of post mortem times on measured pollutant levels is an important component of phase I of the POLLUTION 2000+ programme.

The age, and sometimes the sex composition, of stranded cetaceans can reflect a pattern of the specific mortality rate rather than the actual age and sex structure of the population. This occurs because neonates and senescent animals are more common among stranded animals than are juveniles and young adults. Moreover, the sex ratio or age-class composition of strandings can be affected by a number of factors, such as the occurrence of sex-specific disease or the geographical segregation of sexes or certain age groups (Sigurjónsson and Rorvik, 1983; Reijnders, 2003). A bias of this type may also occur in bycaught animals. For example, the data sets of dolphins killed by net entanglements usually show a severely skewed distribution, often with a higher representation of males and young individuals (Gearin *et al.*, 1994; Kinze, 1994; Silvani *et al.*, 1999). Comparisons between datasets should, whenever possible, ensure that the age and sex composition of the sub-samples are homogeneous. If this is not possible, comparisons should only be made between comparable groups (i.e. newborns, juveniles, adult males, adult females) or should be statistically adjusted. Since OC concentrations increase with age in males, the range of variability in this gender may be as high as six-fold (Aguilar *et al.*, 1999). If age of specimens cannot be determined, comparisons between female sub-samples are less likely to be affected by the heterogeneity in age structure.

Biopsy sampling

Biopsies are 'non-destructive' techniques for obtaining tissue samples from live, 'free-ranging' cetaceans (Fossi *et al.*, 1997). Biopsy samples are small sections (0.2-4g) of epidermis and underlying blubber. Samples can be obtained from smaller cetaceans (captured and released) using a biopsy punch or a surgical procedure. In addition, remote sampling of larger cetaceans can be conducted using a modified gun or crossbow to shoot a biopsy dart (5-9mm diameter and 20-100mm long) into the side of a free-ranging animal (Aguilar and Borrell, 1994a; Fossi *et al.*, 1997; Weller *et al.*, 1997). The sex of the animal sampled can be determined in the laboratory by PCR amplification of sex-specific DNA regions of the sex chromosomes (Bérubé and Palsbøll, 1996).

Biopsy sampling is often the primary means of obtaining samples from cetacean species that rarely strand, are not usually harvested or have been designated a protected species. In addition, these samples are easier to obtain on a sustained basis, provide better quality samples than those from stranded animals (because tissues, as well as certain contaminants and physiological parameters, degrade following death) and can be considered a reasonable representation of OC concentrations in the specimen sampled (Aguilar and Borrell, 1994a; Aguilar *et al.*, 1999). Unfortunately, there are limitations to this technique, particularly when biopsy darts are used. For example, most currently used darts are only able to extract epidermis and the outer layer of blubber from large whales. Researchers have demonstrated that the lipid content and composition differ between the outer and inner blubber layers in some species (Sigurjónsson and Rorvik, 1983; Aguilar and Borrell, 1991; Koopman *et al.*, 1996), so biopsy samples may not provide results identical to those of 'full-thickness' blubber samples in these species (see the discussion in the section 'Blubber stratification' below). A biopsy tip that avoids this limitation by sampling the whole blubber thickness in large whales has been designed (Lambertsen *et al.*, 1994), but has so far been used on very limited occasions because of its invasive nature.

The age of a cetacean cannot be determined from a biopsy sample alone and this information is important, because lipophilic contaminants can be accumulated throughout the entire life span of marine mammals. The sex and reproductive state of a cetacean are also significant, because females can transfer contaminants to offspring through gestation and lactation and therefore a stabilisation or a decrease in contaminant concentrations takes place (Aguilar *et al.*, 1999). Some techniques have been developed to determine sex from muscle and potentially other tissues, although they have not yet been widely applied to field studies (Yoshioka *et al.*, 1994). If, however, biopsy sampling is combined with photo-identification techniques and long-term tracking of animals (Smith *et al.*, 1999; Ylitalo *et al.*, 2001), both the sex and reproductive status of individuals can often be determined. Having this information greatly improves the ability to interpret data obtained from biopsy samples.

ANALYTICAL METHODS FOR DETERMINING CONTAMINANTS

Detailed methods

Detailed methods are employed to determine tissue concentrations of numerous individual OC contaminants (i.e. PCB congeners and pesticides) using a single analytical procedure. The multi-step procedure generally includes extraction, clean-up and instrumental determination of analytes. In the extraction step, each sample is weighed and then a solvent (e.g. dichloromethane or hexane) and a drying agent (e.g. sodium sulphate) are added and the mixture is 'homogenised', i.e. cut into very small pieces through use of an apparatus such as a 'Tissumiser' (Krahn *et al.*, 1988). Alternative extraction methods use a Soxhlet extractor (Schantz *et al.*, 1996) or an automated accelerated solvent extractor (Weichbrodt *et al.*, 2000) to extract the contaminants and lipid from tissue.

Since some endogenous components (e.g. lipid) are extracted along with the contaminants, a two-step clean-up process is generally used, because a single step is usually insufficient to remove all the interfering compounds. One step in sample clean-up is generally elution of the extract through a gravity-flow column of silica/alumina (Krahn *et al.*, 1988) or florisil (Muir *et al.*, 1988; 1990; Newman *et al.*, 1994; Stern *et al.*, 1994) to remove some of the endogenous materials. Size-exclusion chromatography (SEC) is often used as another step in removing co-extracted materials (Krahn *et al.*, 1988; Muir *et al.*, 1988; 1990; Stern *et al.*, 1994; Schantz *et al.*, 1996) and can be carried out before or after the gravity-flow column step. An alternative one-step clean-up method consists of treating the extract with an acid (e.g. sulfuric acid) which degrades the cellular material (Murphy, 1972; Smith *et al.*, 1990). This method is fast and effective, but has definite limitations. In particular, the acid degradation also oxidizes certain pesticides (e.g. aldrin,

dieldrin, heptaclor, heptaclor epoxide) and these contaminants cannot be determined because their degradation products remain on the clean-up column. This method is of most use when the target compounds are acid resistant (e.g. PCBs, DDTs, hexachlorocyclohexanes (HCHs)).

Finally, gas chromatography (GC), an analytical technique that separates individual contaminants from each other, is used for resolving the contaminants (Wells *et al.*, 1993). In GC, compounds pass through the column (i.e. elute) at different rates. This allows the identification of various contaminants by their 'retention time' (time between injection and detection). Usually, the GC is equipped with either an electron capture detector (Krahn *et al.*, 1988; Muir *et al.*, 1988; 1990; Newman *et al.*, 1994; Stern *et al.*, 1994; Schantz *et al.*, 1996) or mass spectrometer (MS) detector (Reddy *et al.*, 1998) in order to measure the retention times and quantities of the compounds. In addition, if a MS detector is used, each compound is fragmented and these fragments form a pattern ('mass spectrum') that can be matched with an electronic library of spectra in order to identify the compound.

Detailed methods are advantageous because concentrations are provided for numerous individual OC contaminants and can be used with various statistical techniques (e.g. principal component analysis) to recognise variations in 'patterns' or 'fingerprints' of OCs in cetaceans from different stocks or geographical regions (Muir *et al.*, 1996; Krahn *et al.*, 1999). In addition, measuring concentrations of as many individual PCB congeners as feasible can increase the accuracy of 'total PCBs' (see 'Summed PCBs' below).

Rapid (screening) methods

A major challenge confronting environmental scientists is developing accurate and cost-effective analyses for measuring persistent contaminants in marine biota (Krahn *et al.*, 1994). Often, analytical time and costs can be greatly reduced by first employing rapid 'screening' methods to estimate contaminant concentrations (Krahn *et al.*, 1993; 1994). Based on the results of the screening analyses, a subset of samples can then be selected for detailed analyses (e.g. GC/MS) that can confirm the concentrations and identities of the contaminants. Screening methods often use high-performance liquid chromatography (HPLC) with ultraviolet photodiode array (PDA) (Krahn *et al.*, 1994; Ylitalo *et al.*, 2001) or fluorescence detection (Krahn *et al.*, 1993).

Rapid methods generally have fewer steps in the analytical procedure. Tissues are extracted using any of the methods described in 'Detailed methods' above. Next, a single clean-up step is used to remove endogenous materials and lipid, providing considerable savings in time and materials. For example, an extract can be chromatographed on a gravity-flow clean-up column, consisting of acidic, basic and neutral silica gel, to separate the analytes from interfering compounds in the tissue (Krahn *et al.*, 1994; Ylitalo *et al.*, 2001). Finally, the OCs are chromatographed on an HPLC column that separates the analytes according to their planarity and chlorination level and a UV photodiode array detector is used to measure the retention times, quantities and spectra of the compounds (Krahn *et al.*, 1994; Ylitalo *et al.*, 2001).

Although rapid methods are more cost-effective than detailed methods, they are generally limited to measuring fewer analytes than can be determined by GC/MS (Krahn *et al.*, 1994; Ylitalo *et al.*, 2001). However, this limitation can be overcome by reanalysing the extract prepared for the rapid method using GC/MS to measure additional analytes (Herman, pers. comm.). This GC/MS procedure does add some time and expense, but it still is cost-effective. The greatest drawback of rapid procedures that measure OCs is that certain pesticides (e.g. dieldrin and heptaclor epoxide) are degraded during the clean-up step.

Comparing results when analytical methods differ

There are procedures available to ensure that comparable data are obtained from two or more laboratories that use substantially different methods (or indeed similar methods). The first is to have each laboratory analyse splits of the same samples and then compare the results. Often, this approach is not reasonable (e.g. split samples may not be homogeneous) or possible (e.g. insufficient sample available). Furthermore, such analyses provide information on whether results are comparable among laboratories, but not on their accuracy. Ideally, each laboratory providing datasets should also provide related quality assurance information (see the section on 'Quality assurance procedures'). Then, quality assurance results (e.g. performance for analysing standard reference materials (SRMs) or results from Interlaboratory Comparison Exercises) can be assessed to determine each laboratory's accuracy. If the results from SRMs are similar among the laboratories, it is reasonable to assume that other data from these groups would also be comparable. However, this assumption should be made only for groups of similar analytes; thus, two laboratories may produce highly comparable PCB results for the SRM, but the determination of DDTs may differ substantially.

Conventions for summing groups of contaminants

The result of the analyses for certain OC groups are usually presented – not as the concentration of the individual chemicals – but as the sums of the various individual chemicals of a group (e.g. summing the PCB congeners to determine a 'total PCB' concentration). This is an important element to be taken into account when comparing sample sets analysed by laboratories using different procedures for calculating 'total' concentrations.

Summed PCBs

Summed PCBs (ΣPCBs or total PCBs) is the most difficult summed group to compare among studies, because there are many conventions for expressing this quantity. In the 1970s and 1980s, PCBs were commonly measured by comparing the pattern from a low resolution GC chromatogram to that of a commercial Aroclor product (e.g. 1254, 1260) and calculating how much Aroclor was present ('Aroclor equivalents'). When high resolution GC columns came into common usage in the 1980s, laboratories started measuring individual PCB congeners. Ideally, a laboratory should have the ability to measure all 209 PCB congeners individually and total their quantities to arrive at ΣPCBs. Unfortunately, it is exceptionally difficult to resolve the 209 PCB congeners by GC, unless a complicated 'multi-dimensional' GC technique is used (Schultz *et al.*, 1989). Furthermore, only recently have commercial standards been available for all PCB congeners. Thus, most laboratories routinely measure only a fraction of the total congeners (e.g. numbers of PCB congeners measured: 10 (AMAP, 1998), 88 (Muir *et al.*, 1996) or 136 (Ross *et al.*, 2000)), sum the congeners measured and then report the sum as ΣPCBs or 'total PCBs'. In most instances, the sum represents the major congeners (those present in largest quantities), but the total is still underestimated. Alternatively, the US National Status and

Trends Program recommended summing 17 particular PCB congeners and then multiplying this sum by 2 to provide a reasonable estimate of the total PCBs by measuring only a few congeners (Lauenstein *et al.*, 1993). However, this method tends to overestimate 'total PCBs' in marine mammals that are capable of metabolising PCB congeners, because the more recalcitrant PCBs are among the 17 measured. Thus, ΣPCBs is an estimate of total PCBs present in the sample and the degree of accuracy of ΣPCBs depends on the method used to calculate this sum.

Summed DDTs

Summed DDTs (ΣDDTs) is most often calculated by summing *o,p'*-DDD, *p,p'*-DDD, *o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDT and *p,p'*-DDT. However, some investigators report the sum of *p,p'*-DDD, *p,p'*-DDE and *p,p'*-DDT instead, so care must be taken to determine which compounds are included in the total before comparisons are made. Unfortunately, some publications report only *p,p'*-DDE and others do not detail the exact method used to calculate ΣDDTs. However, *p,p'*-DDE occurs in the greatest proportion; thus, reporting only *p,p'*-DDE generally does not contribute to large errors when datasets are compared.

Summed chlordanes

Summed chlordanes (Σchlordanes) is usually calculated by summing *cis*-chlordane, *trans*-chlordane, oxychlordane, *cis*-nonachlor, *trans*-nonachlor, nonachlor III, heptachlor and heptachlor epoxide. However, some laboratories measure fewer chlordane-related compounds for Σchlordanes. Again, it is necessary to determine which compounds are included in the total before comparisons are made.

Summed HCHs

Technical HCH is a mixture of α-, β-, γ- (lindane) and ∂-HCH. Many laboratories do not determine all these isomers, so they report only two or three, usually α-HCH and γ-HCH, sometimes adding β-HCH. Thus, caution should be exercised when comparing sums of the HCHs.

Summed toxic equivalents

Exposure of marine mammals to and possible health risks from polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and other compounds that exhibit 'dioxin-like' toxicity (i.e. certain PCB congeners) can be measured by calculating 'toxic equivalents' (TEQs). TEQs are calculated according to an additive model of toxicity (Safe, 1990) in which the molar concentration of each dioxin-like congener is multiplied by the appropriate toxic equivalency factor (TEF; a measure of toxicity relative to that of 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin) (Van den Berg *et al.*, 1998). The individual TEQs are then summed to determine ΣTEQs. Not all laboratories routinely measure PCDDs and PCDFs, so TEQs may be reported based on dioxin-like PCBs only. In marine mammal samples, concentrations of PCDDs and PCDFs are generally low, so the majority of the ΣTEQ contribution is from PCBs (often > 80%) (Ross *et al.*, 2000), so ΣTEQs derived from PCBs may be a reasonable measure of total TEQs in cetaceans. Because several different TEF schemes have been developed in the last decade, determining which set of TEFs each study used to calculate TEQs is very important. The TEFs established by Safe (1990) were revised in 1994 following international agreement at a workshop sponsored by the World Health Organisation-European Centre for Environment and Health (WHO-ECEH) and the International Programme on Chemical Safety (IPCS) (Ahlborg *et al.*, 1994). Subsequently, these TEFs were refined at an expert meeting organised by WHO in 1997 (Van den Berg *et al.*, 1998). Thus, publications before 1999 generally use the older TEFs. Therefore, care must be taken when comparing the older studies to those using newer TEFs, because some of the TEFs have been revised for several compounds and some formerly 'toxic' PCBs (congeners 170 and 180) have been withdrawn from the list.

ANALYTICAL METHODS FOR DETERMINING PERCENT LIPID

Gravimetric methods

There are a number of lipid extraction and analysis procedures available (Wells, 1993), but the method most often used as the 'gold standard' was reported by Bligh and Dyer (1959). This method involves extraction of the sample with chloroform and methanol in carefully controlled ratios. Another commonly used procedure measures the percent lipid in an aliquot of the extraction solvent used for contaminant determinations, quite often made using dichloromethane or hexane as solvents (Schantz *et al.*, 1993; Honeycutt *et al.*, 1995; Krahn *et al.*, 2001). The solvent is evaporated and the lipid is weighed. Because this technique extracts other endogenous material besides lipid, this is often called the 'total extractable' method. Researchers should be aware that different solvents are likely to produce different amounts of extractable material, which can markedly affect 'percent lipid' values (see below).

Instrumental method

A relatively recent innovation in lipid methods has been the use of a thin layer chromatography/flame ionisation detection (TLC/FID) micromethod (Iatroscan) in which total lipids, as well as lipid classes, can be determined concurrently (Parrish, 1987; 1998; Shantha, 1992; Krahn *et al.*, 2001). The advantages of this method include rapid analytical times, improved precision and accuracy and high sensitivities. There are some limitations to this method, such as the need for careful instrument calibration to achieve accurate quantitation (Crane *et al.*, 1983; Rao *et al.*, 1985; Shantha, 1992).

Comparing results when lipid methods differ

When percent lipid data are produced using different lipid methods, it is necessary to exercise care when comparing data presented as 'lipid weight' (see 'Lipid weight' discussion below). Solvents are particularly critical in this respect. For example, the chloroform-methanol mixture is more polar, and therefore able to extract a larger fraction of polar lipids (e.g. phospholipids), whereas hexane, a more neutral solvent, has a higher affinity for neutral lipids (e.g. triglycerides). For example, Randall *et al.* (1998) reported that a three- to five-fold variation in OC concentrations in fish muscle (lipid weight basis) is introduced using different lipid extraction methods. In general, lipid percentages determined by TLC/FID are lower than those determined using the 'total extractables' method. In one study, Delbeke *et al.* (1995) determined lipid in tissues of various marine species by both the 'total extractables' and TLC/FID methods. Although the lipid values determined by both methods were correlated, the lipid concentrations determined by TLC/FID were, on average, half as great as those determined by the gravimetric method. Another study (Krahn *et al.*, 2001), which measured percent lipid in blubber

of gray whales (*Eschrichtius robustus*) by both the TLC/FID and 'total extractables' methods, found that the results were not statistically different.

CONCENTRATION UNITS COMMONLY USED IN CONTAMINANT ANALYSES

Before making a comparison of datasets from two or more sources, it is essential to establish that the concentration units used are the same for each dataset. If not, the numbers must be converted into like units (Table 1). Usually, an investigator will report the units that are conventionally used for the analytes measured or, alternatively, the units preferred by their laboratory. Investigators must provide units (e.g. ng/g wet weight) when entering numbers into text, tables and figures. Otherwise, the data cannot be properly interpreted, particularly if the units cannot be determined from information provided elsewhere in the document.

Three common formats for describing contaminant concentrations (wet, dry and lipid weight) are presented below. Investigators who report data using only one of these formats should be encouraged to provide enough additional information (i.e. percent lipid and/or percent dry weight), so that other researchers can recalculate the data in one of the other formats. This reformatting of data may be necessary so that data from two studies can be compared, or to allow a researcher to use the information for a purpose different from that of the original investigator (e.g. for use in human health risk assessments or for determining temporal or spatial trends). Again, it is critically important that every measurement reported have an associated set of units, including the designation 'wet weight', 'dry weight' or 'lipid weight'. If this information is missing, no comparisons can be made with other data.

Wet weight

Contaminant concentrations are calculated by measuring the amount of a contaminant in a weighed portion of a tissue (as received, i.e. wet weight — sometimes called fresh weight). For example, if 50ng of hexachlorobenzene (HCB) is measured in a 2g tissue sample, the concentration of HCB would be reported as 50ng HCB/2g sample = 25ng/g, wet weight (w.w.).

The wet weight format is used in studies where the tissue is consumed (either by humans or other predators), as well as for other toxicological interpretations. It should be noted, however, that this format is particularly sensitive to tissue freshness because water — which is a main component in many tissues — is rapidly lost not only during decomposition, but also during freezer storage (where the potential for water loss increases as the storage temperature increases).

Dry weight

Contaminant concentrations can be calculated on a dry weight basis to offset the variability in concentrations caused by variations in water content of tissues. First, the dry weight of a tissue is determined by weighing a sample (as received, but a different portion from that used for contaminant determinations) and then the water is evaporated in a drying oven. The dried tissue is re-weighed and the percent dry weight is calculated as follows: Percent dry weight = weight of tissue after drying/weight of tissue (wet) $\times$ 100.

The dry weight of a contaminant is then calculated by dividing the wet weight concentration by the dry weight fraction (percent dry weight/100). For example, if the percent dry weight of the tissue in the first example is 20% then: 25ng/g HCB (wet weight)/0.20 (dry weight fraction) = 125ng/g, dry weight (d.w.).

Note that the dry weight determination is always conducted on a different portion of the sample from that used for contaminant determinations, because the heat used to dry the sample could result in loss of volatile analytes. The dry weight format is generally not used for blubber samples of marine mammals (because blubber has a low water content and can be difficult to dry), although it is often used for liver, kidney, muscle and other tissues, particularly when comparing contaminant concentrations among tissues. Note that some laboratories report 'percent water' in tissues and a calculation must be performed to obtain percent dry weight: percent dry weight = 100 – percent water.

Lipid weight

Lipid is extracted from a tissue, the solvent is removed and the remaining lipid is weighed (see the section 'Analytical methods for determining percent lipid'). Lipid weight is then calculated by dividing the wet weight concentration of a contaminant by the lipid fraction (percent lipid/100).

For example, if the tissue sample in the example above contains 50% lipid, the concentration of HCB expressed in lipid weight would be: 25ng/g HCB (wet weight)/0.50 (lipid fraction) = 50ng/g, lipid weight (l.w.).

Lipid weight is often used to compare contaminant concentrations among different tissues in the same animal (see 'Lipid normalisation' below). In addition, comparisons of contaminant concentrations among different species or different studies are often made on a lipid weight basis to control for varying lipid content. This may be particularly useful when comparing specimens with dissimilar body condition or when analysing non-fresh samples. However, comparisons of different studies on a lipid weight basis should be made cautiously, because there are a number of analytical methods used to determine percent lipid and the lipid values can vary appreciably, depending on method selected (see 'Comparing results when lipid methods differ'). In addition, some investigators believe (Hebert and Keenleyside, 1995) that contaminant concentrations should not be reported on a lipid weight basis unless these two

Table 1

Equivalencies for commonly used analytical units.

Equivalent units			Formulas for converting between units	Comments
ppm* (part per million)	μg/g ($\mu g*g^{-1}$)	mg/kg ($mg*kg^{-1}$)	# ppm = # ppb/1,000 = # ppt/1,000,000	Used for concentrations of organics and metals
ppb (part per billion)	ng/g ($ng*g^{-1}$)	μg/kg ($\mu g*kg^{-1}$)	# ppb = # ppm x 1,000 = # ppt/1,000	Most common units for concentrations of organics
ppt (part per trillion)	pg/g ($pg*g^{-1}$)	ng/kg ($ng*kg^{-1}$)	# ppt = # ppb x 1,000 = # ppm x 1,000,000	Used for toxic equivalents (TEQs)

*1 ppm = 1,000 ppb = 1,000,000 ppt.

factors are correlated (see the section on 'Lipid normalisation' below). Finally, when percent lipid information is not available, comparisons of contaminant data can still be made on a wet or dry weight basis.

LIPID 'NORMALISATION'

Concentrations of lipophilic contaminants are often 'adjusted' for variation in tissue lipid content (see the section on 'Lipid weight' above). This adjustment is performed because it is assumed that lipophilic contaminants such as OCs accumulate in proportion to tissue lipid content. Lipid-adjusted ('normalised') data are used in a variety of applications, such as modelling biomagnification in food webs and examining differences in contaminant levels among tissues or species. Lipid normalisation is usually accomplished by dividing tissue contaminant concentrations by the corresponding lipid fraction to form a ratio (ratio approach; see the section 'Lipid weight' above) and it is assumed that this procedure eliminates the influence of lipid covariation. However, previous studies (Hebert and Keenleyside, 1995) have shown that this normalisation of contaminant concentrations to total lipid does not correct for variation in the lipid unless these factors are correlated. There is some evidence that contaminant concentrations do not correlate with total lipid when a marine mammal undergoes cyclic deposition and mobilisation of lipid stores, because the rates of mobilisation and deposition of pollutants are not identical to those of lipids (Aguilar, 1987). Moreover, even if the lipid content is similar among different tissues, their qualitative composition may markedly vary (see below). Even when contaminants and lipids are correlated, using the ratio approach to normalisation may lead to misleading conclusions and then an alternative normalisation approach (e.g. analysis of covariance) should be used (Hebert and Keenleyside, 1995).

The normalisation approach also has other limitations resulting from: (1) variations in lipid extraction and analysis procedures; (2) differences in contaminant partitioning among different lipid classes (e.g. neutral or polar); (3) very low lipid content of tissues; and (4) seasonal or physiological changes in lipid percentages or composition. Randall *et al.* (1998) reported that the three- to five-fold variation introduced by different lipid extraction methods may exceed any reduction in variation from lipid normalisation of contaminant concentrations (see the section 'Comparing results when lipid methods differ' above).

Various lipid classes have different affinities for PCBs (e.g. concentrations in brain are lower, partly due to high percent of phospholipids; Reijnders, 1986), with higher affinities observed for neutral (storage) lipids (de Boer, 1988). Thus, some researchers have begun to use certain lipid classes, rather than total lipid, in the study of pollutants in marine mammals. For example, Aguilar (1985) examined the relationship between the lipophilicity of various OCs and their distribution pattern among tissues and recommended triglycerides as the lipid class that best 'matches' the polarity of DDTs and PCB congeners most frequently occurring in cetaceans. In addition, Bergen *et al.* (2001) reported that the sum of PCBs in ribbed mussels was correlated to neutral lipids ('triacyclglycerol' = triglyceride) but not to total lipids, thus demonstrating that the current practice of normalising contaminant concentrations to total lipid may not be appropriate as a standard practice.

When low concentrations of lipids are present (< 5%), normalisation of OCs to lipid content results in a substantial increase in lipid weight concentrations compared to wet weight concentrations (20-fold increase for 5% lipid). Thus, lipid-normalised OC concentrations can be artificially inflated when lipid values are very low — for example, in blood where percent lipid generally ranges from 0.01-2.2% (50-10,000-fold increases from wet weight to lipid weight), because small errors in determination of percent lipid will have a large effect on concentrations expressed in lipid weight. Furthermore, when lipid percents are low, differences in analytical methods (e.g. gravimetric vs. TLC/FID) for lipids can result in very different lipid weight concentrations of OCs. In these instances, comparing datasets based on wet weight concentrations would be more accurate.

Investigators have found that OC concentrations differ among the blubber layers in certain cetacean species and this may be explained, in part, by different proportions of lipid classes (Sigurjónsson and Rorvik, 1983; Aguilar and Borrell, 1991; Koopman *et al.*, 1996) (see the section on 'Blubber stratification' below). Furthermore, lipid composition and distribution are influenced by seasonal or physiological changes. Some baleen whales increase their body weight (primarily through fat accumulation) by 50-100% between the beginning and the end of the feeding period (Lockyer and Brown, 1981). For example, the gray whales' annual cycle of feeding, migration and reproduction causes marked changes in lipid content of blubber (Krahn *et al.*, 2001). Changes of this nature are expected to result in substantial variations in the lipid-contaminant relationship. Unfortunately, the resulting changes in contaminant concentrations can be misinterpreted (see 'Lipid content and nutritive condition').

QUALITY ASSURANCE PROCEDURES

The most important and relevant measures of data comparability, whether comparisons are made among datasets from several laboratories or between datasets from different analytical methods in the same laboratory, come from an assessment of each study's Quality Assurance Programme. A comprehensive quality assurance plan (the foundation of a Quality Assurance Programme) is essential for producing and evaluating data (Topping, 1992; Wells *et al.*, 1993; Chidi Ibe and Kullenberg, 1995). The quality assurance plan is designed to monitor the performance of a laboratory's analytical results and to provide rapid feedback so that corrective measures can be taken before data quality is compromised. In addition, the plan spells out procedures that will determine if reported data are sufficiently complete, accurate, comparable, representative, unbiased and precise. Many elements must be present in a quality assurance plan to assure that comparisons of the data produced by the study will be accurate and comparable to data from other studies. Typically, a laboratory's protocols will include specification of the analytes to be determined, the minimum sensitivity of the analytical methods, and whenever possible, will specify the use of certified calibration solutions and standard/certified reference materials, as well as analysis of replicate samples and method blanks. In addition, a laboratory must continue to demonstrate its analytical proficiency through participation in refereed intercomparison exercises.

The quality of a chemical analysis is considered 'assured' when the analysis is performed in a technically competent manner by qualified personnel using appropriate methods and equipment. In addition, the precision and accuracy of the measurement must be within the expected ranges for the technique. Acceptable quality can vary by analyte, matrix,

concentration level of analyte being measured (especially when levels are very high or near detection limits), analysis technique and quantitation method. The following quality control techniques should be applied to every set (generally 10-20 samples) of analyses, but these are not all the measures that are routinely applied to assure good quality (e.g. others are instrument calibration and maintenance, standard operating procedures that include field sampling procedures). Table 2 provides examples of the criteria from a laboratory's Quality Assurance Programme that are applied in evaluating laboratory performance.

Table 2

Selected Quality Assurance Programme criteria for evaluation of laboratory performance in analysing for OCs in tissue samples.

Surrogate/internal standards	60-130% recovery
Method blanks	One method blank should be analysed for every 20 samples. No more than 3 analytes in the method blanks are to exceed 3x the limit of detection.
Replicate samples	One sample should be analysed in duplicate for every 20 samples. The relative (percent) standard deviation between these two analyses must be < 25%.
Standard Reference Materials (SRMs)	One Standard Reference Material or appropriate control material must be analysed with each 20 samples. Concentrations of individual OC analytes must be within 30% of either end of the 95% confidence interval range of the certified values.
Spiked samples	60-130% recovery
Inter-laboratory comparisons	As defined by NIST, NRC, QUASIMEME or other certifying organisation; generally once a year.

Surrogate or internal standards are compounds that are not expected to be found in samples and are added in the beginning of the analysis to measure losses in laboratory processing procedures. The quantity of surrogate standard remaining at completion of the analysis is measured and compared to the amount originally added to determine the percent recovery. Percent recoveries of surrogate standards are evaluated in accordance with laboratory criteria.

Method blanks provide a check on the quality of the reagents and solvents and reflect any system contamination. The blank is an empty sample container to which all of the reagents, chemicals, or solvents used in an analysis are added and then the blank is processed in the same manner as the rest of the samples.

Replicate samples are analysed to provide a measure of the precision for the methods used by analysing two separate sub-samples. Most often, sub-sampling occurs following homogenisation of the samples, particularly when the sample to be analysed is a composite (from two or more animals). Replicate analyses are evaluated in accordance with laboratory criteria.

Standard or Certified Reference Materials (SRMs/CRMs) are analysed to provide evidence that the analytical method works with the naturally incorporated analyte and that the results are comparable to those obtained by a certifying organisation (e.g. National Institute of Standards and Technology (NIST, USA), National Research Council (NRC, Canada) or Quality Assurance of Information for Marine Environmental Monitoring in Europe (QUASIMEME)). Reference materials are pre-homogenised samples that are certified to contain a stated amount of analyte. Great care is taken by the certifying organisation in the homogenising step, because it is critical that each sub-sample of the SRM/CRM is identical in concentrations of contaminants. Furthermore, a laboratory should choose an SRM/CRM with tissue characteristics as close to the samples to be analysed as possible. For example, NIST whale blubber SRM 1945 should be used with cetacean samples and NIST mussel SRM 1974b with invertebrate analyses. SRMs/CRMs are evaluated in accordance with the precision and accuracy that would be expected for the amount of analyte present and with the known error in the certification.

Spiked matrices are analysed to provide a measure of the accuracy of the methods used when no reference materials are available for specific analytes. After a sample is homogenised, two separate sub-samples are taken; one is processed as a sample and a known quantity of analyte is added to the other sub-sample before analysis. Recoveries of the spiked analytes are determined and evaluated in accordance with laboratory criteria.

Interlaboratory comparisons on at least an annual basis, i.e. participation in a refereed interlaboratory comparison, is essential (Chidi Ibe and Kullenberg, 1995; Davies and Wells, 1997). In this intercomparison, each laboratory analyses a portion of the same 'blind' sample and the results are compared by the organiser of the exercise – generally one of the certifying organisations (e.g. NIST or QUASIMEME) listed under 'Standard Reference Materials' (above). If any significant differences occur from the 'consensus value' (typically, the mean of all participating laboratories, excluding outliers), corrective action should be initiated by laboratories who do not meet the criteria.

BIOLOGICAL FACTORS TO CONSIDER WHEN COMPARING DATA

Age, sex and reproductive status

Age, sex and reproductive status are important factors to be taken into account when making intra- and interspecies comparisons of OCs in cetaceans (Aguilar *et al.*, 1999). Bioaccumulative contaminants, such as many OCs, would be expected to increase progressively with age in individual animals. However, the rate of increase tends to level off in older individuals as a result of reduced daily feeding, as well as increased metabolism and excretion rates when OCs accumulate (Aguilar *et al.*, 1999). In most of the studies reviewed by Aguilar *et al.* (1999), a positive correlation was found between age and OC concentrations in male cetaceans, indicating bioaccumulation of these contaminants. Similarly, OC concentrations increased with age in male white whales from the Arctic (Krahn *et al.*, 1999) and in male harbour porpoises (*Phocoena phocoena*) from Scandinavia (Kleivane *et al.*, 1995). In contrast, no similar correlation was found between age and OC concentrations in adult female cetaceans, because transfer of contaminants from mother to offspring during gestation and lactation affect OC concentrations in tissues of reproductive females (Aguilar *et al.*, 1999). Studies of mother-calf pairs have shown that significant quantities of lipid-soluble contaminants can be transferred to the young via lactation (Aguilar and Borrell, 1994b; Ridgway and Reddy, 1995) and the amount of contaminants transferred is directly related to the duration of lactation (Aguilar *et al.*, 1999). This transfer was also demonstrated in juvenile killer whales – especially in first-recruited animals, which were found to contain much higher concentrations of PCBs and DDTs in blubber than were found in their mothers (2.8-28 times and 3.2-54 times,

respectively) (Ylitalo *et al.*, 2001). This initial contaminant dose can be further concentrated as the weaned whale metabolises fat to provide energy, resulting in unusually high OC concentrations in young animals. For the mother, this transfer represents a loss of contaminants, evidenced by the fact that actively reproducing females have notably lower OC concentrations than those in mature males and in some instances, tissue concentrations may even decrease with age (Aguilar *et al.*, 1999).

Health status

Information on the health status of cetaceans is important for interpreting observed tissue pollutant levels. For example, diseases affecting hepatic and renal functions are likely to affect OC metabolism or excretion, and could result in increased accumulation (Aguilar *et al.*, 1999). However, this should not be considered a rule, because some diseases may also activate hepatic enzymatic activity and this would eventually result in increased metabolism of pollutants and consequently in reduced OC burdens (Aguilar *et al.*, 1999). Furthermore, reduced reproduction in females limits pollutant transfer during gestation and lactation, thereby altering the usual age-related decrease in OC concentrations in maternal tissues. For example, in reproductively impaired populations of white whales and ringed seals (*Phoca hispida*), it has been difficult to establish whether abnormally high OC levels observed in adult females were a consequence of the reduced reproductive activity (caused by other factors) or, actually, the cause of impaired reproduction (Helle *et al.*, 1983; 1990; Martineau *et al.*, 1987). One plausible hypothesis suggests a synergistic process – elevated OC concentrations can result in reproductive failure and then as a result of reproductive failure, OC levels increase further. Some diseases may also cause reproductive failure (e.g. abortions). An example of an infectious agent of this nature is *Brucella*, which has been recently reported as widespread among marine mammals (Miller *et al.*, 1999; Nielsen *et al.*, 2001). In individuals affected by this disease, the pollutant transfer associated with gestation and lactation does not take place and consequently, OC concentrations in the female increase abnormally. Thus, it is important that the health status of cetaceans is assessed and then considered when datasets are evaluated.

Lipid content and nutritive condition

Due to the lipophilic (hydrophobic) nature of OCs, their dynamics in marine food webs is closely related to the dynamics of lipids in marine organisms. Concentrations of OCs in organisms are correlated with OC levels in lipids of prey that they consume and by the efficiency of lipid absorption (AMAP, 1998). Fat comprises a large proportion of the body mass of many cetaceans and is consolidated as a blubber layer. In species that have seasonal migratory and feeding regimes (e.g. most baleen whales), lipid content may vary throughout the year; seasonal fluctuations in condition do not appear to be as large in odontocetes as in mysticetes (Aguilar *et al.*, 1999).

Data to assess overall body condition of the specimens sampled (e.g. body girth, body mass and/or blubber thickness at selected body sites) should be collected and examined in connection with the OC concentrations observed. The lipid content of the tissues surveyed, which is routinely determined during the analyses for OCs, is also a significant variable to use in this respect. Lipid content and thickness of blubber can be indicative of the nutritive condition of marine mammals (Sigurjónsson and Rorvik, 1983) and nutritive status can have dramatic effects on OC concentrations. When animals lose weight (i.e. lipid is mobilised and metabolised) due to migration, food shortage, reproductive activity, disease or other stresses, two processes are possible: either contaminants remain in the blubber or are redistributed to vital organs such as the central nervous system (Henrikson *et al.*, 1996). Aguilar *et al.* (1999) reported that the actual process is probably somewhere between these extremes. The total amount of many OCs increases in body tissues as lipid is metabolised, but are not as high as would be predicted if all the contaminants were concentrated in the remaining lipid. This increase of OCs in tissues following depletion of lipid stores has been observed in a number of species. For example, PCB concentrations (lipid basis) in Mediterranean striped dolphins (*Stenella coeruleoalba*) were negatively correlated to lipid content, indicating a build-up of contaminants in dolphins in poor nutritive condition (Aguilar *et al.*, 1999). Thus, it is important when comparing contaminant concentrations in cetaceans to consider nutritive status, as well as to compare animals of similar status, e.g. males or juveniles during a single season.

Blubber stratification

As noted above, collecting biopsies from the blubber of free-ranging cetaceans through surgical or punch biopsies on captured/released small cetaceans and through remote biopsy darting of larger cetaceans, is becoming more frequent as part of an effort to develop non-destructive techniques for contaminant monitoring. There are only a few studies that have tested whether these small samples are representative of the contamination in the entire blubber layer (Aguilar and Borrell, 1991; Gauthier *et al.*, 1997). Examination of this is an important component of the POLLUTION 2000+ research programme. Unfortunately, studies that assess differences among blubber layers do not provide unequivocal answers. Among the odontocetes, Koopman *et al.* (1996) found that vertical stratification was evident between the inner and outer blubber layers in harbour porpoises, suggesting that the inner blubber layer is more active metabolically than the outer layer in terms of lipid deposition and mobilisation and thus that stratification could affect contaminant distribution. Another study (Tilbury *et al.*, 1997) found no differences in lipid content among three blubber layers in harbour porpoises, but the study included just three animals. In addition, distribution of lipid-normalised OCs in the blubber layers was inconsistent among the animals studied. Two of the animals had significantly higher OC concentrations in the inner layer nearest the muscle than in the other two layers and the third showed no differences among layers.

Baleen whales are much larger than most odontocetes and their blubber is more markedly layered. In addition, the intensity of feeding varies seasonally for many baleen whales, so that the lipid reserves in blubber vary throughout the year. Two studies of blubber stratification in baleen whales have led to different conclusions. Aguilar and Borrell (1991) have found that the lipid content of blubber differs among the three layers in balaenopterid whales and that this may result in differential distribution of lipophilic contaminants. They studied the stratification of OCs in the blubber of 89 fin whales (*Balaenoptera physalus*) and 23 sei whales (*B. borealis*) and found significantly higher OC concentrations in the outer layer compared to the inner layer of blubber in both species (Aguilar and Borrell, 1991). They concluded that blubber samples collected from cetaceans for pollutant analyses should include all layers in order to be

representative of an individual animal's pollutant load. In contrast, Gauthier *et al.* (1997) found no statistically significant differences in lipid-normalised OCs among the outer, middle or inner blubber layers in balaenopterid whales: minke (*B. acutorostrata*) and blue (*B. musculus*). However, this study was limited to only three minke whales and one blue whale, so no statistical conclusions could be drawn due to the reduced sample size.

As a result of these inconsistent findings for stratification of lipid classes or OC distributions, it will be necessary to determine whether stratification is species-specific and if it changes seasonally. Surgical biopsies should take a full-thickness sample of blubber whenever possible. In addition, designing biopsy darts to sample deeper into the blubber strata may help obtain more representative biopsy samples (Lambertsen *et al.*, 1994; Gauthier *et al.*, 1997), although this technique has the potential for producing a higher impact on the sampled individual.

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