Persistent organic pollutants and heavy metals in the green sea turtle, *Chelonia mydas*

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Abstract

The chemical contamination of sea turtles is an emerging area of conservation research. Chemicals, such as persistent organic pollutants (POPs) and heavy metals, have a wide range of harmful effects on animals and humans and are beginning to be reported in sea turtle populations around the world. However, prior to the present study, research on chemical contamination in sea turtles had generally been limited to studies on deceased animals. Furthermore, the analytical methods used in these studies had limited sensitivity and reported small numbers of compounds. The main objectives of this thesis were therefore to further develop methods for measuring POPs in sea turtles and to systematically investigate some of the important aspects of accumulation and transfer of POPs and heavy metals in the green sea turtle, *Chelonia mydas*.

Gas chromatography with electron capture detection (GC-ECD) has generally been used to analyse POPs in sea turtle studies. However, GC-ECD relies on relative retention time for identification and can therefore not distinguish between co-eluting compounds. Furthermore, the limit of detection is relatively high (> 1 ng g^{-1}) and these methods are therefore often unable to detect POPs in low trophic level organisms. More recent methods combining GC-ECD and gas chromatography with mass spectrometry (GC-MS) have reported a large number of POPs in sea turtles at trace concentrations. However, these methods required multiple injections of each sample into a complex arrangement of multiple gas chromatographs. This can only be replicated in well equipped and highly funded laboratories. It was therefore the first objective of this thesis to develop an equally accurate and sensitive method requiring a single injection of each sample onto a simple instrument setup.

A method using gas chromatography with coupled mass spectrometry (GC-MS/MS) was developed on a Varian 3800 gas chromatograph fitted with a Saturn 2200 mass spectrometer and a 1079 programmable temperature vapourising (PTV) injector. Using calibrants and mass-labelled internal standard and recovery solutions obtained from the National Institute of Standards and Technology (NIST), South Carolina, USA, a GC-MS/MS method was established for 83 polyclorinated biphenyls (PCBs), 23 organochlorine pesticides (OCPs) and 19 polybrominated diphenyl ethers

(PBDEs). Sample preparation was modified from previous studies and involved accelerated solvent extraction with dichloromethane, followed by gel permeation chromatography and Florisil clean-up procedures. Recoveries were generally > 60% and standard reference materials were reported to within 60 and 70% of the reference and certified values, respectively. The coefficients of variation of pooled samples were < 20%, although generally < 5%, and the limit of detection ranged from 5 to 35 pg g^{-1}. This method therefore provided an accurate way of measuring a large number of POPs at trace concentrations in *C. mydas*. This method was then used to investigate a number of important accumulation and transfer aspects of chemical contamination in *C. mydas*.

The earliest studies on chemical contamination in sea turtles sampled tissue from dead and stranded animals. However, it is of more interest to investigate the contamination of living sea turtle populations. Blood and carapace sampling have increasingly been used as non-lethal methods for analysing POPs and metals in sea turtles. However, there was very little information on how well blood and carapace samples represented the chemical contamination of internal tissues. To address this issue, blood, carapace, liver, kidney and muscle samples from 16 *C. mydas* that died at the Sea World Sea Turtle Rehabilitation Program were analysed for POPs and heavy metals. Heavy metal and POP levels in the blood and carapace were significantly correlated with internal tissue concentrations. Furthermore, these relationships were not affected by sex or age. While it must be considered that these *C. mydas* were in rehabilitation, blood and carapace samples are good predictors of the internal contamination of *C. mydas*. This information therefore provides scientists with reliable non-lethal methods for estimating chemical contamination in living sea turtle populations.

Information on the accumulation of chemical contaminants in sea turtles was also limited prior to this study. The biology of sea turtles indicates that chemicals may accumulate through feeding and/or maternal transfer during vitellogenesis. The present study investigated the chemical contamination of *C. mydas* from different foraging areas. Satellite telemetry tracked the movement of three *C. mydas* nesting at Ma'Daerah, Peninsular Malaysia to three different foraging areas in Southeast Asia. Furthermore, the egg chemical contamination profiles of the *C. mydas* from these different foraging areas were significantly different. This suggested the use of

multivariate contaminant analysis to assess the variation of foraging area locations of a nesting *C. mydas* population.

The use of contamination profiles to assess foraging ground variation in a nesting population was further supported by chemical analysis of eggs from 11 *C. mydas* nesting at Ma'Daerah, Peninsular Malaysia. The egg POP contamination profiles from the 11 turtles were separated into six groups. This indicated that these *C. mydas* nesting at Ma'Daerah may have migrated from six distinct foraging areas. However, investigation into the variation in chemical profiles of *C. mydas* from the same foraging areas must be investigated before this concept can be validated. There are a number of factors such as age, sex and specific foraging range that may lead to variations in *C. mydas* contamination from the same foraging area.

The accumulation of POPs in *C. mydas* via maternal transfer to eggs and hatchlings was also investigated in the present study. Maternal blood, eggs and hatchling blood were collected from the 11 *C. mydas* nesting at Ma'Daerah. There were significant correlations in POP concentrations between maternal blood and eggs, indicating transfer of these chemicals to eggs during vitellogenesis. These results also indicated that egg sampling could be used as a relatively non-invasive method for determining POPs in adult female *C. mydas*. There were also significant correlations in POP concentrations between eggs and hatchling blood, indicating further transfer of these chemicals to hatchlings during development. Furthermore, as egg POP concentration increased the mass:length ratio of hatchlings decreased. This indicated a subtle influence of POPs on the development of *C. mydas* hatchlings that may compromise the duration of offshore dispersal and affect predator avoidance.

The implications of chemical contamination on the conservation of *C. mydas* populations and on human health in communities that consume sea turtle eggs were investigated in Peninsular Malaysia. A sample of 55 *C. mydas* eggs was purchased from markets on the east coast of Peninsular Malaysia. According to the vendors, the eggs had been collected from sites ranging from adjacent beaches to thousands of kilometres away in Borneo Malaysia. The concentrations of POPs and metals in the eggs were variable among nesting areas and markets. A screening risk assessment indicated that the reported arsenic concentrations posed a relatively high risk of

disruption to embryonic development. Furthermore, the large number of compounds detected was thought to increase the risks to embryonic development of *C. mydas* eggs. The concentrations of coplanar PCBs also posed a considerable risk to human health, with the consumption of a single *C. mydas* egg estimated to represent 3-300 times the acceptable daily intake (ADI) of these compounds. The large number of compounds detected was also expected to further contribute to the health risks of consuming *C. mydas* eggs. Incidentally, the human health risks associated with consuming *C. mydas* eggs may contribute to the conservation of this species in Southeast Asia. Public awareness campaigns highlighting the dangers associated with consuming *C. mydas* eggs could reduce the collection of eggs for human consumption. However, this is unlikely to be completely beneficial to this population if the harmful effects of these chemicals are further realised.

Overall, this thesis has further advanced knowledge in chemical contamination of sea turtles. The GC-MS/MS method allows fast and inexpensive detection of 125 POP compounds at trace concentrations. Blood and carapace sampling have been validated as non-lethal methods for predicting contamination in the internal tissues of sea turtles. The use of contamination profiles has been proposed as a way of investigating the foraging ground variability of a nesting sea turtle population and there is now evidence of maternal transfer of POPs from nesting females to eggs and hatchlings. Finally, *C. mydas* egg contamination has been found to be potentially detrimental to the development of hatchlings and human health by communities that consume sea turtle eggs. However, chemical contamination of *C. mydas* eggs has the potential to reduce the impact of egg collection for human consumption on sea turtle populations.

Statement of Originality

This work has not previously been submitted for a degree of diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Jason van de Merwe

September 26, 2008

Animal ethics

All research in this thesis was conducted in accordance with protocols approved by the Griffith University Animal Ethics Committee (approval number: EAS/04/04/aec).

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1.1 Sea turtles

Sea turtles are oviparous reptiles that adapted to the marine environment during the late Jurassic period, over 150 million years ago (Pritchard 1997). Currently, there are seven sea turtle species, grouped into two families, Dermochelyidae and Cheloniidae (Bustard 1972; Cogger 2000). Dermochelyidae is represented by a single species, the leatherback turtle, *Dermochelys coriacea,* and Cheloniidae includes six species of hard-shelled sea turtles, including the green turtle, *Chelonia mydas* (Cogger 2000). Morphologically, *C. mydas* is characterised by four pair of post-occular scutes, one pair of prefrontal scutes and four costal scutes on either side of the carapace. They have a single claw on each flipper and nesting females grow up to 120 cm (straight carapace length) and 230 kg. *Chelonia mydas* hatchlings are further distinguished from other morphologically similar chelonid hatchlings by having a shiny green/black carapace and white plastron (Bustard 1972; Lutz and Musick 1997; Pritchard and Mortimer 1999).

Due to life cycle traits such as high juvenile mortality, late sexual maturation and low fecundity, sea turtle populations are susceptible to increasing anthropogenic impacts in coastal areas (Table 1.1). Of the 33 IUCN indexed *C. mydas* populations around the world, ten are increasing, six are stable and 17 are declining (Seminoff 2002). As a result, *C. mydas* is currently listed as "Endangered" in the IUCN Red Book of Threatened Species and in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (IUCN 2007). The accumulation and effects of chemical pollutants such as organochlorine pesticides (OCPs), polychrorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and heavy metals in chelonid sea turtles have been identified as a major current threat to sea turtle populations and is the focus of this thesis.

Table 1.1. Summary of documented anthropogenic impacts affecting marine turtle populations throughout the world.

Threat	Life cycle stage	Impact	References
Introduction of feral animals	Eggs; hatchlings	Feral animals dig up nests	Witham (1982); Mrosovsky (1983); Seabrook (1989); Broderick and Godley (1996)
Beach front development	Hatchlings; nesting females	Hatchlings disoriented by lighting; nesting habitat altered	Witham (1982); Mrosovsky (1983)
Fishing gear entanglement	Hatchlings; sub-adults; adults	Entanglement causes suffocation and injuries	Broderick and Godley (1996); Poiner and Harris (1996)
Boat strikes	Hatchlings; sub-adults; adults	Injuries and death caused by propeller damage and fuselage impact	Limpus (1997)
Harvesting	Sub-adults; adults	Death	Limpus (1997)
Ocean debris	Hatchlings; sub-adults; adults	Entanglement of hatchlings; ingestion by adults and sub-adults	Carpenter and Smith (1972); Carr (1987); Laist (1987); Hutchison and Simmonds (1992)
Egg poaching	Eggs	Death	Hendrickson and Alfred (1961); Bustard (1972)
Poorly managed tourism	Nesting females; hatchlings	Disturbance to natural nesting behaviour	Venizelos (1991); Limpus (1997)
Chemical pollution	Hatchlings; sub-adults; adults	Immunosuppression, fibropapilloma disease	See Tables 1.3 and 1.5

1.2 Sea turtle biology and ecology

The life cycle of chelonid sea turtles involves a number of distinct stages that occur in a range of oceanic and coastal habitats (Figure 1.1). All chelonid turtle species follow the same general cycle, although the timing of the different stages varies among species and even among populations of the same species.

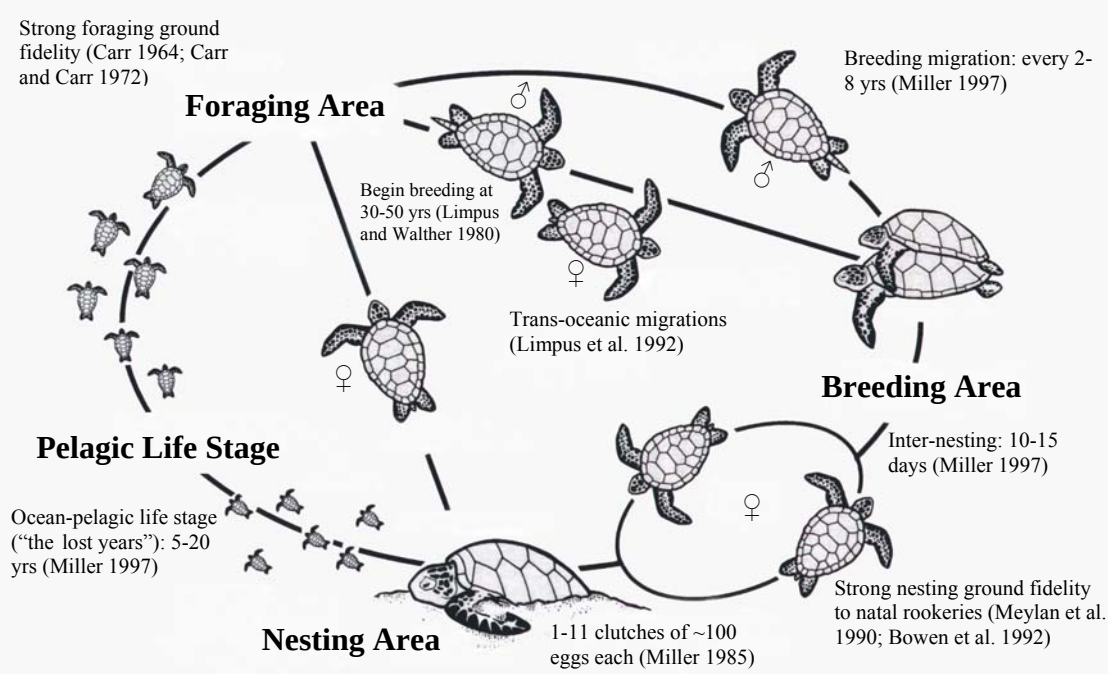

Figure 1.1. General life-cycle of chelonid sea turtles with details specific to *Chelonia mydas*. Modified from Miller (1997).

Of particular relevance to chemical contamination of sea turtles are feeding habits, nesting and foraging ground fidelity and the mobilisation of stored lipids for migration and egg production (vitellogenesis). Hatchling and juvenile sea turtles feed on gelatinous zooplankton during the open-ocean pelagic life stage (Bjorndal 1985; Limpus and Miller 1993; Zug and Glor 1998; Reich et al. 2007). At approximately 3-6 years of age they take up residence in neritic foraging grounds and switch to an almost exclusively herbivorous diet of seagrass and algae (Mortimer 1982; Bjorndal 1985, 1997; Musick and Limpus 1997). Persistent organic pollutants and heavy metals from agricultural, industrial and domestic sources are assimilated by these food sources and can therefore accumulate in sea turtles through feeding.

At the age of 30-50 years *C. mydas* become sexually mature (Limpus and Walther 1980) and begin seasonal migrations from their foraging grounds to breeding areas, which can be thousands of kilometres away (Carr 1964; Carr and Carr 1972; Limpus et al. 1992; Lohmann et al. 1999). Turtles from a single foraging area can migrate to many different breeding areas and a breeding population can be comprised of turtles from many foraging areas (Balazs 1994; Balazs et al. 1994; Cheng 2000; Godley et al. 2002; Seminoff et al. 2008). However, individual sea turtles show strong fidelity to their foraging and nesting areas, returning to and from the same sites throughout their adult years (Carr 1964; Carr and Carr 1972; Meylan et al. 1990; Bowen et al. 1992; Limpus et al. 1992; Bowen et al. 1993; Lohmann et al. 1997; Lohmann et al. 1999). Sexually mature female marine turtles generally do not reproduce every year as it can take some time to accumulate the energy reserves to reach breeding condition (Hirth 1980; Limpus and Nicholls 1988). The range in remigration intervals for mature female *C.mydas* is from two to > 9 years, at an average of three years (Hirth 1980; Van Buskirk and Crowder 1994; Miller 1997). Sea turtles do not feed during the migration or while in the breeding/nesting grounds (Bjorndal 1982, 1985, 1997). Accumulation of chemicals through feeding will therefore be dependent on the contamination of the foraging areas. Identification of foraging areas of a nesting population is therefore critical in understanding the threat of chemical contamination in sea turtles.

Prior to reproduction, nesting females must accumulate enough energy reserves to support vitellogenesis, migration, multiple egg laying episodes and remigration back to the foraging grounds (Bjorndal 1982). The process of vitellogenesis involves the conversion of stored lipid into egg yolk and is stimulated by estrogen in the green turtle (Owens 1976, 1999). In a single nesting season, female *C. mydas* will lay one to 11 clutches of, on average, 110 eggs at 10-15 day intervals (Miller 1985, 1997). Egg yolks are produced with similar lipid content over the entire nesting season (Hamann et al. 2002) and aerobic metabolism of nesting *C. mydas* and *D. coriacea* females is reduced during nesting (Prange and Jackson 1976; Paladino et al. 1996), indicating that breeding females are reliant on lipid reserves to support the metabolic and reproductive costs during this period.

Green turtle eggs are buried 40 to 100 cm below the surface of the sand and generally take between 50 and 90 days to incubate (Miller 1985; Ackerman 1997). The development of the embryo is a metabolic process, where differentiation and growth are fuelled by the energy stored in the egg yolk (Ackerman 1997). Sea turtles also exhibit temperature dependent sex determination. Warmer nests incubate faster and produce more females, while cooler nests produce more males (Mrosovsky and Yntema 1980; Yntema and Mrosovsky 1982; Standora and Spotila 1985; Spotila et al. 1987; Ewert et al. 1994; Georges et al. 1994). For *C. mydas*, the pivotal temperature, one that produces a 1:1 ratio of males to females, is between ~ 28 and 30 $^{\circ}$C, varying slightly between populations (Miller and Limpus 1981). Sea turtle hatchlings use the energy adsorbed from the yolk to fuel emergence from the nest and offshore dispersal (Miller 1985). During this period, the hatchlings maintain a "frenzy period" during which they run directly to the water and swim continuously without feeding or sleeping until they reach the open ocean and the sanctuary of *Sargassum* rafts (Dial 1987; Wyneken and Salmon 1992; Lohmann et al. 1997).

The mobilisation of lipids in adult turtles for migration and vitellogenesis and the transfer of these lipids to hatchlings for development and energy for offshore dispersal highlight the importance of investigating lipophilic contaminants (e.g. POPs) in sea turtles. Due to their strong affinity to lipids, it is probable that these chemicals are mobilised and transferred with the lipids and therefore have the potential to compromise migration, embryonic development and hatchling dispersal.

1.3 Persistent organic pollutants (POPs)

Persistent organic pollutants (POPs) are compounds that are resistant to chemical, biological and photolytic breakdown in the environment. The majority of POPs are chlorinated and brominated aromatic hydrocarbons, including the organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), dioxins and furans (Jones and de Voogt 1999). Of particular interest to this study are the OCPs, PCBs and PBDEs. The OCPs include hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane (DDT), hexachlorocyclohexanes (HCHs), aldrin, dieldrin (and isomer endrin), heptachlor, chlordanes and endosulfan (Baird 1995). There are theoretically 209 congeners of both PCBs and PBDEs, containing from one

to ten chlorine and bromine atoms, respectively. However, due to structural constraints, only about half of the congeners can actually be produced.

Persistent organic pollutants have been manufactured in recent history as insecticides, fungicides, pharmaceuticals, heat exchange fluids, paint additives, sealants, plasticisers and flame-retardants (Table 1.2). However, in the early 1960s, their adverse effects on wildlife were realised and publicised (Carson 1962). Soon after this, the production and use of many of these chemicals was banned or restricted in many countries around the world. And in 2001, the Stockholm Convention on Persistent Organic Pollutants was established to control and monitor the global manufacture and use of these chemicals. However, many of these chemicals are still produced and used around the world both legally and illegally. Furthermore, due to their resistance to degradation, many POPs are still considered to be extremely toxic to the environment.

Table 1.2. A list of common POPs and their uses.

Compound	Uses
Organochlorine Pesticides	
DDT	mosquito control for spread of malaria; insecticide
Chlordane	termite control; general insecticide for crops
Mirex	ant and termite control; fire retardant in plastic, rubber, paper, paint and electrical goods
Hexachlorocyclohexanes (HCHs)	agricultural insecticide; pharmaceutical for treatment of headlice and scabies
Endosulfan	agricultural pesticide (aphids, leafhoppers, beetles)
Heptachlor	soil insect and termite control; crop insects; malaria
Hexachlorobenzene	crop fungi; by-product of chemical synthesis
Aldrin	soil insecticide (termites, grasshoppers)
Dieldrin	termite control; insecticide for soils
Endrin	insecticide for cotton and grain crops; rodent control; bird control
Polychlorinated biphenyls (PCBs)	heat exchange fluids, in electric transformers and capacitors; paint additives; carbonless copy paper; sealants; plasticisers
Polybrominated diphenyl ethers (PBDEs)	flame retardants

1.3.1 Fate of POPs in the environment

The wide use of OCPs in agriculture results in these chemicals entering the environment directly. Furthermore, a number of these compounds are degraded or metabolised once they are released into the environment and their metabolites and by-products can be equally as toxic or persistent. For example, DDT is degraded in the environment to *para*-dichlorodiphenyldichloroethane (DDD). Once DDD is taken up by organisms it is quickly metabolised to dichlorodiphenyldichloroethane (DDE), which is virtually biologically non-degradable (Baird and Cann 2005). The PCBs and PBDEs make their way into the environment through disposal of items containing these compounds. Also, when PCBs are incinerated in the presence of oxygen, toxic polychlorinated dibenzofurans (PCDFs) are produced (Baird 1995).

Once released into the aquatic environment, POPs bind to organic particulate matter suspended in the water column and in sediments. From here they enter food webs as they are assimilated with their associated organic matter into aquatic primary producers and filter feeding animals (Connell et al. 1999). POPs are very persistent in the environment due to their low vapour pressure (and hence slow rate of evaporation), low reactivity with light and other environmental chemicals and low solubility in water. Furthermore, they are lipophilic, resulting in bioaccumulation and biomagnification in food webs (Baird and Cann 2005).

While environmental contamination is usually greatest near the point of release, POPs can also travel thousands of kilometres from their original source (Baird and Cann 2005). Most POPs are sufficiently volatile to evaporate slowly from the surface of soil and water bodies. This evaporation is greater in warmer equatorial regions of the world due to the vapour pressure of chemicals increasing exponentially with temperature. Once in the vapour form, these chemicals are transported by air currents until they reach an area where the temperature is cool enough to recondense them back into the aquatic and terrestrial environments (Baird and Cann 2005). This results in the widespread movement of many POPs and contamination occurring in even remote areas of the world.

1.3.2 Effects of POPs - endocrine disrupting chemicals

The accumulation of POPs in animals and humans has the potential to disturb the development and/or operation of the vertebrate endocrine system (Colborn et al. 1993). Exposure to these endocrine disrupting chemicals (EDCs) has been associated with abnormal thyroid function, decreased fertility, decreased hatching success, sex reversal and alterations in immune function in a number of fishes, birds, reptiles and mammals (Colborn et al. 1993). The EDCs can disrupt normal endocrine function by altering: 1) hypothalamic-pituitary axis of endocrine control, 2) activity of hormones, 3) function of hormone binding molecules, 4) activity of hormone receptors by acting as a hormone agonist or antagonist, or 5) hepatic clearance rate of hormones (Crain and Guillette 1997).

The mode of action of particular EDCs can vary significantly between animal groups and even between species (Guillette et al. 1996). For example, *p,p'*-DDE can have estrogenic activity, no estrogenic activity and anti-androgenic activity, depending on the species examined (Guillette et al. 2000). The interaction between different EDCs can also have an influence on the effects of these chemicals. The insecticide *o,p'*-DDT binds weakly to alligator estrogen receptors (ERs) when present alone, but binds strongly in the presence of *p,p'*-DDE, which does not bind to ERs (Crain et al. 2000). This results in a species-specific complex interaction of EDC effects in living organisms. This indicates that all species should be treated independently when investigating the effects of EDCs.

The majority of the current research on endocrine disrupting chemicals has focussed on a specific group of compounds known as ecoestrogens. Ecoestrogens are chemicals that are released into the environment from anthropogenic sources and mimic estrogen activity once they accumulate in wildlife and humans (Guillette et al. 1996). The potency of an ecoestrogen is determined by: 1) functional similarity to estrogen, 2) magnitude of exposure, 3) timing of exposure, and 4) availability to the target cell (Guillette et al. 1996). Despite having a low binding affinity to the estrogen receptor when compared to the natural estrogen, 17β-estradiol, the lipophilic nature and resistance to metabolic breakdown of ecoestrogens result in their bioconcentration in organisms. Whereas natural estrogens have a half-life of minutes to hours (Tait and Tait 1991), ecoestrogens can have half-life of over 50 years. Ecoestrogens also

generally have low affinity for plasma binding proteins, a characteristic that allows them to freely enter cells, thus increasing their biological availability (Colborn et al. 1993). This bioconcentration and increased bioavailability, combined with biomagnification down the food chain results in ecoestrogens reaching concentrations in the cells capable of eliciting physiological effects in many animals (Guillette et al. 1996). Ecoestrogens can also elicit an estrogen mimicking affect through less direct and more antagonistic pathways. For example, in rats, the DDT metabolite, *p,p'*-DDE, inhibits binding of androgen to its receptor and androgen-induced transcriptional activity (Kelce et al. 1995; Gray 1998).

1.3.3 Persistent organic pollutants in sea turtles

In recent years, there has been limited research on POPs in sea turtles (Table 1.3). The majority of these studies have been on adults and sub-adults and have been opportunistic in nature, sampling deceased and stranded animals. Although concentrations of POPs in these studies are highly variable, there are a number of trends that can be observed through interpretation of these data.

The carnivorous and omnivorous loggerheads (*Caretta caretta*), ridleys (*Lepidochelys olivacea* and *Lepidochelys kempii*) and leatherbacks (*Dermochelys coriacea*) generally have higher POP contamination than the herbivorous green sea turtles (*Chelonia mydas*), even when inhabiting the same foraging area (McKenzie et al. 1999; Gardner et al. 2003). This is likely due to these species being at a higher trophic level and experiencing greater bioaccumulation and biomagnification. However, evidence of bioaccumulation should be supported by increasing POP concentrations with age, which is not observed in a number of these studies. Juvenile *C. caretta* in Italy had higher concentrations of PCBs and DDE than adults and adult males had higher concentrations than adult females (Storelli and Marcotrigiano 2000a). This pattern was also observed in *C. mydas* from Cyprus and Scotland (McKenzie et al. 1999). An explanation for reduced POP contamination observed in adult females could be maternal offloading of lipophilic POPs during egg production. However, this has received limited attention in the current literature and warrants further investigation.

Table 1.3. Persistent organic pollutants in sea turtles (ng g^{-1} wet mass unless stated otherwise). Data presented in mean ± SD or range; sample sizes in parentheses.

Tissue	Species	Location	Organochlorine Pesticides						PCBs	Reference
			HCB	Dieldrin	Chlordanes	DDD	DDE	DDT		
Liver	*Caretta caretta*	VA and NC, USA	-	-	-	5.5 ± 3.6 (7)	50.8 ± 100.5 (19)	3.44 (1)	177.7 ± 182.1 (21)	Rybitski et al. (1995)
		Italy	400 ± 300 (6)	-	-	-	410 ± 250 (6)	-	350 ± 210 (6)	Storelli and Marcotrigiano (2000a)[b]
		Italy	-	-	-	<LOD - 61.2 (11)	3.6 - 217.3 (11)	<LOD - 70.6 (11)	7.6 - 247.3 (11)	Perugini et al. (2006)
		Cyprus, Greece and Scotland	-	1.8 ± 1.3 (3)	3.0 ± 1.8 (3)	0.19 ± 0.27 (3)	78.2 ± 41.3 (5)	0.36 ± 0.25 (3)	-	McKenzie et al. (1999)
		Italy	-	-	-	-	-	-	119 ± 60 (3)	Corsolini et al. (2000)
		FL, USA	-	-	-	-	21.5 ± 32.8 (9)	-	64.4 ± 61.1 (9)	McKim and Johnson (1983)
		Italy	-	-	-	-	-	18.3 ± 18.4 (19)[d]	52 ± 75 (19)	Storelli et al. (2007)
		Mexico	<LOD (1)	<LOD (1)	<LOD (1)	-	-	<LOD (1)[d]	-	Gardner et al. (2003)
	Dermochelys coriacea	Cyprus, Greece and Scotland	-	2.8 ± 0.4 (2)	2.3 ± 0 (2)	0.7 ± 0.1 (2)	4.1 ± 3.4 (2)	0.7 ± 0.1 (2)	-	McKenzie et al. (1999)
	Lepidochelys kempii	VA and NC, USA	-	-	-	8.7 ± 3.4 (3)	55.5 ± 1.3 (3)	-	375.3 ± 225.4 (3)	Rybitski et al. (1995)
		NY, USA	-	-	-	-	29.8 - 547 (22)	-	43.4 - 1950 (22)	Lake et al. (1994)
	Lepidochelys olivacea	Mexico	3.5 (1)	7.3 (1)	45.3 (1)	-	-	10.4 (1)[d]	-	Gardner et al. (2003)
	Chelonia mydas	Cyprus, Greece and Scotland	-	1.5 ± 1.0 (7)	1.3 ± 1.2 (8)	0.8 ± 0.3 (7)	4.9 ± 6.4 (9)	0.8 ± 0.3 (7)	-	McKenzie et al. (1999)
		FL, USA	-	-	-	-	4.3 ± 4.9 (3)	-	65.3 ± 15.7 (4)	McKim and Johnson (1983)
		Hawaii, USA	-	-	-	-	-	-	51.6 ± 6.7 (3)	Miao et al. (2001)
	Chelonia agassizzi	Mexico	<LOD - 18.6 (7)	<LOD (7)	<LOD - 10.4 (7)	-	-	<LOD - 7.8 (7)[d]	-	Gardner et al. (2003)
Kidney	*Caretta caretta*	Italy	200 ± 100 (6)	-	-	-	220 ± 190 (6)	-	340 ± 70 (6)	Storelli and Marcotrigiano (2000a)[b]
		Italy	-	-	-	-	-	5.7 ± 6.5 (19)[d]	19.1 ± 27.2 (19)	Storelli et al. (2007)
	Chelonia agassizzi	Mexico	<LOD (7)	<LOD - 4.8 (7)	<LOD - 22.9 (7)	-	-	<LOD - 10.2 (7)[d]	-	Gardner et al. (2003)
	Lepidochelys olivacea	Mexico	<LOD (1)	7.3 (1)	17.2 (1)	-	-	18.3 (1)[d]	-	Gardner et al. (2003)

a, ng g^{-1} lipid; b, mg kg^{-1} lipid; c, μg g^{-1} dry wt.; d, sum of DDTs (ie. DDDs + DDEs + DDTs); e, unhatched embryos
LOD, limit of detection

Table 1.3. (Cont'd.)

Tissue	Species	Location	Organochlorine Pesticides						PCBs	Reference
			HCB	Dieldrin	Chlordanes	DDD	DDE	DDT		
Muscle	*Caretta caretta*	Italy	300 ± 200 (6)	-	-	-	180 ± 100 (6)	-	160 ± 60 (6)	Storelli and Marcotrigiano (2000a)[b]
		Italy	-	-	-	-	-	-	15 ± 4 (3)	Corsolini et al. (2000)
		Italy	-	-	-	<LOD - 1.6 (11)	0.4 - 20.2 (11)	<LOD - 2.6 (11)	1.5 - 19.5 (11)	Perugini et al. (2006)
		Mexico	<LOD (1)	<LOD (1)	<LOD (1)	-	-	<LOD (1)[d]	-	Gardner et al. (2003)
		Italy	-	-	-	-	-	1.45 ± 1.1 (19)[d]	4.7 ± 5.6 (19)	Storelli et al. (2007)
		FL, USA	-	-	-	-	8.0 ± 14.4 (9)	-	12.9 ± 13.0 (9)	McKim and Johnson (1983)
	Lepidochelys olivacea	Mexico	4.5 (1)	ND (1)	9.0 (1)	-	-	8.6 (1)d	-	Gardner et al. (2003)
	Chelonia agassizzi	Mexico	<LOD (7)	<LOD - 4.8 (7)	<LOD - 13.5 (7)	-	-	<LOD - 14.0 (7)[d]	-	Gardner et al. (2003)
	Chelonia mydas	FL, USA	-	-	-	-	1 ± 0 (2)	-	6.8 ± 1.8 (4)	McKim and Johnson (1983)
Adipose	*Caretta caretta*	NC, USA	2.57 ± 6.40 (12)	35 ± 87.2 (38)	246 ± 412 (43)	<LOD	445 ± 643 (41)	7.03 ± 13.1 (11)	2010 ± 2960 (44)	Keller et al. (2004a)[a]
		VA and NC, USA	-	-	-	12.6 ± 10.8 (20)	194.8 ± 248.8 (23)	5.0 ± 2.4 (11)	565 ± 452 (23)	Rybitski et al. (1995)
		Cyprus, Greece and Scotland	-	5.7 ± 3.7 (3)	19.7 ± 11.6 (3)	4.8 ± 2.6 (3)	509.0 ± 173.3 (3)	4.9 ± 3.0 (6)	-	McKenzie et al. (1999)
		Italy	-	-	-	<LOD - 39.6 (11)	1.5 - 621 (11)	<LOD - 52 (11)	2.9 - 1472 (11)	Perugini et al. (2006)
		Italy	-	-	-	-	-	-	334 ± 179 (3)	Corsolini et al. (2000)
		Mexico	<LOD (1)	<LOD (1)	<LOD (1)	-	-	<LOD (1)[d]	-	Gardner et al. (2003)
	Dermochelys coriacea	Cyprus, Greece and Scotland	-	16.0 ± 4.2 (2)	17.0 ± 7.1 (2)	7.2 ± 1.3 (2)	33.5 ± 33.2 (2)	7.2 ± 1.3 (2)	-	McKenzie et al. (1999)
	Lepidochelys kempii	NC, USA	15.1 ± 13.2 (9)	51.7 ± 66.2 (10)	240 ± 331 (10)	2.96 ± 5.11 (5)	254 ± 332 (10)	257 ± 332 (10)[d]	1110 ± 1030 (10)	Keller et al. (2004a)[a]
		VA and NC, USA	-	-	-	27.3 ± 13.5 (3)	193.9 ± 98.2 (3)	5.19 (1)	659.7 ± 332.5 (3)	Rybitski et al. (1995)
		NY, USA	-	-	-	-	64.1 - 720 (22)	-	149 - 1960 (13)	Lake et al. (1994)
	Lepidochelys olivacea	Mexico	<LOD (1)	<LOD (1)	8.1 (1)	-	-	5.1 (1)[d]	-	Gardner et al. (2003)
	Chelonia mydas	Cyprus, Greece and Scotland	-	2.8 ± 0.8 (3)	2.7 ± 0.7 (3)	2.7 ± 0.7 (3)	9.1 ± 8.7 (3)	2.7 ± 0.7 (3)	-	McKenzie et al. (1999)

a, ng g^{-1} lipid; b, mg kg^{-1} lipid; c, μg g^{-1} dry wt.; d, sum of DDTs (ie. DDDs + DDEs + DDTs); e, unhatched embryos
LOD, limit of detection

Table 1.3. (Cont'd.)

Tissue	Species	Location	Organochlorine Pesticides						PCBs	Reference
			HCB	Dieldrin	Chlordanes	DDD	DDE	DDT		
Adipose	*Chelonia mydas*	Hawaii, USA	-	-	-	-	-	-	284.6 ± 329.9 (3)	Miao et al. (2001)
	Chelonia agassizzi	Mexico	<LOD (7)	<LOD (7)	<LOD - 65.1 (7)	-	-	<LOD - 12.2 (7)[d]	-	Gardner et al. (2003)
Blood	*Caretta caretta*	NC, USA	<LOD	20.1 ± 25.8 (26)	102 ± 151 (43)	4.09 ± 6.45 (20)	300 ± 578 (41)	0.286 ± 1.9 (1)	2490 ± 3700 (44)	Keller et al. (2004a)[a]
	Dermochelys coriacea	NC, USA	-	-	-	-	-	34.1 - 220 (6)[d]	28.2 - 1670 (6)	Stewart et al. (2008)[a]
	Lepidochelys kempii	NC, USA	1.33 ± 2.25 (2)	17.9 ± 16.3 (6)	77.2 ± 81.6 (8)	5.97 ± 1.79 (8)	166 ± 147 (8)	172 ± 147 (8)[d]	985 ± 1250 (8)	Keller et al. (2004a)[a]
Eggs	*Caretta caretta*	Cyprus, Greece and Scotland	-	0.6 (1)	1.8 (1)	0.3 (1)	154 (1)	0.4 (1)	89 (1)	McKenzie et al. (1999)
		FL, USA	-	-	-	-	66 ± 63 (9)	-	-	Clark and Krynitsky (1980)
		FL, USA	-	-	5 - 8 (16)	-	56 - 150 (55)	-	-	Clark and Krynitsky (1985)
		FL, USA	-	1.7 - 44 (22)	4 - 685 (22)	0.1 - 1.7 (4)	0.5 - 1330 (22)	8 - 1340 (22)[d]	7 - 3930 (22)	Alava et al. (2006)
		FL, USA	<LOD	<LOD	<LOD	776.5 ± 33.2 (2)	<LOD	<LOD	1155 - 16730 (20)	Alam and Brim (2000)[c]
	Dermochelys coriacea	NC, USA	-	-	-	-	-	9.96 - 61.9 (6)[d]	7.82 - 388 (6)	Stewart et al. (2008)[a]
	Chelonia mydas	FL, USA	-	-	-	-	2.0 ± 2.0 (2)	-	-	Clark and Krynitsky (1980)
		Cyprus, Greece and Scotland	-	<LOD	<LOD	<LOD	2.3 (1)	0.5 (1)	6.1(1)	McKenzie et al. (1999)
		Ascension Island	-	-	-	-	<LOD - 9 (10)	-	20 - 220 (10)	Thompson et al. (1974)
		Queensland	-	-	-	-	1.7 ± 0.3 (15)	-	-	Podreka et al. (1998)
Hatchling	*Caretta caretta*	Cyprus, Greece and Scotland	-	3.0 ± 4.2 (4)	3.7 ± 3.0 (4)	0.50 ± 0.34 (4)	43.9 ± 44.2 (4)	0.83 ± 0.76 (4)	-	McKenzie et al. (1999)
		SC, USA[e]	-	-	-	-	-	-	1188 ± 1200 (16)	Cobb and Wood (1997)[a]
	Chelonia mydas	Cyprus, Greece and Scotland	-	0.4 ± 0.1 (3)	0.4 ± 0 (3)	0.4 ± 0.1 (3)	1.4 ± 1.7 (3)	0.4 ± 0 (3)	-	McKenzie et al. (1999)

a, ng g^{-1} lipid; b, mg kg^{-1} lipid; c, μg g^{-1} dry wt.; d, sum of DDTs (ie. DDDs + DDEs + DDTs); e, unhatched embryos
LOD, limit of detection

The distribution of POPs in the tissues of sea turtles is highly correlated with tissue lipid content, with highest concentrations of most POPs found in the adipose tissue followed by liver, kidney and muscle (McKim and Johnson 1983; Lake et al. 1994; Rybitski et al. 1995; McKenzie et al. 1999; Corsolini et al. 2000; Storelli and Marcotrigiano 2000a; Miao et al. 2001; Gardner et al. 2003; Storelli et al. 2007). However, as aforementioned, these studies have generally sampled from deceased and stranded animals. Of more interest to the management and conservation of sea turtles is determining the concentrations of POPs in wild populations. This would require non-lethal sampling that represents the contamination of internal tissues. The use of blood samples as a non-lethal method for estimating internal body burdens has recently been investigated in juvenile loggerhead (*C. caretta*) and Kemp's ridley (*L. kempii*) turtles (Keller et al. 2004a). Blood POP concentrations were significantly correlated with concentrations in adipose for both *C. caretta* and *L. kempii*. This indicated that blood may be a useful method for predicting the concentrations of internal tissues in sea turtle. However, further investigation into the specific relationships between blood and internal tissue POP concentrations is required before the use of blood as a predictor of internal burdens in sea turtles can be justified.

The concentration of POPs in sea turtle eggs has also been investigated in a limited number of studies (Clark and Krynitsky 1980, 1985; Cobb and Wood 1997; Podreka et al. 1998; McKenzie et al. 1999; Alam and Brim 2000; Alava et al. 2006). Sea turtle eggs may also provide a non-lethal way for predicting contamination of sea turtles, particularly if unhatched eggs are used. In a study on *D. coriacea*, strong correlations were found between POP concentrations in nesting female blood and eggs (Stewart et al. 2008). This provides evidence of maternal transfer of POPs to eggs and validates the use of eggs to predict contamination of nesting female *D. coriacea*. However, these patterns need to be investigated in other species and the study of further transfer of POPs to hatchlings is also warranted. Furthermore, the effects of POP transfer to eggs on hatchling development could also be investigated to assess the risk of these chemicals to sea turtle populations.

1.4 Heavy metals

There are more than 40 elements in nature that are classified as metals. Heavy metals make their way into the environment from a variety of domestic, industrial and agricultural sources (Table 1.4: Clark 1986; Connell et al. 1999). A number of these, including chromium, cobalt, copper, iron, magnesium, manganese, molybdenum, selenium and zinc are considered essential nutrients and play important roles in functioning and homeostasis of living organisms (Chang 1996). However, these essential elements must be present within a certain concentration range to be beneficial to organisms. Deviations above this range result in the metal becoming toxic, while concentrations below this range can also be detrimental to the functioning of the organism. For metals such as copper and selenium, there is less than one order of magnitude between normal and toxic concentrations (Suzuki and Suzuki 1996). There is also another class of metals known as the toxic metals that are often present in organisms but play no known beneficial role. The toxic metals include aluminium, antimony, arsenic, bismuth, cadmium, silver, lead, lithium, mercury, nickel, platinum and thallium (Suzuki and Suzuki 1996).

Table 1.4. Sources of marine pollution by common heavy metals.

Heavy metal	Source
Cobalt	Pesticides; industrial incinerators; chemical plants; motor vehicle exhaust; combustion of fossil fuels
Copper	Antifouling paint; wood preservatives; algicides; chemical industries; incorrect disposal of electrical equipment
Zinc	Paint additives; rubber manufacture; by-products of dye, wood preservative and ointment manufacture
Selenium	Copper, nickel and zinc smelting by-product; incorrect disposal of photoelectric components; procurement and refinement of oil
Arsenic	Copper, zinc and lead smelting; by-product of chemical and glass manufacture; pesticides
Mercury	Chlorine industry; paper and pulp mills; agricultural pesticides
Cadmium	Zinc smelting by-product; iron and steel industry; zinc galvanising; wear from automobile tyres; sewage sludge; incorrect disposal of batteries and plastics; pesticides
Lead	Motor vehicle exhaust; sewage sludge; lead pipes

1.4.1 Fate of heavy metals in the environment

Once in the marine environment, heavy metals can enter plants and animals through passive diffusion and through animals consuming metal-contaminated food (Clark 1986). The bioavailability of consumed metals depends on the delivery to the systemic circulation. Gastrointestinal uptake of metals is dependent on the solubility of the metal complexes consumed. This is influenced by the natural ligands present in food that bind to the metal ions (Nieboer and Fletcher 1996). Once in the system, the metals are transported throughout the body to the different organs. Positively charged metal ions either bind through electrostatic attraction to electronegatively charged biological membranes or are chelated at the membrane by a number of reactive sites, such as the sulfhydral groups (Foulkes 1996). This interaction with the membrane can be either direct or indirect and results in an alteration of membrane function, such as inhibiting transmembrane transport of electrolytes, sugars, amino acids and solutes (Foulkes 1996). Metals can also enter cells as either free cations, anionic metal compounds (e.g. oxyanions) or in complexes with ligands such as metallothionein (Nieboer and Fletcher 1996).

1.4.2 Effects of heavy metals

The major effects of metals occur at a cellular level. Inside cells, metals can generate free radicals and disrupt cell functioning by binding to the functional sites of proteins. Many of the toxic metals alter the functioning of DNA polymerase, an enzyme responsible for the repair of damaged DNA (Sanders et al. 1996). However, the damage to cellular proteins by toxic metals activates a stress response within the cell. This involves a rapid synthesis of stress proteins that repair and protect the targeted proteins (Sanders et al. 1996). There is also a metal specific stress response, involving the production of metallothioneins (MTs), lysosomes, mineralised and organic-based concretions. These compounds bind to the metals and limit their binding capacity, and hence ability to damage targeted proteins. The synthesis of MT is induced by the presence of many toxic metals in a protective feedback mechanism that occurs at the level of transcription of the MT gene (Sanders et al. 1996).

Despite the cellular response to metal toxicity, protection from toxic metals is not complete. Many metals have been associated with cancer as well as neurological,

cardiovascular, respiratory, gastrointestinal, endocrinological, developmental, renal, hepatic, cognitive, reproductive and immunological pathologies (Chang 1996).

1.4.3 Heavy metals in sea turtles

The presence of heavy metals has been reported in a number of sea turtle populations around the world. Although the elements and tissues analysed vary between studies, zinc (Zn), copper (Cu), cadmium (Cd), mercury (Hg), arsenic (As) and lead (Pb) are the most common essential and toxic metals investigated. Liver, kidney, muscle, blood and eggs are the most commonly analysed tissues (Table 1.5). Like studies on POPs in sea turtles, these studies have generally sampled tissue from deceased and stranded animals. Only three known studies to date have analysed the blood of a wild population (Kenyon et al. 2001; Day et al. 2005; Day et al. 2007). The differences in metal concentrations between sea turtle species are minimal. Furthermore, there are tissue-specific trends in metal accumulation, although these trends vary among species. In *C. mydas*, the concentrations of the essential metals, copper and zinc, were lowest in the muscle. Copper concentrations were higher in liver than in kidney, although the opposite was reported for zinc. For the toxic metals, arsenic was generally highest in muscle followed by kidney, which was higher than liver. Cadmium and lead concentrations were highest in the kidney followed by liver, which was higher than muscle. However, there was no apparent tissue-specific pattern of mercury accumulation apart from muscle concentrations generally being lower than both liver and kidney.

Only three studies to date have investigated metal concentrations in sea turtle blood (Kenyon et al. 2001; Day et al. 2005; Day et al. 2007). Blood concentrations of heavy metals were generally lower than other tissue and egg concentrations for the same species. However, as with the POP studies, there is limited evidence on the correlations between blood and internal tissue concentrations for essential and toxic metals. In the only study of its kind to date, Day et al. (2005) found strong correlations between blood mercury levels and mercury concentrations in the muscle and spinal cord. Furthermore, they found strong correlations between scute mercury levels and mercury concentrations in the liver, muscle, spinal cord and kidney. These findings indicate that both blood and carapace scute sampling may be good indicators of the mercury concentrations in the internal tissues of sea turtles. However,

relationships for essential metals and other toxic metals would further validate the use of non-lethal blood and carapace samples to predict internal metal burdens of sea turtles.

Heavy metal pollution in sea turtle eggs has also received limited attention in the literature (Table 1.5: Stoneburner et al. 1980; Sakai et al. 1995; Vazquez et al. 1997; Godley et al. 1999; Lam et al. 2006). Furthermore, there is very little information about the maternal transfer of metals to eggs and hence the effectiveness of using eggs to predict internal contamination of sea turtles. In the only study to date that sampled eggs and tissue from the same individual female, Sakai et al. (1995) collected eggs from the oviduct and tissue from *C. caretta* caught in fishing nets in Japan. This study found that heavy metal concentrations in the eggs represented < 5% of the entire body burden, concluding that egg laying is not a major route of metal elimination in *C. caretta* (Sakai et al. 1995). Furthermore, the organ:egg ratio was found to be approximately one for essential metals and between 26 and 2000 for toxic metals. This indicated that essential metals were transferred freely from the mother to the eggs, while toxic metals were not (Sakai et al. 1995). Maternal transfer of toxic metals may therefore not be an important factor in the contamination of sea turtle eggs.

The distribution of heavy metals in the different components of *C. mydas* eggs has also been investigated in Hong Kong (Lam et al. 2006). This study revealed that the majority of arsenic, mercury and lead were sequestered in the yolk with much smaller amounts in the albumin and shell. Cadmium however, was highest in the eggshell for this population of *C. mydas*. Analysis of egg contents may therefore not accurately reflect cadmium concentration in sea turtle eggs. Furthermore, arsenic, mercury and lead are more likely to be transferred to developing hatchlings during incubation. As these toxic metals have a number of adverse effects on humans and wildlife, even at trace levels, the analysis of heavy metals in sea turtle eggs is an important area of conservation research.

Table 1.5. Heavy metal concentrations in sea turtles (μg g^{-1} wet mass, unless stated otherwise). Data presented in mean ± SD or range; sample sizes in parentheses.

Tissue	Species	Location	Copper	Zinc	Cadmium	Arsenic	Mercury	Lead	Reference
Liver	*Caretta caretta*	Japan	17.9 ± 8.17 (7)	27.9 ± 10.4 (7)	9.29 ± 3.3 (7)	-	1.51 ± 2.93 (7)	-	Sakai et al. (1995)
		Cyprus	-	-	5.14 - 12.97 (4)	-	0.82 - 7.50 (5)	<LOD - 4.90 (5)	Godley et al. (1999)[a]
		Mexico	16.6 - 58.98 (5)	42.45 - 91.87 (5)	<LOD - 30.62 (5)	-	-	<LOD (5)	Gardner et al. (2006)[a]
		Mexico	-	-	-	-	0.12 - 0.18 (4)	-	Kampalath et al. (2006)
		Mexico	-	-	13.12 ± 1.5 (7)	-	-	13.3 ± 2.1 (7)	Frias-Espericueta et al. (2006)[a]
		Italy	9.4 - 41.8 (14)	23.8 - 178 (14)	1.6 - 114 (14)	-	0.42 - 8.8 (22)	-	Maffucci et al. (2005)[a]
		Italy	1.43 - 17.8 (19)	18.8 - 46.5 (19)	1.1 - 6.55 (19)	-	0.13 - 1.26 (19)	<LOD - 0.29 (19)	Storelli et al. (2005)
		Italy	7.4 ± 3.9 (30)	27.9 ± 6.5 (30)	2.84 ± 0.72 (30)	-	-	-	Franzellitti et al. (2004)
		SC, USA	-	-	-	-	0.59 ± 0.16 (6)	-	Day et al. (2005)
		Queensland	-	22.8 ± 3.0 (5)	16.4 ± 3.3 (8)	0.46 ± 0.24 (6)	0.015 ± 0.0006 (6)	-	Gordon et al. (1998)
		Italy	-	-	-	6.70 ± 4.49 (7)	-	-	Storelli and Marcotrigiano (2000b)
		Canary Islands	0.01 - 65.57 (78)	0.09 - 91.38 (78)	0.04 - 21.98 (78)	0.08 - 131.9 (78)	0.001 - 0.47 (78)	0.05 - 33.09 (78)	Torrent et al. (2004)
		Japan	-	-	-	6.3 ± 1.6 (4)	-	-	Saeki et al. (2000)[a]
		Italy	-	-	7.60 ± 6.05 (12)	21.7 ± 17.2 (12)	1.68 ± 1.04 (12)	1.23 ± 1.01 (12)	Storelli et al. (1998)[a]
		France	8.25 ± 6.59 (7)	25.0 ± 9.5 (7)	2.58 ± 4.12 (7)	-	-	-	Caurant et al. (1999)
		Japan	17.7 ± 8.93 (6)	28.1 ± 4.73 (6)	9.74 ± 3.37 (6)	-	400 ± 155 (6)	0.08 ± 0.03 (6)	Sakai et al. (2000b)
	Eretmochelys imbricata	Queensland	-	17.7 - 30.3 (3)	2.4 - 6.2 (3)	0.18 - 1.85 (2)	0.036 - 0.048 (2)	-	Gordon et al. (1998)
		Japan	-	-	-	0.66 - 7.5 (5)	-	-	Fujihara et al. (2003)
		Japan	54.9 ± 116 (22)	109 ± 54 (22)	7.05 ± 6.37 (22)	-	0.87 ± 1.87 (22)	0.169 ± 0.130 (22)	Anan et al. (2001)[a]
		Mexico	2.47 (1)	25.89 (1)	0.49 (1)	-	-	<LOD	Gardner et al. (2006)[a]
		Japan	-	-	-	15.3 ± 8.8 (4)	-	-	Saeki et al. (2000)[a]
	Lepidochelys olivacea	Queensland	-	-	6.4 (1)	-	-	-	Gordon et al. (1998)
		Mexico	-	-	-	-	0.057 - 0.795 (6)	-	Kampalath et al. (2006)
		Mexico	16.99 - 100 (6)	18.66 - 85.75 (6)	4.98 - 148 (6)	-	-	<LOD	Gardner et al. (2006)[a]
	Dermochelys coriacea	France	8.61 ± 4.40 (18)	29.2 ± 4.1 (18)	6.84 ± 3.66 (18)	-	-	-	Caurant et al. (1999)
		U.K.	0.15 ± 0.04	2.62 ± 0.15	0.22 ± 0.02	0.58 ± 0.11	0.39 ± 0.04	0.12 ± 0.02	Davenport and Wrench (1990)[a]
	Chelonia mydas	Cyprus	-	-	2.53 - 10.73 (6)	-	0.27 - 1.37 (6)	<LOD - 1.84 (6)	Godley et al. (1999)[a]
		Japan	-	-	-	0.8 - 2.3 (5)	-	-	Fujihara et al. (2003)
		Mexico	6.79 - 128 (8)	41.81 - 109 (8)	<LOD - 72.57 (8)	-	-	<LOD - 0.07 (8)	Talavera-Saenz et al. (2007)[a]
		Mexico	-	-	-	-	0.026 - 0.15 (11)	-	Kampalath et al. (2006)
		Mexico	6.79 - 133 (11)	1.32 - 166 (11)	<LOD - 102 (11)	-	-	<LOD	Gardner et al. (2006)[a]
		Queensland	-	39.7 ± 3.0 (30)	12.5 ± 2.0 (38)	0.26 ± 0.04 (23)	0.021 ± 0.003 (23)	-	Gordon et al. (1998)
		Japan	50.2 ± 31.6 (50)	30.3 ± 7.13 (50)	5.58 ± 4.05 (50)	-	0.287 ± 0.156 (46)	<LOD	Sakai et al. (2000a)
		Japan	-	-	-	1.76 ± 0.95 (19)	-	-	Saeki et al. (2000)[a]
		Hong Kong[e]	133.0 ± 148.6 (2)	128.9 ± 63.9 (2)	1.1 ± 1.0 (2)	4.65 ± 3.96 (2)	0.781 ± 0.193 (2)	0.152 ± 0.043 (2)	Lam et al. (2004)[a]
		Japan	139 ± 86 (26)	87.2 ± 30.6 (26)	18.2 ± 9.7 (26)		0.42 ± 0.19 (26)	0.507 ± 0.412 (26)	Anan et al. (2001)[a]
		Hawaii	87.6 ± 64.2 (13)	30.6 ± 10.4 (13)	8.66 ± 8.89 (13)	3.65 ± 3.89 (2)	-	-	Aguirre et al. (1994)
		Hong Kong	9.168 (1)	211.6 (1)	0.1445 (1)	19.57 (1)	0.126 (1)	0.826 (1)	Lam et al. (2004)[a]

a, μg g^{-1} dry weight; b, egg yolk only; c, egg albumin only; d, egg shell only; e, juveniles only

Table 1.5. (Cont'd.)

Tissue	Species	Location	Copper	Zinc	Cadmium	Arsenic	Mercury	Lead	Reference
Kidney	*Caretta caretta*	Japan	1.30 ± 0.20 (7)	25.8 ± 4.17 (7)	39.4 ± 16.2 (7)	-	0.25 ± 0.13 (7)	-	Sakai et al. (1995)
		Cyprus	-	-	18.8 - 42.2 (2)	-	0.13 - 0.80 (2)	<LOD - 4.90 (2)	Godley et al. (1999)[a]
		Canary Islands	0.13 - 49.06 (78)	0.07 - 38.53 (78)	0.01 - 61.1 (78)	1.16 - 122.1 (78)	0.01 - 0.33 (78)	0.02 - 17.3 (78)	Torrent et al. (2004)
		Italy	0.36 - 2.12 (19)	16.6 - 27.9 (19)	1.26 - 16.4 (19)	-	0.06 - 0.31 (19)	<LOD - 0.21 (19)	Storelli et al. (2005)
		Italy	1.7 - 4.7 (19)	62.4 - 206 (21)	10.9 - 158 (19)	-	0.37 - 3.41 (20)	-	Maffucci et al. (2005)[a]
		SC, USA	-	-	-	-	0.214 ± 0.046 (6)	-	Day et al. (2005)
		Queensland	-	18.4 ± 0.9 (5)	28.3 ± 5.7 (5)	0.71 ± 0.26 (3)	0.045 ± 0.011 (3)	-	Gordon et al. (1998)
		Mexico	-	-	-	-	0.075 - 0.108 (2)	-	Kampalath et al. (2006)
		Mexico	1.39 - 8.23 (5)	2.68 - 130 (5)	13.7 - 140 (5)	-	-	<LOD - 69.9 (5)	Gardner et al. (2006)[a]
		Mexico	-	-	15.8 ± 1.2 (7)	-	-	13.4 ± 1.9 (7)	Frias-Espericueta et al. (2006)[a]
		Japan	-	-	-	9.47 ± 5.37 (4)	-	-	Saeki et al. (2000)[a]
		Italy	-	-	24.2 ± 21.4 (12)	29.9 ± 39.5 (12)	0.65 ± 0.34 (12)	0.7 ± 0.35 (12)	Storelli et al. (1998)[a]
		France	2.21 ± 0.46 (5)	23.6 ± 6.9 (5)	13.3 ± 13.6 (5)	-	-	-	Caurant et al. (1999)
		Japan	1.30 ± 0.13 (6)	25.4 ± 4.39 (6)	38.3 ± 17.5 (6)	-	237 ± 140 (6)	0.16 ± 0.05 (6)	Sakai et al. (2000b)
	Eretmochelys imbricata	Queensland	-	13.2 - 20.9 (3)	3.6 - 6.7 (3)	0.13 - 0.93 (2)	0.034 - 0.038 (2)	-	Gordon et al. (1998)
		Mexico	3.89 (1)	82.45 (1)	4.2 (1)	-	-	<LOD	Gardner et al. (2006)[a]
		Japan	-	-	-	28.3 ± 9.82 (4)	-	-	Saeki et al. (2000)[a]
		Japan	7.04 ± 2.79 (19)	120 ± 32 (19)	93.7 ± 76.3 (19)		1.3 ± 1.2 (19)	0.270 ± 0.236 (19)	Anan et al. (2001)[a]
	Lepidochelys olivacea	Queensland	-	-	29.8 (1)	-	-	-	Gordon et al. (1998)
		Mexico	-	-	-	-	0.028 - 0.372 (3)	-	Kampalath et al. (2006)
		Mexico	0.81 - 53.4 (6)	0.43 - 114 (6)	0.81 - 274 (6)	-	-	<LOD - 2.63 (6)	Gardner et al. (2006)[a]
	Dermochelys coriacea	France	2.68 ± 0.33 (5)	25.7 ± 7.7 (5)	30.3 ± 28.1 (5)	-	-	-	Caurant et al. (1999)
	Chelonia mydas	Queensland	-	21.3 ± 0.7 (30)	15.3 ± 2.5 (38)	0.19 ± 0.05 (23)	0.02 ± 0.004 (23)	-	Gordon et al. (1998)
		Mexico	-	-	-	-	0.003 - 0.31 (10)	-	Kampalath et al. (2006)
		Mexico	1.98 - 11.6 (8)	102 - 281 (8)	65.08 - 653 (8)	-	-	<LOD - 1.74 (8)	Talavera-Saenz et al. (2007)[a]
		Mexico	1.59 - 20.36 (11)	1.59 - 330 (11)	6.09 - 653 (11)	-	-	<LOD - 0.36 (11)	Gardner et al. (2006)[a]
		Japan	2.15 ± 0.86 (23)	29.6 ± 7.39 (23)	38.5 ± 21.3 (23)	-	0.132 ± 0.077 (21)	0.18 ± 0.07 (18)	Sakai et al. (2000a)
		Japan	-	-	-	5.72 ± 2.99 (19)	-	-	Saeki et al. (2000)[a]
		Hong Kong[e]	15.20 ± 7.22 (2)	143.1 ± 12.42 (2)	2.49 ± 1.75 (2)	6.97 ± 0.05 (2)	0.342 ± 0.038 (2)	0.311 ± 0.189 (2)	Lam et al. (2004)[a]
		Japan	8.27 ± 4.06 (25)	169 ± 61 (25)	142 ± 64 (25)	-	0.30 ± 0.14 (25)	0.813 ± 0.559 (25)	Anan et al. (2001)[a]
		Hawaii	3.6 ± 2.7 (13)	22.3 ± 7.5 (13)	26.0 ± 21.1 (13)	6.8 (1)	-	-	Aguirre et al. (1994)
Muscle	*Caretta caretta*	Japan	0.83 ± 0.26 (7)	24.2 ± 3.8 (7)	0.06 ± 0.03 (7)	-	0.11 ± 0.05 (7)	-	Sakai et al. (1995)
		Cyprus	-	-	0.30 - 1.43 (4)	-	<LOD - 1.78 (7)	<LOD - 5.53 (4)	Godley et al. (1999)[a]
		Italy	0.8 - 7 (26)	76.4 - 177 (24)	0.06 - 0.78 (26)	-	0.14 - 1.92 (26)	-	Maffucci et al. (2005)[a]
		Italy	0.19 - 1.35 (19)	19.8 - 35.1 (19)	<LOD - 0.13 (19)	-	0.03 - 0.66 (19)	<LOD - 0.09 (19)	Storelli et al. (2005)
		Italy	1.5 ± 0.4 (17)	30.9 ± 8 (17)	0.36 ± 0.11 (17)	-	-	-	Franzellitti et al. (2004)

a, μg g^{-1} dry weight; b, egg yolk only; c, egg albumin only; d, egg shell only; e, juveniles only

Table 1.5. (Cont'd.)

Tissue	Species	Location	Copper	Zinc	Cadmium	Arsenic	Mercury	Lead	Reference
Muscle	*Caretta caretta*	Italy	-	-	-	15.5 ± 11.9 (7)	-	-	Storelli and Marcotrigiano (2000b)
		Canary Islands	0.01 - 27.25 (78)	0.05 - 32.37 (78)	0.15 - 12.48 (78)	1.55 - 67.2 (78)	-	0.22 - 21.1 (78)	Torrent et al. (2004)
		SC, USA	-	-	-	-	0.155 ± 0.07 (6)	-	Day et al. (2005)
		Mexico	<LOD - 3.44 (5)	0.63 - 100 (5)	<LOD - 1.45 (5)	-	-	<LOD - 1.57 (5)	Gardner et al. (2006)[a]
		Mexico	-	-	-	-	0.003 - 0.059 (4)	-	Kampalath et al. (2006)
		Mexico	-	-	2.48 ± 0.4 (7)	-	-	8.9 ± 1 (7)	Frias-Espericueta et al. (2006)[a]
		Japan	-	-	-	20.6 ± 13.1 (4)	-	-	Saeki et al. (2000)[a]
		Italy	-	-	0.55 ± 0.63 (12)	68.9 ± 45.8 (12)	0.69 ± 0.46 (12)	0.54 ± 0.17 (12)	Storelli et al. (1998)[a]
		France	0.73 ± 0.45 (21)	19.6 ± 5.7 (21)	0.08 ± 0.05 (21)	-	-	-	Caurant et al. (1999)
		Japan	0.81 ± 0.28 (6)	25.0 ± 3.49 (6)	0.064 ± 0.028 (6)	-	94.4 ± 36.1 (6)	0.02 ± 0.03 (6)	Sakai et al. (2000b)
	Eretmochelys imbricata	Japan	-	-	-	153 ± 65.1 (4)	-	-	Saeki et al. (2000)$_a$
		Mexico	3.68 (1)	102 (1)	1.02 (1)	-	-	0.38 (1)	Gardner et al. (2006)[a]
		Japan	0.96 ± 0.32 (9)	48.6 ± 26.1 (9)	0.068 ± 0.039 (9)	-	0.04 ± 0.03 (9)	0.043 ± 0051 (9)	Anan et al. (2001)[a]
	Lepidochelys olivacea	Mexico	0.7 - 4.37 (6)	49.89 - 107 (6)	<LOD - 8.85 (6)	-	-	<LOD (6)	Gardner et al. (2006)[a]
		Mexico	-	-	-	-	0.015 - 0.144 (6)	-	Kampalath et al. (2006)
	Dermochelys coriacea	France	0.95 ± 0.49 (16)	25.9 ± 5.9 (16)	0.35 ± 0.20 (16)	-	-	-	Caurant et al. (1999)
		U.K.	0.26 ± 0.05	1.89 ± 0.10	0.06 ± 0.01	0.21 ± 0.07	0.12 ± 0.06	0.31 ± 0.03	Davenport and Wrench (1990)[a]
	Chelonia mydas	Cyprus	-	-	0.12 - 0.78 (6)	-	<LOD - 0.37 (5)	<LOD - 2.45 (6)	Godley et al. (1999)[a]
		Mexico	-	-	-	-	0.003 - 0.059 (10)	-	Kampalath et al. (2006)
		Mexico	<LOD - 13.8 (11)	10.44 - 134 (11)	<LOD - 39.2 (11)	-	-	<LOD - 1.23 (11)	Gardner et al. (2006)[a]
		Japan	0.35 ± 0.41 (47)	8.79 ± 5.51 (47)	0.05 ± 0.08 (45)	-	0.019 ± 0.03 (46)	<LOD	Sakai et al. (2000a)
		Japan	-	-	-	24.1 ± 13.1 (19)	-	-	Saeki et al. (2000)[a]
		Hong Kong[e]	3.735 ± 1.643 (2)	147.7 ± 12.62 (2)	<LOD - 0.096 (2)	14.45 ± 4.9 (2)	0.426 ± 0.211 (2)	0.082 ± 0.103 (2)	Lam et al. (2004)[a]
		Japan	0.88 ± 0.42 (12)	47.7 ± 18.6 (12)	0.24 ± 0.17 (12)	-	0.04 ± 0.07 (12)	0.09 ± 0.05 (12)	Anan et al. (2001)[a]
		Hong Kong	1.56 ± 0.11 (3)	238.7 ± 101.6 (3)	0.17 ± 0.06 (3)	14.6 ± 7.5 (3)	0.053 ± 0.036 (3)	0.26 ± 0.11 (3)	Lam et al. (2004)[a]
	Lepidochelys kempii	France	0.98 ± 0.50 (6)	16.4 ± 3.3 (6)	0.09 ± 0.09 (6)	-	-	-	Caurant et al. (1999)
Blood	*Lepidochelys olivacea*[e]	TX, LA, USA	0.22 - 1.3 (106)	3.28 - 18.9 (106)	-	-	0.005 - 0.07 (106)	<LOD - 0.034 (106)	Kenyon et al. (2001)
	Caretta caretta[e]	SC, USA	-	-	-	-	0.005 - 0.188 (34)	-	Day et al. (2005)
		SC, GA, FL	-	-	-	-	0.006 – 0.077 (66)	-	Day et al. (2007)

a, μg g^{-1} dry weight; b, egg yolk only; c, egg albumin only; d, egg shell only; e, juveniles only

Table 1.5. (Cont'd.)

Tissue	Species	Location	Copper	Zinc	Cadmium	Arsenic	Mercury	Lead	Reference
Eggs	*Caretta caretta*	Japan	1.05 ± 0.199 (5)	14.7 ± 1.44 (5)	0.013 ± 0.004 (5)	-	0.006 ± 0.002 (5)	<0.03	Sakai et al. (1995)
		FL, USA[b]	5.97 ± 0.79 (27)	77.10 ± 9.29 (27)	0.112 ± 0.08 (27)	-	1.36 ± 0.04 (27)	2.19 ± 2.0 (27)	Stoneburner et al. (1980)
		GA, USA[b]	4.97 ± 1.12 (33)	73.54 ± 3.64 (33)	0.195 ± 0.07 (33)	-	1.39 ± 0.11 (33)	1.14 ± 0.84 (33)	Stoneburner et al. (1980)
		NC, USA[b]	5.44 ± 1.11 (15)	78.51 ± 6.70 (15)	0.04 ± 0.002 (15)	-	0.64 ± 0.014 (15)	1.77 ± 1.15 (15)	Stoneburner et al. (1980)
		NC, USA[b]	6.61 ± 1.29 (15)	80.51 ± 5.55 (15)	0.026 ± 0.01 (21)	-	0.41 ± 0.013 (15)	1.24 ± 1.04 (15)	Stoneburner et al. (1980)
		Cyprus	-	-	0.23 - 0.56 (3)	-	0.16 - 0.57 (3)	<LOD - 3.93 (3)	Godley et al. (1999)[a]
	Chelonia mydas	Hong Kong[b]	-	25 - 68 (30)	<LOD (30)	1.4 - 5.0 (30)	0.001 - 0.002 (30)	0.025 - 0.14 (30)	Lam et al. (2006)
		Hong Kong[c]	-	0.038 - 0.45 (30)	<LOD (30)	0.095 - 0.34 (30)	<LOD - 0.0004 (30)	<LOD - 0.014 (30)	Lam et al. (2006)
		Hong Kong[d]	-	0.31 - 2.6 (30)	<LOD - 156 (30)	0.13 - 0.37 (30)	0.001 - 0.002 (30)	0.029 - 0.28 (30)	Lam et al. (2006)
		Cyprus	-	-	0.05 - 1.22 (17)	-	<LOD - 0.19 (17)	<LOD - 1.61 (24)	Godley et al. (1999)[a]
	Dermochelys coriacea[d]	Mexico	8.90 ± 1.26 (15)	11.9 ± 10.0 (15)	0.90 ± 0.61 (15)	-	-	11.6 ± 26.0 (15)	Vazquez et al. (1997)[a]

a, μg g^{-1} dry weight; b, egg yolk only; c, egg albumin only; d, egg shell only; e, juveniles only

1.5 Focus of this thesis

The overall aim of this study was to investigate the accumulation of POPs and heavy metals in the green sea turtle, *Chelonia mydas*. These chemicals have known harmful effects on mammals, birds, fishes and reptiles and may affect the health of sea turtle populations. Therefore, in light of the potential impacts of chemical pollutants on sea turtle populations and the indigenous and traditional cultures that consume sea turtle products, this is an important area of research.

The literature on POPs and heavy metals in sea turtles has been limited to dead or stranded sea turtles. Furthermore, these studies have generally reported a limited number of POP compounds, with many compounds undetected due to poor method sensitivity. In addition, studies on wild populations have been limited by the absence of validated non-lethal sampling methods for sea turtles. The initial objective of this thesis was therefore to develop an analytical method that could detect a large number of POPs at trace concentrations in sea turtle blood, egg and tissue. Once established, this method was used to validate the use of blood, carapace and egg samples as predictors of internal tissue contamination in *C. mydas*. These methods were then used to investigate the chemical contamination of *C. mydas* from different foraging areas as well as the maternal transfer of these chemicals to eggs and hatchlings. Peninsular Malaysia was used as a case study to investigate the extent of chemical contamination within a single management unit and to consider the effects on the *C. mydas* populations and the human communities that are consuming sea turtle products in this region. Finally, the response of *C. mydas* eggs to the topical administration of the pesticide DDE was investigated in a pilot study. This was done to explore the use of this technique in future studies on the effects of chemicals on sex determination and embryonic development in sea turtles. This thesis is therefore divided into the following components:

- Development of a gas chromatography with coupled mass spectrometry (GC-MS/MS) method for analysing 125 POP compounds in *C. mydas* egg, tissue and blood at a limit of detection < 35 pg g^{-1} (Chapter 3).
- Investigation into blood and carapace sampling as non-lethal methods for predicting internal tissue contamination in *C. mydas* (Chapter 4).

- The use of satellite telemetry and egg contaminant analysis to identify conservation issues for a major C. *mydas* breeding population in Peninsular Malaysia (Chapter 5).
- An investigation into maternal transfer, nesting population variation and effects on hatchling development of persistent organic pollutants in *C. mydas* eggs (Chapter 6).
- The chemical contamination of *C. mydas* eggs in Peninsular Malaysia: Implications for conservation and public health (Chapter 7).
- The response of *C. mydas* eggs to the topical administration of the pestcide DDE (Appendix C).

Chapter 2 - General methods

2.1 Study sites and sources of samples

Sea World Sea Turtle Rehabilitation Program

The Sea World Research and Rescue Foundation operates a turtle rehabilitation program at Sea World (27° 57' 37" S, 153° 25' 27" E) on the Gold Coast (Queensland, Australia). This was the site for the collection of blood, carapace and tissue samples for investigating their use as non-lethal indicators of internal tissue contamination (Chapter 4). The Sea World Sea Turtle Rehabilitation Program (SWSTRP) receives ~ 50-70 sea turtles each year from Moreton Bay and the surrounding Gold Coast areas. This region has important sea turtle habitats, with foraging grounds in Moreton Bay, and *Chelonia mydas* and *Caretta caretta* nesting on the surrounding Moreton, North Stradbroke and South Stradbroke Islands. Sea turtles arrive at the SWSTRP with various injuries and ailments ranging from severe boat strikes to general lethargy and buoyancy difficulties. Many of these turtles are rehabilitated and released back into the wild. However, a number of turtles each year with severe injuries and ailments are either euthanased by the veterinary staff or die at the centre. In most cases, post-mortems are performed on deceased animals in an attempt to understand the cause of death.

Peninsular Malaysia

Peninsular Malaysia was the location for studies on variation in egg contamination in *C. mydas* from different foraging grounds (Chapter 5), maternal transfer of persistent organic pollutants (POPs) (Chapter 6), and the chemical contamination of eggs collected from markets (Chapter 7). Peninsular Malaysia is situated in Southeast Asia between Thailand and Singapore and is separated from Borneo Malaysia by the South China Sea (Figure 2.1). Historically, there have been large numbers of nesting *C. mydas* on the coastline and islands of Peninsular Malaysia. Estimates made from egg collection licenses, field surveys and surveys of local residents in 1956, found > 770,000 green turtle eggs laid in the state of Terengganu in that year (Hendrickson and Alfred 1961). This nesting population of *C. mydas* is concentrated around the east-coast state of Terengganu, where 92% of all marine turtle nesting in Malaysia occurs (Hendrickson and Balasingham 1966).

During the 1950s, nearly 100% of all marine turtle eggs laid on the beaches of Malaysia were being collected for human consumption, at a rate of roughly two million eggs per year (Hendrickson and Alfred 1961; Siow and Moll 1981). There has also been a high rate of adult mortality from incidental by-catch in fishing gear due to the overlap of fishing zones and sea turtle inter-nesting habitats (Chan et al. 1988). As a result of decades of egg harvesting and high adult mortality, the nesting population of green turtles in Peninsular Malaysia has declined by > 80% since the 1950s (Limpus 1993a; Ibrahim 1994; Ibrahim et al. 2003).

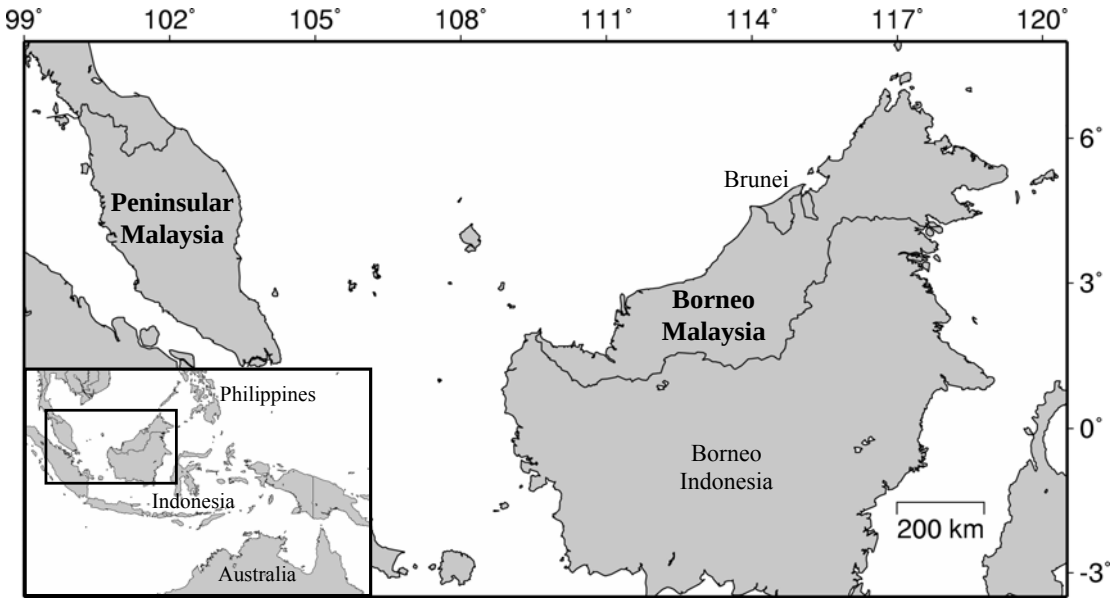

Figure 2.1. Locations of Peninsular and Borneo Malaysia (Inset map: Southeast Asia). Map generated using Maptool (SEATURTLE.ORG 2002).

Currently, there are six major nesting sites for *C. mydas* on the east coast of Peninsular Malaysia, with occasional nesting between these sites and at a small number of locations on the west coast (Figure 2.2). Approximately 1000 - 3000 nests are laid on these beaches each year, with the majority at Ma'Daerah and Redang Island (Kamarruddin Ibrahim, pers. comm.). Although the protection of *C. mydas* eggs in Peninsular Malaysia has increased over recent years, nearly 10% of eggs are still collected for human consumption under a government regulated permit system.

Two studies of this thesis were carried out at the Ma'Daerah Sea Turtle Sanctuary: 1) the variation in egg contamination of *C. my*das from different feeding grounds, as determined by satellite telemetry (Chapter 5); and 2) the maternal transfer of POPs to

eggs and hatchlings (Chapter 6). The Ma'Daerah Sea Turtle Sanctuary (4° 32' 17" N, 103° 28' 14" E) is a 1.7 km sandy beach situated between the coastal towns of Paka and Kerteh, Terengganu. The sanctuary is operated by the Department of Fisheries, Malaysia and is also home to the World Wildlife Fund's (WWF) Sea Turtle Education Program. *Chelonia mydas* nesting in this area has declined by > 80% since the 1950s (Ibrahim 1994; Ibrahim et al. 2003) due to harvesting of eggs for human consumption, beachfront development and interactions with local and regional fishing practices (Hendrickson and Alfred 1961; Siow and Moll 1981; Chan et al. 1988; Limpus 1993a). Currently, 300-700 *C. mydas* nests are laid at Ma'Daerah each year, the majority of which are relocated into hatcheries that have been in use at Ma'Daerah since 1999 (Figure 2.3).

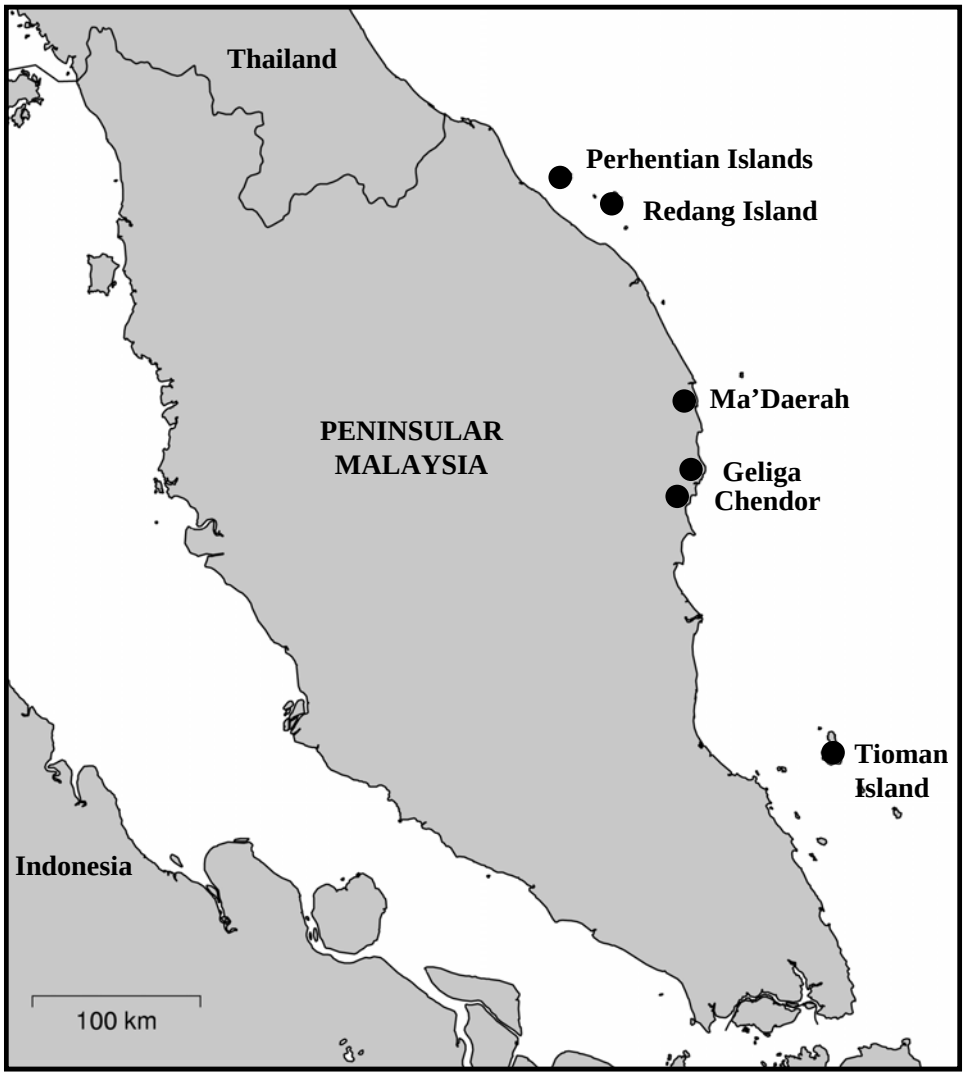

Figure 2.2. Major *Chelonia mydas* nesting sites in Peninsular Malaysia. (Source: Department of Fisheries, Malaysia). Map generated using Maptool (SEATURTLE.ORG 2002).

Figure 2.3. Internal view of a typical hatchery used in Peninsular Malaysia for protection and incubation of sea turtle eggs. Each tag represents a single clutch of *C. mydas* eggs.

2.2 Blood sampling

Animal preparation

In Peninsular Malaysia, blood samples were taken from nesting female *C. mydas* during the nest covering process, following the completion of oviposition. The sand was removed from under the head, which was then gently pushed forward to extend and expose the neck. Hatchlings in Peninsular Malaysia were sampled immediately after emergence from the nest. They were held on a 45° angle between the first and second fingers with the head facing down. The middle finger and thumb were used to extend the head forward, exposing the neck.

Adult, sub-adult and juvenile green turtles sampled from the Sea World Sea Turtle Rehabilitation Program were removed from the pools by hand and placed on a foam mattress with their heads hanging over the edge to expose the neck.

Immediately prior to blood procurement, the skin on the dorsal surface of the neck was cleaned with a 70% isopropyl alcohol swab (Tyco Healthcare Group, Mandfield, MA, USA).

Blood procurement

All blood samples were taken from the dorsal cervical sinuses in the neck, according to methods developed by Owen and Ruiz (1980). For adults and sub-adults, a single-use sterile, non-toxic, non-pryogenic syringe and needle (Terumo, Philippines) was used. For larger individuals (curved carapace length > 30 cm) a 10 mL syringe was used with a 21G x 1½" needle and for smaller individuals (curved carapace length < 30 cm) a 10 mL syringe was used with a 22G x ¾" needle. For hatchlings, a 29G x ½" fixed needle on a 0.5 mL insulin syringe (BD, Franklin Lakes, NJ, USA) was used for blood procurement.

The needle was inserted perpendicular to the dorsal surface of the neck, ~ 1 cm either side of the dorsal-cervical midline and about half way between the head and the carapace (Figure 2.4). A small amount of suction was applied and the depth of the needle was adjusted until the syringe started to fill with blood. The needle and syringe were kept still until the required volume was taken. In cases where the sinus was not initially located, the needle was removed and reinserted either slightly more laterally, or contralaterally on the neck.

A.

B.

Figure 2.4. Photographs of blood procurement procedure. **A**. Lateral view **B**. Dorsal view.

A sample of 10 mL was taken from adults and sub-adults and ~ 350 µL from hatchlings. Samples were immediately transferred to 5 mL glass vacutainers (BD, Franklin Lakes, NJ, USA). Samples for persistent organic pollutant analysis were transferred to lithium heparin tubes and frozen immediately (-20 °C) until analysis. Samples for metal analysis were transferred to EDTA tubes and kept at ~ 4 °C until analysis.

2.3 Turtle morphology

Hatchling measurements

For hatchlings, the maximum straight carapace length (max SCL) was measured from the junction of the skin and nuchal scute to the posterior tip of the post-vertebral scutes (Figure 2.5). The maximum straight carapace width (max SCW) was measured between the lateral carapace-plastron junctions at the widest point. The head length (HL) was measured from the tip of the pipping tooth to the posterior end of the skull (located under the skin of the neck). Head width (HW) was measured directly posterior to the eyes. All measurements were taken with digital callipers (Mitutoyo, Neuss, Germany) to the nearest 0.01 mm. Due to the flexibility of the hatchling carapace, care was taken not to flex the ends with the callipers during measurement.

Adult and sub-adult measurements

For adult and sub-adult turtles the minimum curved carapace length (min CCL) was measured to the nearest 0.1 cm using calibrated flexible fibreglass tape. The min CCL was measured from the junction of the skin and nuchal scute to the posterior tip of the dorsal surface along the centre line of the carapace (Figure 2.5). The carapace width was measured between the lateral carapace-plastron junctions at the widest point.

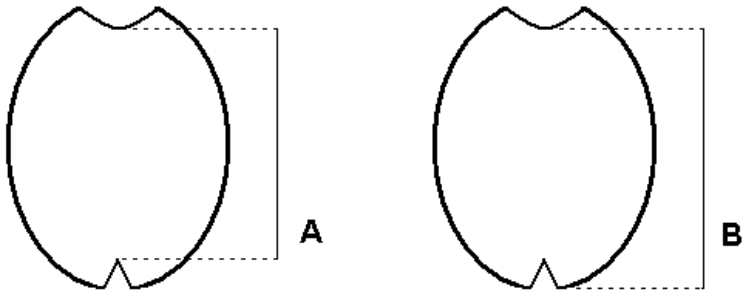

Figure 2.5. Carapace diagrams depicting the different measurements for carapace length. **A.** Minimum curved carapace length (min CCL) used to measure adults and sub-adults. **B.** Maximum straight carapace length (max SCL) used to measure hatchlings.

Mass

The mass of adults and juveniles was measured to the nearest kg by wrapping the turtle in a specially designed harness and suspending the animal from a spring balance. Weighing was done immediately after the nesting process was completed for nesting turtles, and upon arrival for turtles at the Sea World Sea Turtle Rehabilitation Program. For hatchlings, mass was measured to the nearest 0.02 g on a Precisa 3000 electronic balance (Precisa, Switzerland) with a 30 second integrative function to adjust for hatchling movement.

Scute nomenclature

The scutes of all sea turtles used in this thesis were investigated to determine abnormalities in development (Figure 2.6). For adults and sub-adult turtles, only carapace and head scutes were recorded. For hatchlings, carapace, head and plastron scutes were recorded. All individuals were assigned a scute abnormality index, which was calculated as the total number of scute deviations from the normal state (see Figure 2.6). For example, an individual with two extra vertebral scutes and one less left marginal scute was assigned an abnormality index of three. For paired categories (e.g. left and right costals), each side was considered separately.

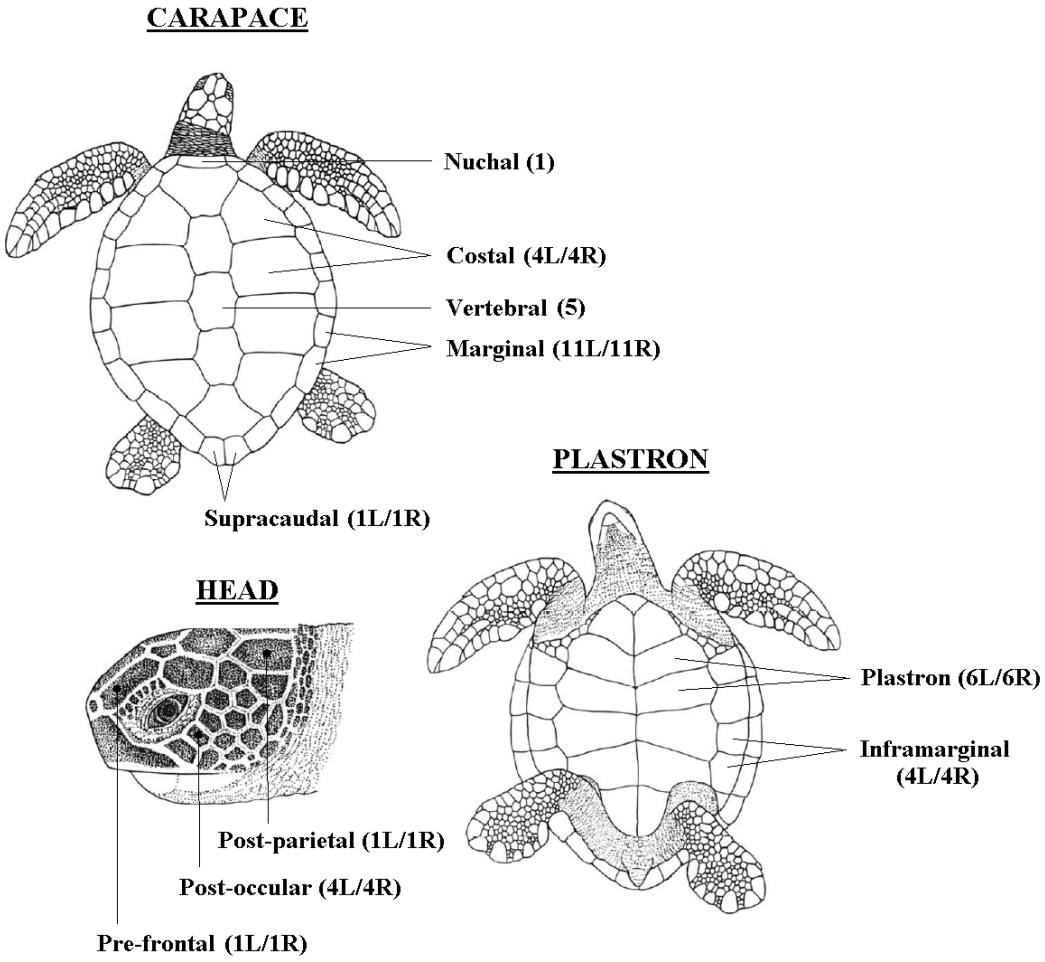

Figure 2.6. Scute nomenclature used for assessment of green turtle abnormalities. A green turtle was considered to have no abnormalities if scute counts matched the figures in brackets (L - left; R - right). Adapted from Pritchard and Mortimer (1999).

2.4 Analysis of heavy metals in *Chelonia mydas* eggs and tissue

Analysis of heavy metals in *C. mydas* eggs and tissue was performed at the Inorganics Section of Queensland Health Scientific Services, Coopers Plains, Queensland, Australia. Cobalt (Co), copper (Cu), zinc (Zn), selenium (Se), arsenic (As), cadmium (Cd), and lead (Pb) were quantified using HNO_3 digestion and inductively coupled plasma mass spectrometry (ICP-MS), based on methods modified from Scheelings (2002), Tinggi et al. (2004) and Sakao and Uchida (1999). Mercury (Hg) was quantified using acid digestion (H_2SO_4, HNO_3, HCl) and cold vapour atomic absorption spectrometry (CV-AAS), using methods modified from Tinggi and Craven (1996).

2.4.1 Sample collection and preparation

Immediately after collection, whole eggs and tissue samples were stored in plastic zip-lock bags and kept frozen at -20 °C until analysis. Prior to homogenisation, eggshells were rinsed with deionised water and the egg contents (albumin and yolk) were emptied into polypropylene containers that had been soaked in 2% HCL overnight, rinsed twice with deionised water and dried. For tissue samples (kidney, liver and muscle), the outer layer that had been in contact with the zip-lock storage bag was removed with a sterile stainless steel scalpel and discarded. Approximately 4 g of each tissue sample was added to an acid washed polypropylene container. Samples were homogenised with a domestic blender (Bamix, Switzerland) immediately prior to digestion. Each sample was prepared in duplicate – one for analysis of Co, Cu, Zn, As, Se, Cd, and Pb with ICP-MS and one for analysis for Hg with CV-AAS.

2.4.2 Quantification by ICP-MS

Sample digestion

Advanced composite Teflon digestion vessels and lids (CEM, NC, USA) were cleaned with dilute detergent (5% Decon 90), rinsed with de-ionised water and soaked (for 2 hours or more) in heated (~ 90 °C) 20% HNO_3. The vessels were then rinsed twice with de-ionised water and left to dry at room temperature. Each sample (1-2 g) was accurately weighed (± 1 mg) into a separate digestion vessel. Concentrated HNO_3 (4 mL; ultra pure, 70% w/w; Australian Chemical Reagents, Australia) was added to each vessel and samples were allowed to stand at room temperature for 20 mins for slow digestion and expulsion of excess gases. Vessels were loaded onto carousels (12 vessels per carousel) and the lids were hand-tightened, ensuring that the ventilation tube of each vessel was properly secured to the collection container. The carousels (3 per batch) were placed inside a microwave digestion system (MD2100; CEM, NC, USA) and digested for 28 min (Table 2.1).

Table 2.1. Microwave digestion heating program.

Stage	1	2	3	4	5
Power (%)*	20	60	70	85	90
Pressure (psi)	20	40	60	85	95
Time (min)	6	6	6	5	5
TAP**	5	5	5	5	3
Temperature (°C)	85	95	100	110	120

*1 % is equivalent to 9.5 watts.
**TAP – time required to reach a pre-set pressure.

Following digestion, the vessels were allowed to cool and the pressure was allowed to drop to < 60 psi. Samples were then transferred to acid washed graduated polypropylene containers and made up to 40 mL with deionised water. Any samples containing undigested tissue were filtered.

Preparation of reagents

A number of solutions and dilutions were prepared from reagents obtained from Agilent, California, USA (Table 2.2). Standards of 10 μg L^{-1} and 1000 μg L^{-1} were prepared by diluting multi-element stock standard solution 2A (Agilent, Santa Clara, CA, USA) in deionised water. An internal standard solution was prepared by adding 10 mL internal standard stock (Agilent, Santa Clara, CA, USA), 20 mL of 100 ppm gold (Au) solution and 40 mL concentrated HNO_3 to a clean acid-leached plastic bottle. This was further diluted in deionised water to produce a solution that contained Au at 100 μg L^{-1} and internal standard elements at 5 μg L^{-1} in 0.2% HNO_3.

Table 2.2. Reagents for preparation of solutions used for metal ICP-MS quantification.

Reagent	Concentration	Elements
Agilent multi-element standard 2A	10 mg L^{-1}	Co, Cu, Zn, As, Se, Cd, Pb
Agilent internal standard mix	10 mg L^{-1}	Li, Sc, Ge, Y, Tb, Bi

ICP-MS analysis

The quantitative analysis of cobalt, copper, zinc, arsenic, selenium, cadmium, and lead was performed with a Hewlett-Packard 4500*plus* ICP-MS with a Neslab Chiller and CETAC ASX-500 autosampler (Hewlett-Packard, Palo Alto, CA, USA). Operating and data acquisition parameters for the ICP-MS are outlined in Table 2.3.

Table 2.3. ICP-MS operating and data acquisition parameters.

Rf power (W)	1300
Carrier gas flow rate (L min^{-1})	1.03
Make-up gas flow rate (L min^{-1})	0.34
Sampler and skimmer cone composition	Ni
Oxide ratio ^{156}CeO:^{140}Ce	< 0.5%
Doubly charged ratio $^{70}Ce^{2+}$:$^{140}Ce^{+}$	< 2.0%
Mode of data acquisition	Quantitative
Points/spectral peak	3
Sweeps/reading	7

Calibration standards ranging from 0.05 to 1000 μg L^{-1} were prepared by spiking 0.5 mL of 5% high purity HNO_3 with the series of prepared multi-element standards (Table 2.4). These mixtures were vortex mixed and filled to 10 mL with deionised water. Immediately prior to analysis, 0.1 mL of internal standard solution was added to each calibration standard and mixed well.

Table 2.4. The volume of multi-element standard 2A dilutions added to 5% HNO_3 to prepare the calibration standards.

Calibration Standard	**Volume of multi-element standard 2A added (mL)**		
(μg L^{-1})	**10 μg L^{-1}**	**1000 μg L^{-1}**	**10 mg L^{-1}**
0.05	0.05	-	-
0.10	0.10	-	-
0.50	0.50	-	-
1	1	-	-
5	-	0.05	-
10	-	0.10	-
50	-	0.50	-
100	-	1	-
500	-	-	0.5
1000	-	-	1

A reagent blank was prepared by adding 0.5 mL of 5% HNO_3 and 0.1 mL internal standard solution to 9.5 mL deionised water in a 10 mL acid washed tube. A calibration blank was prepared by adding 0.5 mL of 5% HNO_3 to 9.5 ml of deionised water and 0.1 mL of internal standard. Samples for analysis were prepared by adding 2.5 mL of sample acid digest to 7.5 mL deionised water and 0.1 mL internal standard solution to a 10 mL acid washed tube. This solution was mixed thoroughly and centrifuged to remove any precipitate prior to analysis in the ICP-MS. For quality

control, at least 10% of samples were done in duplicate and each carousel contained at least one blank (containing only 4 mL of digestion acid) and one in-house seafood mix standard reference material (QAC 180 or FFM 04).

Concentration calculations

The ICP-MS returned concentrations (μg L^{-1}) for all samples, blanks and reference materials. The concentration (μg g^{-1} wet weight) of each element in the tissue samples and reference materials were calculated (Equation 2.1).

$$\text{Concentration (}\mu\text{g g}^{-1}\text{ wet weight)} = \frac{(C_S - C_B) \times V_S}{M_S \times 1000} \qquad \text{……. Equation 2.1}$$

C_S = concentration of sample digestion solution from ICP-MS (μg L^{-1})
C_B = concentration of blank digestion solution from ICP-MS (μg L^{-1})
V_S = volume of sample digestion solution (= 40 mL)
M_S = mass of sample digested (g)

Limit of detection (LOD, in μg L^{-1}) of the ICP-MS for each element was determined as three times the standard deviation of the blank replicates. These values were previously established through Queensland Health Scientific Services in-house quality control analysis (Table 2.5; Scheelings 2002). The limit of reporting (LOR) at a confidence level of 90% for the samples (μg g^{-1} wet weight) was calculated from the LOD of the ICP-MS, the mass of sample digested, the final volume of the digest and the dilution of the digest prior to injection in the ICP-MS (Equation 2.2). Based on a sample mass of 1.5 g and the established LODs, the LORs ranged from 0.01 to 0.05 μg g^{-1} (Table 2.5). However, the LOR varied slightly between runs and samples due to the variation in actual sample mass digested and the quantification of the blanks.

$$\text{Limit of Reporting (}\mu\text{g g}^{-1}\text{ wet weight)} = \frac{LOD_I \times V_S \times D_S}{M_S \times 1000} \qquad \text{…… Equation 2.2}$$

LOD_I = limit of detection of the ICP-MS (μg L^{-1})
V_S = volume of sample digestion solution (= 40 mL)
M_S = mass of sample digested (g)
D_S = dilution factor of the digest (= 4)

Table 2.5. The limit of detection (LOD) of the ICP-MS and the estimated limit of reporting (LOR) for a 1.5 g sample.

	Cu	Zn	Se	As	Cd	Pb
LOD of ICP-MS (μg g^{-1})	0.5	0.5	0.5	0.5	0.1	0.5
LOR of 1.5 g sample (μg g^{-1})	0.05	0.05	0.05	0.05	0.01	0.05

Accuracy and precision

The accuracy of the method was validated by determining metal concentrations in 12 and four replicates of the in-house seafood mix standard reference materials QAC 180 and FFM 04, respectively (Table 2.6). The accuracy of the method was between 86 and 111%.

Table 2.6. Accuracy of the ICP-MS method for determining heavy metal concentrations in standard reference materials QAC 180 and FFMO4. All concentrations presented as mean ± SE (μg g^{-1}).

	Cu	Zn	As	Se	Cd	Pb
QAC 180						
Analysis (n = 12)	3.7 ± 0.3	19.2 ± 1.5	29.2 ± 1.1	2.2 ± 0.1	0.045 ± 0.005	N/A
Reference	3.5 ± 0.3	19.8 ± 1.8	28.8 ± 1.7	2.3 ± 0.2	0.048 ± 0.007	N/A
Accuracy (%)[a]	106	97	101	96	94	N/A
FFM 04						
Analysis (n = 4)	3.0 ± 0.3	14.0 ± 2.8	4.5 ± 0.6	3.4 ± 0.5	0.54 ± 0.11	10.5 ± 2.2
Reference	3.5 ± 1.2	13.0 ± 1.7	4.1 ± 0.4	3.2 ± 0.4	0.49 ± 0.05	10.2 ± 1.6
Accuracy (%)[a]	86	108	109	106	111	103

a, accuracy (%) calculated as: (analysed mean/reference mean) x 100

Precision of the method was estimated by the coefficient of variation (standard deviation/mean x 100) for each element. The coefficient of variation was calculated from a pooled egg sample that was quantified in duplicate in each of the eight analytical runs over the duration of the study. The coefficient of variation ranged from 0.5 to 1.9% (Table 2.7).

Table 2.7. The mean coefficient of variation (± SE) of pooled egg quantified in duplicate over the course of the study (n = 8).

	Co	Cu	Zn	As	Se	Cd	Pb
Coefficient of variation (%)	0.9 ± 0.2	0.8 ± 0.5	1.7 ± 1.1	0.5 ± 0.2	0.8 ± 0.4	1.9 ± 1.0	1.9 ± 0.4

2.4.3 Quantification of mercury in sea turtle egg and tissues using CV-AAS

Sample digestion

Each sample (250 mg) was accurately weighed (± 0.01 g) in duplicate into a 120 mL acid washed (as in 2.4.2) PFA-Teflon pressure release type digestion vessel capable of withstanding pressures up to 250 psi (CEM, Matthews, NC, USA). Concentrated HCl (0.5 mL) and concentrated HNO_3 (2.5 mL) were added to the sample, mixed gently and allowed to stand for 5 minutes. Sulfuric acid (1 mL) was carefully added to the digest by running the acid down the side of the digestion vessel. The digest was mixed well, covered with glass marbles and placed in a block digester at 65 $^{\circ}$C for 2 hr or until completely digested. Following digestion, the vessels were cooled in ice water for 20 min and each sample digest was transferred to an acid washed test tube, made up to 20 mL with deionised water and covered with PVC film.

Preparation of reagents

A mercury stock standard solution (1000 mg L^{-1}) was prepared by dissolving 1.354 g of mercuric chloride in 10 mL of concentrated HNO_3 (BDH, Canada) and making up to 1 L with deionised water. Working standard A (10 mg L^{-1}) was prepared by adding 10 mL of the stock standard solution (1000 mg L^{-1}) to 10 mL concentrated HNO_3 and making up to 1 L with deionised water. Working standard B (100 μg L^{-1}) was prepared by adding 10 mL of working standard A (10 mg L^{-1}) to 5 mL of concentrated HNO_3 and making up to 1 L with deionised water. A reagent blank was prepared by adding 0.5 mL concentrated HCl acid, 2.5 mL concentrated HNO_3 and 1 mL H_2SO_4 to a digestion vessel.

CV-AAS analysis

The concentration of mercury in the samples was quantified with a Varian Techtron SpectrAA 300 atomic absorption spectrophotometer equipped with a mercury hollow cathode lamp (4 mA) and a cold vapour apparatus (Varian, Palo Alto, CA, USA). The band pass was set at 0.5 nm and the resonance wavelength at 253.7 nm.

Calibration standards containing 50, 100, 150 and 200 ng of mercury were prepared by adding 0.5, 1, 1.5 and 2 mL of Standard B, respectively, to separate cleaned digestion vessels. For quality control, the reagent blank, an in house seafood mix

(QAC 150) and a DORM-I dogfish muscle standard reference material (National Research Council, Canada) were run with every ten samples.

Immediately prior to analysis, two drops of anti-foam solution (Dow Corning, Midland, MI, USA) were added to each digest and the signal baseline was stabilised using the signal optimisation parameter option in the software. To create the reduced mercury vapour for cold vapour atomic absorption spectrometry (CV-AAS), 2 mL of stannous chloride was added to each sample. The tube was capped, placed in the CV-AAS system and vortex mixed for 60 s. Immediately following vortex mixing, the tube was opened allowing the carrier gas to sweep the mercury vapour through the AAS. After each sample, an open empty tube was placed in the system until the signal returned to a stable baseline.

Concentration calculations

The CV-AAS returned a value for each sample in nanograms based on the height of the signal peak. The concentration ($\mu g\ g^{-1}$) of each sample was then calculated (Equation 2.3).

$$\text{Mercury Concentration } (\mu g\ g^{-1} \text{ wet mass}) = \frac{M_{AAS}}{M_S \times 10^3} \qquad \text{..... Equation 2.3}$$

M_{AAS} = mass of mercury returned from the CV-AAS (ng)
M_S = mass of sample digested (g)

Limit of detection (LOD) of the CV-AAS method was determined as three times the standard deviation of the reagent blank replicates. The limit of reporting LOR at a confidence level of 90% for the samples ($\mu g\ g^{-1}$ wet mass) was calculated from the LOD of the CV-AAS, the mass of sample digested and the final volume of the digest (Equation 2.4). The LOR was 0.01 $\mu g\ g^{-1}$, although it was sample specific and varied slightly between samples and batches.

$$\text{Limit of Detection } (\mu g\ g^{-1} \text{ wet weight}) = \frac{LOD_C}{M_S \times 10^3} \quad \ldots\ldots \text{ Equation 2.4}$$

LOD_C = limit of detection of the CV-AAS (ng)
M_S = mass of sample digested (g)

Accuracy and precision

The accuracy of the method was validated by determining mercury concentrations in four replicates of DORM-I dogfish muscle (National Research Council, Canada) standard reference material (Tinggi and Craven 1996). The analysed mean was 95% of the certified value. The precision of the method was estimated as the coefficient of variation (standard deviation/mean x 100). The coefficient of variation was calculated from a pooled egg sample that was quantified in duplicate in each of the eight analytical runs over the duration of the study. The coefficient of variation was 1.3 ± 0.2%.

2.5 Quantification of heavy metals in *Chelonia mydas* blood

Analysis of heavy metals in *C. mydas* blood was performed at the Inorganics Section of Queensland Health Scientific Services, Coopers Plains, Queensland, Australia. Cobalt (Co), copper (Cu), zinc (Zn), selenium (Se), arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) were quantified using inductively coupled plasma mass spectrometry (ICP-MS) using methods modified from IUPAC (1976) and Francis and Olszowy (2002).

Sample preparation

Blood samples collected in EDTA tubes were kept at 4 $^{\circ}$C until time of analysis. At the time of sampling, small amounts of excess blood was taken from *C. mydas* and was pooled to form the blood blank used in the preparation of calibration standards and calibration blanks.

Preparation of reagents

Standards of 10 μg L^{-1} and 1000 μg L^{-1} were prepared by diluting a 1:1 mixture multi-element standard 2A (10 mg L^{-1}) and mercury stock standard solutions (10 mg L^{-1}; Agilent, Santa Clara, CA, USA) in deionised water. An internal standard solution was

prepared by adding 10 mL internal standard stock (10 mg L^{-1}; Agilent, Santa Clara, CA, USA), 20 mL of 100 ppm gold (Au) solution (Agilent, Santa Clara, CA, USA) and 40 mL concentrated HNO_3 to a clean acid-leached plastic bottle. This was further diluted with deionised water to produce a solution that contained Au at 100 μg L^{-1} and internal standard elements at 5 μg L^{-1} in 0.2% HNO_3. Triton X-100 (0.01%) was prepared by dissolving 0.1 g Triton X-100 (BDH chemicals, Whitehouse Station, NJ, USA) in 1 L of deionised water.

Calibration standards ranging from 0.05 to 1000 μg L^{-1} were prepared by spiking mixtures of 0.5 mL blood blank and 0.5 mL 0.01% Triton X-100 solution with the series of prepared multi-element 2A + mercury standards (see Table 2.4). These mixtures were vortex mixed and filled to 10 mL with deionised water. Immediately prior to analysis, 0.1 mL of internal standard solution was added to each calibration standard and mixed well.

A reagent blank was prepared by adding 0.5 mL of 0.01% Triton X-100 solution and 0.1 mL internal standard solution to 9.5 mL deionised water in an acid washed 10 mL tube. A calibration blank was prepared by adding 0.5 mL of 0.01% Triton X-100 solution and 0.5 mL of blood blank to 9 mL of deionised water and 0.1 mL of internal standard. Samples for analysis were prepared by adding 9 mL of deionised water and 0.1 mL internal standard to a mixture of 0.5 mL blood and 0.5 mL of 0.01% Triton X-100 solution in a 10 mL acid washed tube. This solution was vortex mixed and centrifuged to remove any precipitate prior to analysis in the ICP-MS. For quality control, 10% of samples were analysed in replicate and a human blood standard reference material (Whole Blood Seronorm Trace Elements; SERO, Norway) was run with every batch of blood samples.

ICP-MS analysis and calculations

The quantitative analysis of cobalt, copper, zinc, selenium, arsenic, cadmium, mercury and lead was performed with the same ICP-MS and settings used for metal analysis in egg and tissue (see Section 2.4.2).

The ICP-MS returned concentrations (μg L^{-1}) for all samples, blanks and reference materials. The concentration of each element in the *C. mydas* blood samples and human blood reference materials were calculated (Equation 2.5).

Concentration (μg L^{-1}) = (C_S - C_B) x D ……. Equation 2.5

C_S = concentration of the sample or reference material from ICP-MS (μg L^{-1})
C_B = concentration of the reagent blank from ICP-MS (μg L^{-1})
D = dilution factor (= 20, as 0.5 mL blood was made up to a final volume of 10 mL)

Limit of detection (LOD, in μg L^{-1}) of the ICP-MS for each element was determined as three times the standard deviation of the blank replicates. These values were previously established through Queensland Health Scientific Services in-house quality control analysis (Table 2.8; Francis and Olszowy 2002). The limit of reporting (LOR) at a confidence level of 90% for the samples (μg L^{-1} wet weight) was defined as three times the LOD of the ICP-MS. The LORs ranged from 0.006 to 0.765 μg L^{-1} (Table 2.8). However, the LOR varied slightly between runs and samples due to the variation in actual sample volume digested and the quantification of the blanks.

Table 2.8. The limit of detection (LOD) and limit of reporting (LOR) for ICP-MS methods for measuring heavy metals in blood.

	Co	**Cu**	**Zn**	**Se**	**As**	**Cd**	**Hg**	**Pb**
LOD of ICP-MS (μg L^{-1})	0.002	0.013	0.255	0.144	0.008	0.005	0.014	0.003
LOR for samples (μg L^{-1})	0.006	0.039	0.765	0.432	0.024	0.015	0.042	0.009

Precision and accuracy

The accuracy of the method was validated by determining metal concentrations in eight replicates of Whole Blood Seronorm Trace Elements standard reference material (SERO, Norway). The concentrations of the analysed Seronorm samples were within the certified ranges for all elements (Table 2.9).

Table 2.9. Accuracy of the ICP-MS method for determining heavy metal concentrations in the Whole Blood Seronorm Trace Elements standard reference material (SERO, Norway). All concentrations presented as mean ± SE (μg L^{-1}).

	Co	Cu	Zn	As	Se	Cd	Hg	Pb
Analysis (n = 8)	5.3 ± 0.2	582 ± 10	4758 ± 135	12.1 ± 0.6	119 ± 3	5.8 ± 0.1	7.1 ± 0.3	382 ± 6
Certified range	5.3-6.9	581-665	4669-5407	10.6-15.8	103-143	5.2-6.8	7.0-8.6	351-435

Precision of the method was estimated by the coefficient of variation (standard deviation/mean x 100) for each element. The coefficient of variation was calculated from a pooled *C. mydas* blood sample that was quantified in duplicate in each of the eight analytical runs over the duration of the study. The coefficient of variation ranged from 0.6 to 2.2% (Table 2.10).

Table 2.10. The mean coefficient of variation (± SE) of pooled *C. mydas* blood quantified in duplicate over the course of the study (n = 8).

	Co	Cu	Zn	As	Se	Cd	Hg	Pb
Coefficient of variation (%)	1.2 ± 0.3	0.7 ± 0.4	1.9 ± 1.1	0.6 ± 0.2	0.8 ± 0.5	1.4 ± 0.8	1.3 ± 0.6	2.2 ± 0.7

2.6 Determination of persistent organic pollutants in *Chelonia mydas*

The determination of persistent organic pollutants in *C. mydas* blood, egg and tissue required the development of specific methods. The method development for POP analysis is detailed in Chapter 3.

Chapter 3 - Development of methods for measuring persistent organic pollutants (POPs) in *Chelonia mydas* eggs, blood and tissue

3.1 Introduction

The study of persistent organic pollutants (POPs) such as organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) is an important area of sea turtle health and conservation research. These chemicals are extremely persistent in the environment due to their lipophilic properties and resistance to breakdown and can have a number of harmful effects on the development and functioning of sea turtles and other animals (see Chapter 1). A number of studies have reported POPs in the tissues and eggs sea turtles, although the information for *Chelonia mydas* is limited (Thompson et al. 1974; Clark and Krynitsky 1980; McKim and Johnson 1983; Podreka et al. 1998; McKenzie et al. 1999; Miao et al. 2001). Although omnivorous as juveniles (Bjorndal 1985; Limpus and Miller 1993; Reich et al. 2007), sub-adult *C. mydas* convert to a diet of seagrass and macroalgae, which they maintain for their adult lives (Mortimer 1982; Bjorndal 1985, 1997). Due to their low trophic level, *C. mydas* generally have lower concentrations of POPs compared to other marine vertebrates. As a result, many POP compounds cannot be detected in *C. mydas* using the current analytical methods. Furthermore, previous studies have generally used specific methods that limit reporting to a small number of compounds. There is therefore a current need to further develop methods that can detect a large number of POPs in *C. mydas* at trace concentrations.

Prior to the beginning of this study, POPs in sea turtle tissues and eggs were generally quantified using gas chromatography with electron capture detection (GC-ECD), following various extraction and clean-up procedures (Table 3.1). The advantages of GC-ECD for measuring halogenated compounds are that it is sensitive, rapid and has excellent separation efficiency. However, compound identification using GC-ECD relies completely on relative retention times. Co-eluting compounds therefore cannot be differentiated using GC-ECD. For example, GC-ECD cannot distinguish between polychlorinated biphenyl (PCB) congeners with the same number of chlorine atoms

and can often produce false positives if matrix interferences elute with the retention time of a compound of interest (Harrad et al. 1992; Martinez-Vidal et al. 2002). Furthermore, electron-capture detectors produce a non-linear response across a narrow concentration range, which may produce wide variation in response within a PCB group (Cochran and Frame 1999). Methods using GC-ECD also generally report to a limit of detection of > 0.1 ng g^{-1} with the majority > 1 ng g^{-1} wet mass (Table 3.1). There are therefore a number of limitations in using GC-ECD for the determination of a large number of POP compounds at very low concentrations.

More recently, methods for determining POPs in sea turtle blood, tissue and eggs using gas chromatography with mass spectrometry (GC-MS) have been developed. Using similar extraction and clean-up procedures as previous sea turtle studies and methods modified from quantifying organochlorine compounds in ringed seals (*Phoca hispida*) and polar bears (*Ursus maritimus*) (Kucklick et al. 2002), a large number of POPs have been reported in sea turtles to a limit of detection of 5-10 pg g^{-1} (Keller et al. 2004a; Keller et al. 2004b; Alava et al. 2006; Keller et al. 2006; Stapelton et al. 2007). These methods used accelerated solvent extraction (ASE) with dichloromethane to extract POPs from blood, tissue samples and eggs. Extracts were cleaned by gel permeation chromatography (GPC) and fractionated in semi-preparative aminopropylsilane columns that separated the higher and lower polarity compounds. GC-ECD was initially used to quantify POPs in the fraction containing the lower polarity compounds. Following this, GC-MS in electron-impact (EI) mode with selected ion monitoring (SIM) was used to quantify the POPs in the higher polarity fractions (Keller et al. 2004a; Keller et al. 2004b; Alava et al. 2006; Keller et al. 2006). Furthermore, for quantification of higher brominated PBDEs, the higher polarity fraction was re-injected onto a shorter column on a GC-MS set to negative chemical ionisation (NCI) mode with SIM (Stapelton et al. 2007).

Table 3.1. Methods used for determination of POPs in sea turtle tissue prior to 2004. Quantification by GC-ECD unless noted.

Extraction / Clean-up	Species	Tissue	Study
Soxhlet / Florisil (or Alumina) column	*Chelonia mydas*	Eggs	Thompson et al. (1974)
	C. mydas, Caretta caretta,	Eggs	Clark and Krynitsky (1980; 1985)[a]
	C. caretta	Eggs, Chorioallantonic Membrane	Cobb and Wood (1997)
	C. mydas, C. caretta, Dermochelys coriacea	Liver, Adipose, Hatchlings, Eggs	McKenzie et al. (1999)
	C. caretta, Lepidochelys kempii	Liver, Fat, Muscle, Kidney	Rybitski et al. (1995)[b]
Acetonitrile liquid extraction / C18 and Florisil column	*C. mydas*	Eggs	Podreka et al. (1998)[c]
Supercritical fluid extraction / Sulfuric acid	*C. mydas*	Liver, Adipose	Miao et al. (2001)[d]
1 N KOH ethanol digestion + hexane / silica column, 10% fuming sulphuric acid	*C. caretta*	Liver, Muscle, Fat	Corsolini et al. (2000)
Hexane elution on Florisil / Celite column	*C. mydas, C. caretta,*	Liver, Muscle	McKim and Johnson (1983)
Accelerated Solvent Extraction (50:50 methylene chloride: hexane) / no clean-up	*Chelonia agassizii, Lepidochelys olicvacea, C. caretta*	Muscle, Liver, Adipose, Kidney	Gardner et al. (2003)
Brinkman Polytron (Acetonitrile + pentane) / silica gel column	*L. kempii*	Liver, Fat	Lake et al. (1994)
Tissumizer (dichloromethane) / Alumina and silica gel columns	*C. caretta*	Eggs	Alam and Brim (2000)
Blend in petroleum ether / Florisil column	*C. caretta*	Liver, Kidney, Heart, Muscle, Lung	Storelli and Marcotrigiano (2000a)[e]

a, methods modified from Cromartie et al. (1975)
b, quantification by high resolution GC with electrolytic conductivity detector (ELCD)
c, methods modified from Schenck et al. (1994)
d, quantification by high resolution GC-ECD
e, methods modified from Erney (1983)

Gas chromatography with mass spectrometry (GC-MS) allows the identification of specific compounds through a combination of the retention time on the GC column and the distinct combination of the ions in the mass spectrum resulting from ionisation. Following separation through the GC column, the compounds are ionised by either electrons (EI) or chemicals (NCI), resulting in a distinct set of structurally related ions. These ions are captured by the mass spectrometer to produce a compound specific spectrum that can confirm the presence or absence of a particular compound through reference to a spectra library. However, in a contaminated matrix, other compounds will also be fragmented and their ions will create interference in the spectrum of the compound of interest, hence affecting the sensitivity of this method. This problem is overcome with selective ion monitoring (SIM), which targets one or two of the major ions in each compound's spectrum. This reduces background interference in the spectra by preventing other ions from this compound and other matrix compound ions from hitting the MS detector. The use of SIM therefore greatly increases the sensitivity so that these compounds can be detected at very low concentrations. However, GC-MS methods cannot distinguish between compounds of interest and background analytes that have similar retention times and the same major ion. Therefore, when this occurs, large positive interferences cause overestimations of concentrations.

Although GC-MS methods have eliminated some of the limitations of GC-ECD, the multiple injections involved in current methods for measuring POPs in sea turtle blood, eggs and tissue greatly increase the time and cost required to analyse samples. Furthermore, GC-MS methods cannot separate a compound of interest from a co-eluting background analyte with the same major ion. Quantification of POPs using a single injection, while not compromising selectivity or sensitivity, is therefore required. Gas chromatography with coupled mass spectrometry (GC-MS/MS) has been used to quantify organochlorine compounds and PCBs at trace levels in human milk, fat and serum (Martinez-Vidal et al. 2002; Moreno-Frias et al. 2004) and in the tissues of fish and seals (Serrano et al. 2003; Wang et al. 2005). The GC-MS/MS method isolates one of the major ions for each compound fragmented in EI or NCI ionisation modes and energises it to keep it within the ion trap. It is then further fragmented by EI or NCI, producing a unique set of product ions that is scanned out of the ion trap into the mass spectrometer to produce a spectrum representing that

compound. This method is therefore advantageous over the GC-MS methods in cases where the major ion of a compound of interest and a co-eluting background component have the same mass. The fragmentation of these different ions with the same mass will most likely produce independent sets of product ions that can be used to distinguish the compound of interest from the background compound. In cases where the product ions remain the same, the process can be repeated (GC/MS/MS…) until unique sets of product ions result. Furthermore, similar to the SIM method, the isolation of specific ions in GC-MS/MS reduces the amount of background signal and hence enhances sensitivity.

In this study, it was of interest to screen for a large number of POPs at very low concentrations in *C. mydas*. The objective of this chapter was therefore to develop a GC-MS/MS method capable of measuring the concentration of 125 POP compounds in sea turtle blood, eggs and tissues to a limit of detection of < 35 pg g^{-1} wet mass.

3.2 Development of GC-MS/MS parameters

Instrument parameters

All analyses were performed on a Varian 3800 gas chromatograph fitted with a Saturn 2200 mass spectrometer, a 1079 programmable temperature vapourising (PTV) injector and an 8200 auto-sampler fitted with a 100 μL glass syringe (Varian, Palo Alto, CA, USA). Instrument parameters were modified from GC-MS methods previously used for the analysis of POPs in *Caretta caretta* blood (Keller et al. 2004b). Injector and detector temperatures were 250 °C and 200 °C respectively and the helium carrier gas was set at a constant flow rate of 1.5 mL min^{-1}. The compounds were separated on a 60 m VF-5MS GC capillary column with 0.25 mm interior diameter and 0.32 μm film thickness (Varian, Palo Alto, CA, USA). The column oven was programmed from an initial temperature of 100 °C (1.5 min hold) to 150 °C (25 °C min^{-1}, 5 min hold), 200 °C (0.8 °C min^{-1}, 0 hold) and finally to 280 °C (3 °C min^{-1}, 28 min hold) for a run time of 125 min. Injection volume was set to 20 μL and electron impact (EI) was selected as the mode of ionisation.

Reagents

Calibrants containing 86 PCB congeners, 26 OCPs, 27 PBDE congeners, three hexabromocyclododecane (HBCD) isomers and methyl-triclosan in 2,2,4-trimethyl-pentane (99.6% purity; Burdick and Jackson, NJ, USA) were obtained from the National Institute of Standards and Technology (NIST), South Carolina, USA. Two separate calibrants were prepared at NIST on December 7, 2005 and January 12, 2007 by combining standard reference materials (SRMs) 2261, 2262, 2274 and 2275 with the NIST prepared solutions PCB III, PCB IV, PBDE 26, HBCD mixture, PBDE 209 and octachlorostyrene (see Appendix A).

The preparation and purity of SRMs 2261 (Chlorinated Pesticides in Hexane), 2262 (Chlorinated Biphenyl Congeners in 2,2,4-trimethyl-pentane), 2274 (Chlorinated Biphenyl Congeners in 2,2,4-trimethyl-pentane II) and 2275 (Chlorinated Pesticides in Hexane II) are outlined in the NIST Certificates of Analysis (NIST 2006). The PCB mixtures PCB III (15 PCB congeners) and PCB IV (31 PCB congeners and pentachlorobenzene) and PBDE 26 (mixture of 27 PBDE congeners) were prepared gravimetrically by individually adding pure solid PCB chemical standards and pentachlorobenzene (purity > 99%; Accustandard, CT, USA) to 2,2,4-trimethyl-pentane (99.6% purity; Burdick and Jackson, NJ, USA). Solutions containing octachlorostyrene (99.9% purity; Accustandard, CT, USA) and 3 HCBD isomers (> 98% purity; Wellington Laboratories, Ontario, Canada) were prepared by gravimetrically adding these solid compounds to 2,2,4-trimethyl-pentane (99.6% purity; Burdick and Jackson, NJ, USA). Initial concentrations for each compound in the two calibrants were calculated (Appendix A) and the mass of each calibrant was monitored after each use to track changes in concentration due to evaporation.

An internal standard solution containing mass labelled, deuterated and fluorinated OCP, PCB and PBDE compounds in ethanol was obtained from NIST. This was prepared on June 1, 2006 from commercially available stock solutions (Wellington Laboratories, Ontario, Canada; Cambridge Isotope Laboratories, MA, USA; Chiron, Trondheim, Germany) that were combined gravimetrically in 2,2,4-trimethyl-pentane (Appendix B). This mixture was evaporated to 0.6 mL and diluted to 100 mL in ethanol (ACS grade; Sigma-Aldrich, MO, USA) resulting in concentrations of ~ 140

ng g^{-1} for most compounds (Appendix B). Prior to delivery 1.8 mL aliquots were transferred into autosampler vials and stored at -20 °C until use.

A recovery solution containing ^{13}C-PCB 47 and ^{13}C-PCB 155 in 2,2,4-trimethyl-pentane was obtained from NIST. This was prepared on October 23, 2005 by gravimetrically combining solutions of ^{13}C-PCB 47 and ^{13}C-PCB 155 (CIL, MA, USA) in 2,2,4-trimethyl-pentane (99.6% purity; Burdick and Jackson, NJ, USA) and diluting in 2,2,4-trimethyl-pentane, resulting in concentrations of ~ 30 ng g^{-1} for each compound.

Collision-induced dissociation voltages

Approximately 1 g of calibrant (containing ~ 100 ng of each compound) was accurately weighed in a glass syringe (± 0.00001 g) and transferred to a glass GC-vial. Internal standard solution (~ 50 μL), containing ~ 3-6 ng of each compound, and a recovery standard solution (~ 250 μL), containing ~ 5 ng of recovery compounds ^{13}C PCB-47 and ^{13}C PCB-155, were accurately weighed (± 0.00001 g) and added. The mixture was then evaporated to ~ 100 μL with nitrogen gas. The sample was injected into the GC-MS operating under the parameters outlined above. The chromatograms and spectra were analysed using the MS Workstation software (Varian, Palo Alto, CA, USA), and the compounds present were identified through elution time and the NIST 05 mass spectral reference library software (NIST, South Carolina, USA). A parent (or target) ion was identified from the spectrum of each compound, which was generally the base peak ion (ie. ion with the highest proportion). However, an alternative ion with high proportion was chosen in cases where compounds with the same retention time had base peak ions within three mass units of each other.

The optimal collision-induced dissociation (CID) voltage for each parent ion was determined by creating an automated method development (AMD) time window for each compound using the MS Workstation software. Compounds eluting near each other with the same parent ion (e.g. PCB congeners with the same number of chlorine atoms) were included in the same time window and in cases where compounds with different parent ions co-eluted, a secondary AMD method file was produced and analysed separately. Excitation storage level (RF) was set for each parent ion based on

its mass (Table 3.2). This was the voltage applied to the ring electrode to keep the ions stored in the ion trap during ionisation. Increasing the voltage with mass of the target ions excludes and hence reduces interference from smaller mass ions.

Table 3.2. Excitation storage levels for parent ions.

Parent ion mass (m/z)	Excitation storage level (m/z)
<200	48
200-300	75
300-390	90
>390	100

For each time window, the low and high mass values were set as the excitation storage voltage for that compound and 600 m/z, respectively. Waveform was set to "non-resonant" and eight values were entered as excitation voltages ranging from 30 to 100 V in 10 V increments. The scan time was determined by entering 0.1 s $scan^{-1}$ and using the corrected minimum allowed by the software. The multiplier offset and emission current were set to 200 V and 80 μA, respectively. Count threshold was set to 1 and mass defect was left at 0 mmu/100.

The prepared calibrant containing ~ 100 ng of each compound of interest, 3-6 ng of each internal standard and ~ 5 ng of each recovery compound was reinjected into the GC-MS. The chromatogram for each compound was investigated to determine the 10 V excitation voltage range that resulted in complete dissociation of the parent ion into product ions. The excitation voltage range in the AMD was then modified to include voltages within this 10 V range in 1 V increments. The calibrant mixture was re-injected into the GC-MS and the precise optimal CID voltage was determined as the excitation voltage that dissociated 90-95% of the parent ion (see Table 3.3). In cases where compounds did not dissociate under the "non-resonant" waveform, the "resonant" waveform type was chosen and the method was repeated starting with an excitation amplitude voltage range of 1-10 V.

Multiple reaction monitoring

Following determination of optimal CID voltages for each compound, the GC-MS/MS method was established using the multiple reaction monitoring (MRM) method. Using the MS Workstation software, time windows were set up containing

one to four compounds with similar retention times. A separate experiment was set up for each of the compounds in each time window. The excitation storage level and excitation amplitude were entered for each parent ion as determined by the previous AMD experiments. Low mass was entered as 20 m/z lower than the smallest product ion and the high mass as 20 m/z above the parent ion. Emission current was set to 70 μA and the multiplier offset to 300 V. The scan time was determined as in AMD by entering 0.1 s $scan^{-1}$ and using the corrected minimum allowed by the software. To allow sufficient time for the ion trap to effectively perform all ionisation experiments in each time window, the number of compounds in each window was restricted to four and at least 30 s was allowed between the last peak of a time-window and the first peak of the next window. Due to the large amount of compounds in the calibrant and the limitation of a maximum of four compounds per window, a number of the compounds in the calibrant could not be analysed using this method. Furthermore, only 14 of the internal standard compounds were targeted, which adequately represented the compounds of interest in this study. The product ions and time windows for 23 OCPs, 83 PCBs, 19 PBDEs and 14 mass labelled internal standard and recovery compounds were determined (Table 3.3).

Table 3.3. Assigned time windows and important parameters for the GC-MS/MS method. Target ions were determined by EI full scan; CID voltages and product ions determined by AMD under the assigned excitation storage levels.

Compound	Time window (mins)	Target ion (m/z)	Excitation Storage (m/z)	CID (V)[a]	Major Product Ion (m/z)
PCB 1	16.00 - 20.00	188	48	41	153
pentachlorobenzene		250	75	75	142
alpha-HCH	20.00 - 35.80	219	75	50	181
^{C}13-HCB		290	75	1.44R	253
HCB		284	75	1.6R	247
PCB 8		222	75	50	181
beta-HCH		219	75	50	181
gamma-HCH		219	75	50	181
PCB 18	35.80 - 38.00	256	75	59	186
PCB 29	38.00 - 45.90	256	75	70	186
PCB 50		292	75	59	220
^{13}C-PCB 28		268	75	68	198
PCB 28		256	75	70	186
PCB 31		256	75	70	186
heptachlor	45.90 - 49.60	272	75	59	237
PCB 45		292	75	57	220
^{13}C-PCB 52	49.60 - 53.00	304	90	75	232
PCB 52		292	75	59	220
PCB 49		292	75	59	220
^{13}C-PCB 47		304	90	79	232
aldrin	53.00 - 54.30	263	90	96	191
PCB 104		326	90	79	254
PCB 44	54.30 - 60.50	292	75	59	220
octachlorosytrene		306	80	1.6R	271
^{13}C-oxychlordane	60.50 - 61.90	397	100	72	268
oxychlordane		389	100	68	263
PCB 63		292	75	65	220
heptachlor epoxide		353	90	65	263
PCB 74	61.90 - 64.60	292	75	65	220
PCB 70		292	75	65	220
PCB 95		326	90	78	254
PCB 66		292	75	65	220
PCB 121		326	90	78	254
^{13}C-PCB 155	64.60 - 66.97	372	100	88	300
^{13}C-trans-chlordane		381	100	69	274
trans-chlordane		373	90	62	264
PCB 56		292	75	64	220
PCB 92	66.97 - 67.92	326	90	72	254
2,4'-DDE		246	75	74	176
PBDE 30	67.92 - 69.08	248	75	72	139
PCB 101		326	90	76	254
endosulfan I		195	48	39	159
cis-chlordane		373	90	58	264
PCB 99	69.08 - 69.90	326	90	78	254
^{13}C-trans-nonachlor		419	100	70	310
trans-nonachlor		409	100	70	300

a, all CID voltages determined in non-resonant waveform type unless denoted "R" for resonant waveform type

Table 3.3. (Cont'd.)

Compound	Time window (mins)	Target ion (m/z)	Excitation Storage (m/z)	CID (V)[a]	Major Product Ion (m/z)
PCB 79	69.90 - 71.50	292	75	65	220
PCB 119		326	90	82	254
PCB 112		326	90	82	254
PCB 87	71.50 - 73.75	326	90	75	254
^{13}C-dieldrin	73.75 - 74.50	275	75	68	240
diedrin		263	75	71	191
^{13}C-4,4'-DDE		330	75	54	258
4,4'-DDE		318	90	70	246
PCB 110	74.50 - 75.70	326	90	82	254
PCB 154		360	90	73	288
2,4'-DDD		235	75	60	165
PCB 77		292	75	65	220
PCB 82	75.70 - 76.85	326	90	73	254
PCB 151		360	90	71	288
endrin	76.85 - 77.80	263	75	71	191
PBDE 17		248	75	72	139
PBDE 25		248	75	72	139
PCB 107	77.80 - 79.10	326	90	82	254
PCB 149		360	90	71	288
PCB 106		326	90	82	254
^{13}C-PCB 118		338	100	97	266
PCB 118		326	90	82	254
cis-nonachlor	79.10 - 79.70	409	100	68	300
PBDE 28		248	75	72	139
PBDE 33		248	75	72	139
PCB 114	79.70 - 80.43	326	90	82	254
2,4'-DDT		235	75	65	165
4,4'-DDD		235	75	65	165
PCB 165		360	90	74	288
PCB 188		394	100	83	324
PCB 146	80.43 - 81.00	360	90	72	288
^{13}C-PCB 153	81.00 - 82.50	372	100	86	300
PCB 132		360	90	73	288
PCB 153		360	90	73	288
PCB 105		326	90	83	254
PCB 127		326	90	83	254
PCB 137	82.50 - 83.73	360	90	72	288
PCB 176		394	100	83	324
PCB 130		360	90	72	288
PCB 163	83.73 - 84.50	360	90	74	288
^{13}C-4,4'-DDT		247	75	64	177
PCB 138		360	90	74	288
4,4'-DDT		235	75	65	165
PCB 158		360	90	74	288
PCB 178	84.50 - 86.25	394	90	67	324
PCB 126		326	90	84	254
PCB 175		394	90	67	324
PCB 166		360	90	76	288
PCB 187		394	90	67	324
PCB 159		360	90	76	288
PCB 183		394	90	67	322

a, all CID voltages determined in non-resonant waveform type unless denoted "R" for resonant waveform type

Table 3.3. (Cont'd.)

Compound	Time window (mins)	Target ion (m/z)	Excitation Storage (m/z)	CID (V)[a]	Major Product Ion (m/z)
PCB 128	86.25 - 86.78	360	90	72	288
PCB 167	86.78 - 87.30	360	90	77	288
PCB 185		396	90	67	324
PCB 174	87.30 - 88.64	394	90	67	324
F-PBDE 47		504	100	66	344
PCB 177		394	90	67	324
PCB 202		430	100	79	358
PBDE 75		486	100	67	326
PCB 156	88.64 - 89.35	360	90	76	288
PCB 201		430	100	79	358
PCB 157		360	90	76	288
PBDE 49		484	100	65	324
PCB 172	89.35 - 89.80	394	90	67	324
PCB 197		430	100	79	358
PBDE 71		484	100	63	324
^{13}C-PCB 180	89.80 - 91.20	406	100	81	336
PCB 180		394	90	70	324
PCB 193		394	90	70	324
PCB 191		394	90	70	324
PBDE 47		484	100	67	324
PCB 200		430	100	79	358
PCB 169	91.20 - 91.97	360	90	77	288
PBDE 66	91.97 - 92.50	484	100	64	324
PCB 170		394	90	68	324
PCB 199	92.50 - 93.90	430	100	76	358
mirex		272	75	61	237
PCB 196		430	100	76	358
PCB 203		430	100	76	358
PCB 189	93.90 - 96.20	394	90	70	324
PCB 208		464	100	74	392
PCB 195		430	100	76	358
PCB 207		464	100	74	392
^{13}C-PCB 194	96.20 - 98.20	442	100	76	370
PCB 194		430	100	77	358
PBDE 100		564	100	60	404
PCB 205		430	100	77	358
PBDE 119		564	100	60	404
PBDE 99	98.20 - 100.20	564	100	60	404
^{13}C-PCB 206		476	100	72	404
PBDE 116		564	100	60	404
PCB 206		464	100	73	392
PBDE 85	100.20 - 103.50	564	100	60	404
PBDE 155		484	100	0.75R	482
PBDE 154	103.50 - 120.00	484	100	0.75R	482
PBDE 153		484	100	0.75R	482
PBDE 138		484	100	0.75R	482

a, all CID voltages determined in non-resonant waveform type unless denoted "R" for resonant waveform type

Construction of compound table

A compound table of all of the calibrant, internal standard and recovery compounds was constructed from the chromatogram of the calibrant/recovery/internal standard mixture analysed with the GC-MS/MS method. For each compound, the retention time was set as the point of the peak with the highest proportion of product ion in the spectrum, which was also set as the reference spectrum for that compound. The major product ion was entered as the quantifier ion and the scan function channel was set corresponding to the channel in the MRM method targeting each particular compound. In cases where there was more than one major product ion, secondary ions were entered as qualifier ions. In a few cases chromatogram peaks could not be differentiated between two compounds that had similar retention times and identical parent and product ions (e.g. two PCB congeners with the same number of chlorine atoms). These two compounds were treated as one compound with the area of the curve representing the combined concentration of these two compounds.

3.3 Measurement of POPs in *Chelonia mydas* using GC-MS/MS

Extraction

Blood, eggs and tissue samples were kept frozen (-20 $^{\circ}$C) from time of collection until analysis. Immediately, prior to analysis, blood samples were thawed and sonicated for 20 min and tissue samples were homogenised using a hexane rinsed stainless steel scalpel blade. Eggs were defrosted and the contents (yolk and albumin) were homogenised in separate acetone rinsed glass jars using a stainless steel blender at 11,000 rpm for 2 min.

Homogenised eggs (7-10 g), tissue samples (7-10 g) and sonicated blood (3-9 g) were mixed with 50 g of hydromatrix (Varian, Palo Alto, CA, USA) and 50 g of anhydrous sodium sulfate (AR grade; Mallinckrodt, Kentucky, USA), which had been combusted at 500 $^{\circ}$C and kept dry at 110 $^{\circ}$C until use. The homogenate was packed into a 100 mL accelerated solvent extractor (ASE) cell and ~ 0.04 g of internal standard (NIST, prepared June 1, 2006) was added to each cell, resulting in ~ 3-6 ng of each compound being added to each sample (Table 3.4).

Table 3.4. Approximate concentrations of the compounds in the internal standard (IS) and the approximate mass of each compound added to each sample before extraction.

Compound	Approximate concentration in the IS (ng g^{-1})	Approximate mass added to each sample (ng)
^{13}C-PCB 28	140	5.5
^{13}C-PCB 52	140	5.5
^{13}C-PCB 118	140	5.5
^{13}C-PCB 153	140	5.5
^{13}C-PCB 180	140	5.5
^{13}C-PCB 194	140	5.5
^{13}C-PCB 206	115	4.5
^{13}C-oxychlordane	140	5.5
^{13}C-trans-chlordane	70	3
^{13}C-trans-nonachlor	140	5.5
^{13}C -Dieldrin	140	5.5
^{13}C-4,4'-DDE	140	5.5
^{13}C-4,4'-DDT	140	5.5
F-BDE 47	130	5

Samples were extracted with dichloromethane (OmniSolv grade; Merck, Darmstadt, Germany) in an ASE (Dionex, Salt Lake City, UT, USA). The ASE method parameters were set to: temperature, 125 $^{\circ}$C; static time, 5 min; flush volume, 60 mL; purge time, 250 s; static cycles, 2; cell size, 100 mL. The extract was collected in a glass container, transferred to a 500 mL round bottom flask and rotary evaporated (Buchi, Switzerland) to ~ 10 mL. This was transferred to a tared 15 mL graduated tube, rinsing the round bottom flask twice with 2 mL of dichloromethane. Extracts containing water were put in the freezer overnight and the water layer was removed with a glass pipette the following day.

Lipid determination

The lipid content of the extract was determined gravimetrically. The mass of the extract in the 15 mL tube was weighed. A 1 mL glass syringe was filled with the extract and weighed. This extract was transferred to an acetone washed tared aluminium tray and the empty mass of the syringe was reweighed to determine the exact mass of extract added to the tray. The aluminium tray was left in a fume cupboard overnight (for the DCM to evaporate) and reweighed. All masses were measured to the nearest 0.00001 g on a ME 235S balance (Sartorius, Goettingen, Germany) and the total mass of lipid extracted from the egg sample was calculated

(Equation 3.1). The percent lipid extracted was calculated by dividing the mass of lipid by the mass of sample extracted.

$$\text{Mass of lipid} = \frac{a}{b}(c - b) \qquad \ldots\ldots \text{ Equation 3.1}$$

a = mass of lipid in aluminium tray after evaporation
b = mass of extract added to aluminium tray
c = mass of extract

Ten replicates of each pooled sample (blood, egg, muscle, kidney and liver) were extracted and the mean percent lipid extracted from each sample type was calculated (Table 3.5).

Table 3.5. Mean (± SE) percent lipid (%) extracted from ten replicates of each tissue.

Blood	Egg	Muscle	Kidney	Liver
1.48 ± 0.10	8.42 ± 0.11	0.46 ± 0.03	1.20 ± 0.18	2.48 ± 0.40

Extract clean-up

Lipids in the extracts were removed by gel permeation chromatography (GPC). The extract was evaporated to 7 mL with nitrogen gas on a hot plate (40 °C) and transferred to a 10 mL glass culture tube via a 0.45 μm, 13 mm hydrophobic membrane filter (Millipore, Billerica, MA, USA). Samples were loaded into an autosampler (Isco, Lincoln, Nebraska, USA) and 6 mL of each sample was pumped through a GPC column (300 mm x 19 mm with pre-column 150 mm x 19 mm; particle size: 15 μm with a pore size of 100 Å; Waters, Milford, MA, USA) with a HPLC pump (Millipore, Billerica, MA, USA) at 4.5 mL min^{-1}. Extracts were evaporated to ~ 0.5 mL with nitrogen gas on a hot plate (40 °C) and solvent exchanged to 40 mL hexane (HPLC grade petroleum ether; Merck, Darmstadt, Germany) that had been redistilled in-house at Queensland Health Scientific Services. This was evaporated to ~ 1 mL in preparation for further clean-up using adsorption chromatography on a Florisil™ column.

Activated Florisil™ was removed from the oven (110 °C) and deactivated by adding 50 μL of deionised water for each gram of Florisil™ and placing on a tumbler for 1 h. Approximately 20 g of deactivated Florisil™ were added to separate glass columns (18 mm I.D.) and the column was rattled to settle the Florisil™. Approximately 2 g of

anhydrous sodium sulfate was added to the top of the Florisil™ and the column was rinsed with 50 mL of hexane. When the hexane level reached the top of the sodium sulfate a 500 mL round bottom flask was placed under the column and the extract was added to the column (rinsing twice with 2 mL of hexane). Approximately 150 mL of 6% diethyl ether in hexane was then added to the column and the tap set to a fast drip. The column was allowed to run dry and the extract was rotary evaporated to ~ 10 mL. The concentrated extract was transferred to a 15 mL graduated glass tube, rinsing the round bottom flask twice with hexane. The extract was evaporated to 1 mL with nitrogen gas on a hot plate (40 $^{\circ}$C) and transferred (rinsing once with 1 mL of hexane) to a 2 mL screw cap GC-MS vial (Agilent, Santa Clara, CA, USA). Immediately prior to injection in the GC-MS, 250 μL (~ 0.17 g) of recovery solution containing ^{13}C-PCB 47 and ^{13}C-PCB 155 (NIST, prepared October 23, 2005) was added to each extract gravimetrically. This was further evaporated to 100-150 μL and transferred to a 200 μL glass insert (Varian, Palo Alto, CA, USA) in a 2 mL screw cap GC-MS vial.

Quantification

Six calibration dilutions were prepared gravimetrically from the calibrants obtained from the National Institute of Standards and Technology (prepared 7/12/05 and 12/1/07). The calibration curve ranged from 100 ng to 10 pg (Table 3.6). The mass of each calibrant was monitored before and after use and adjustments were made to the concentration to compensate for evaporation.

Approximately 50 μL of internal standard was added gravimetrically to each calibrant, resulting in the addition of 3-6 ng of each internal standard compound to each calibrant (see Table 3.4). Recovery standard (250 μL) was added to this mixture resulting in ~ 5 ng each compound being added to each calibrant. Each of these mixtures was evaporated to ~ 100 μL and run through the GC-MS/MS method established using the MRM method.

Table 3.6. Calibrants prepared for quantification.

Calibrant	Approx. mass of each compound	Preparation
Cal A	100 ng	NIST Cal prepared 7/12/05 or 1 in 2.5 dilution of NIST Cal prepared 12/1/07
Cal B	10 ng	1 in 10 dilution of Cal A
Cal C	1 ng	1 in 10 dilution of Cal B
Cal D	500 pg	1 in 2 dilution of Cal C
Cal E	50 pg	1 in 10 dilution of Cal D
Cal F	10 pg	1 in 5 dilution of Cal E

Each calibrant compound was assigned an internal standard compound of similar structure. A calibration curve was constructed for each compound by plotting the area ratio (area of compound/area of internal standard) against amount ratio (amount of compound added/amount of internal standard added) for the six calibrants. The linear regression equation and R^2 were calculated for each compound. The mass of each compound in the unknown samples was calculated using the slope and intercept of this calibration curve and the area ratio and amount ratio of the sample (Equations 3.2 and 3.3). The concentration was reported in ng g^{-1} wet mass or ng g^{-1} lipid by dividing this mass by the original wet mass of the extracted sample or the mass of lipid extracted, respectively.

$$y = m\left(\frac{a}{b}\right) + c \qquad \text{……. Equation 3.2}$$

$$a = \left(\frac{y - c}{m}\right) b \qquad \text{……. Equation 3.3}$$

y = area ratio of the sample
m = slope of the calibration curve
$\frac{a}{b}$ = amount ratio of the sample (a = mass of compound in sample, b = mass of IS added to compound)
c = y-intercept of the calibration curve

Recovery and limit of detection

Percentage recovery of the internal standards was calculated for each sample using amount and area ratios of internal standards and recovery standards. Each internal standard compound was assigned a recovery solution compound (see Table 3.7) and the mean area ratio (area of IS compound/area of recovery compound) and mean amount ratio (mass of IS compound added/mass of recovery compound added) were calculated for the six calibrants. A recovery curve was constructed by plotting mean area ratio against mean amount ratio and setting the y-intercept to zero. The mass of each internal standard compound in the sample after extraction and clean up was calculated from the slope of the recovery curve and the area ratio and amount ratio of the sample (Equations 3.4 and 3.5).

$$y = m\left(\frac{a}{b}\right) + c \qquad \text{……. Equation 3.4}$$

$$a = \left(\frac{y - c}{m}\right) b \qquad \text{……. Equation 3.5}$$

y = area ratio of the sample
m = slope of the recovery curve
$\frac{a}{b}$ = amount ratio of the sample (a = mass of IS after extraction and clean-up, b = mass of recovery compound added to sample)
c = y-intercept of the recovery curve (set to 0)

The mass of IS after extraction and clean-up was divided by the original mass of IS added to each sample and multiplied by 100 to give percent recovery. The mean recoveries of 10 replicates of the pooled blood, pooled egg and pooled muscle samples ranged from 30-96%, which was deemed acceptable (Table 3.7). Recoveries of the PCBs generally decreased with increasing chlorination.

Table 3.7. Percent recovery of the internal standard compounds in 10 replicates of pooled *Chelonia mydas* blood, egg and muscle.

Internal Standard	Recovery Compound	Blood	Egg	Muscle
^{13}C-PCB 28	^{13}C - PCB 47	85.6 ± 0.7	87.4 ± 0.8	88.6 ± 0.4
^{13}C-PCB 52	^{13}C - PCB 47	93.4 ± 0.7	95.9 ± 0.8	96.1 ± 0.8
^{13}C-PCB 118	^{13}C - PCB 155	65.9 ± 0.9	63.9 ± 1.0	62.8 ± 0.7
^{13}C-PCB 153	^{13}C - PCB 155	59.1 ± 0.5	57.7 ± 0.8	58.8 ± 0.9
^{13}C-PCB 180	^{13}C - PCB 155	55.2 ± 0.8	55.6 ± 1.1	58.9 ± 1.1
^{13}C-PCB 194	^{13}C - PCB 155	48.6 ± 1.0	49.7 ± 1.3	50.9 ± 1.2
^{13}C-PCB 206	^{13}C - PCB 155	33.9 ± 1.6	30.4 ± 2.9	34.2 ± 1.3
^{13}C-oxychlordane	^{13}C - PCB 155	81.9 ± 1.2	78.1 ± 1.0	77.7 ± 0.9
^{13}C-trans-chlordane	^{13}C - PCB 155	70.6 ± 0.8	69.9 ± 0.5	72.8 ± 0.6
^{13}C-trans-nonachlor	^{13}C - PCB 155	74.9 ± 0.7	76.7 ± 0.5	79.4 ± 0.9
^{13}C-Dieldrin	^{13}C - PCB 155	90.4 ± 0.9	85.9 ± 1.2	88.5 ± 0.6
^{13}C-4,4'-DDE	^{13}C - PCB 155	68.8 ± 0.5	69.2 ± 0.6	72.4 ± 0.9
^{13}C-4,4'-DDT	^{13}C - PCB 155	75.9 ± 1.1	71.1 ± 1.4	72.8 ± 1.3
F-PBDE 47	^{13}C - PCB 155	63.7 ± 1.2	59.1 ± 1.0	61.8 ± 1.0

Limit of detection was compound and sample specific as it was calculated as the mass of the lowest concentration calibrant compound producing a peak at least three times the baseline, divided by the mass of the sample extracted. For most compounds this was between 5 and 35 pg g^{-1}.

Accuracy and precision

For each run of samples, a deionised water blank, a NIST reference material and three sub-samples of pooled *C. mydas* eggs, blood or muscle were extracted and analysed. Egg samples were analysed with pooled *C. mydas* eggs (~ 10 g) from Heron Island (1998, Mark Hamann) and ~ 2 g of a cryohomogenised avian egg control material (QC04-ERM1; common murre, *Uria aalge* and thick-billed murre, *Uria lomvia*) that has been analysed by NIST but not certified (Vander Pol et al. 2007). Tissue samples were analysed with pooled *C. mydas* muscle samples (~ 10 g) from the Sea World Sea Turtle Rehabilitation Program, Queensland, Australia (2006, excess from this study) and cryohomogenised *Salvelinus namaycush* (lake trout) tissue standard reference material, SRM1946 (~ 1 g). Blood samples were analysed with pooled *C. mydas* blood (~ 2 g) from Peninsular Malaysia (2004, excess from this study) and a NIST human serum reference material, SRM 1589a (~ 2 g). Five replicates of each SRM

were analysed and the means were generally within 70% of the mean certified values and 60% of the reference values (Table 3.8).

Intra-run variation was measured by analysing ten replicates of the pooled egg sample and calculating the coefficient of variation (standard deviation/mean x 100) for all compounds present (Table 3.9). Intra-run variation was also monitored for each batch of samples by running three replicates of the pooled egg sample with each run.

Table 3.9. Mean (± SE) and coefficient of variation of the compounds present in ten replicates of the pooled Heron Island *C. mydas* egg sample.

Compound	Concnetration (pg g^{-1})	Coefficient of Variation (%)
pentachlorobenzene	15.99 ± 0.43	8.6
HCB	12.94 ± 0.73	17.9
PCB 18	16.26 ± 0.46	8.9
PCB 28+31	6.77 ± 0.38	17.6
heptachlor epoxide	19.74 ± 0.26	4.2
PCB 74	15.83 ± 0.12	2.4
PCB 95+121	18.57 ± 0.17	2.9
trans-chlordane	20.27 ± 0.40	6.3
PCB 56	13.12 ± 0.05	1.1
BDE 30	134.6 ± 4.2	10.0
endosulfan I	207.1 ± 5.5	8.5
cis-chlordane	6.58 ± 0.07	3.2
PCB 99	14.03 ± 0.15	3.4
diedrin	422.5 ± 3.9	2.9
PCB 107	9.51 ± 0.02	0.6
PCB 118	13.12 ± 0.13	3.2
cis nonachlor	16.34 ± 0.11	2.2
BDE 33+28	12.50 ± 0.06	1.6
PCB 132+153	23.50 ± 0.21	2.8
PCB 138+158	16.76 ± 0.03	0.6
PCB 187	13.08 ± 0.08	2.0
PCB 183	10.04 ± 0.09	2.7
PCB 177	9.97 ± 0.03	1.0
PCB 180+193	32.92 ± 0.16	1.5
BDE 47	45.22 ± 2.61	18.2
PCB 170	13.08 ± 0.07	1.7
PCB 196+203	29.60 ± 0.14	1.5
PBDE 100	23.64 ± 1.09	14.6
PBDE 99	46.83 ± 1.84	12.4
PBDE 154	13.70 ± 0.34	7.9
PBDE 153	33.07 ± 1.41	13.5

Table 3.8. Comparison of measured POP concentrations to certified and reference values of NIST reference materials, SRM 1589a (pg g^{-1} wet mass), SRM 1946 (ng g^{-1} wet mass) and QC04-ERM1 (ng g^{-1} wet mass).

	SRM 1589a (human serum)			SRM 1946 (fish tissue)			QC04-ERM1 (bird egg)	
Compound[a]	Certified[b]	Reference[b]	Measured[c]	Certified[b]	Reference[b]	Measured[c]	Reference[b]	Measured[c]
pentachlorobenzene							1.93 ± 0.5	2.04 ± 0.71
alpha-HCH				5.72 ± 0.65		6.21 ± 0.59	1.16 ± 0.18	1.42 ± 0.14
HCB		76 ± 28	83.8 ± 12	7.25 ± 0.83		8.60 ± 0.59	34.0 ± 5.3	35.5 ± 7.4
beta-HCH		86.2 ± 3.3	100 ± 11				24.9 ± 4.3	30.1 ± 6.7
gamma-HCH				1.14 ± 0.18		1.57 ± 0.16		
PCB 18					0.84 ± 0.11	1.21 ± 0.07		
PCB 50								
PCB 28		40.8 ± 7.1[f]	53.4 ± 6.0[f]		2.00 ± 0.24	4.14 ± 0.14[c]	2.49 ± 0.51	3.07 ± 1.4[f]
PCB 31					1.46 ± 0.20			
PCB 52		45 ± 10	58.8 ± 6.9	8.1 ± 1.0		8.58 ± 0.75	0.234 ± 0.095	0.282 ± 0.05
PCB 49		24 ± 14	26.2 ± 8.1	3.80 ± 0.39		4.13 ± 0.32		
PCB 44		30.7 ± 9.3	40.6 ± 5.7	4.66 ± 0.86		5.27 ± 0.87		
octachlorosytrene							0.913 ± 0.087	0.966 ± 0.128
oxychlordane	125 ± 15		96.1 ± 8.4	18.9 ± 1.5		21.6 ± 2.1	7.16 ± 0.7	8.52 ± 1.6
PCB 63					1.28 ± 0.19	1.53 ± 0.34		
heptachlor epoxide		96.6 ± 2.9	108 ± 3.9	5.50 ± 0.23		6.87 ± 0.17	4.41 ± 0.3	5.13 ± 0.98
PCB 74		169 ± 40	213 ± 23	4.83 ± 0.51		5.54 ± 0.50	1.92 ± 0.061	2.45 ± 0.27
PCB 70		18.8 ± 3.6	24.5 ± 2.3	14.9 ± 0.6		18.0 ± 1.1	0.205 ± 0.018	0.271 ± 0.017
PCB 95 + 121		51 ± 15	55.4 ± 7.5	11.4 ± 1.3[g]		15.3 ± 2.4		
PCB 66		30.2 ± 10.5	36.4 ± 5.3	10.8 ± 1.9		14.1 ± 2.2	2.41 ± 0.12	2.46 ± 0.17
trans-chlordane				8.36 ± 0.91		9.84 ± 1.29		

a, PCB congener numbers based on IUPAC system; **b**, certified and reference values are means ± an expanded uncertainty about the mean (approx. 95% confidence); **c**, measured values are means ± 95% confidence of 5 replications of each SRM analysed in this study; **d**, PCB 132 + 153; **e**, PCB 180 + 193; **f**, PCBs 28 + 31; **g**, PCB 95 only; **h**, PBDE 32 + 28; **i**, PCB 138 + 158; **j**, 2,4'-DDT + 4,4'-DDD

Table 3.8. (Cont'd.)

	SRM 1589a (human serum)			SRM 1946 (fish tissue)			QC04-ERM1 (bird egg)	
Compound[a]	Certified[b]	Reference[b]	Measured[c]	Certified[b]	Reference[b]	Measured[c]	Reference[b]	Measured[c]
PCB 56					5.77 ± 0.93	6.09 ± 1.56	0.746 ± 0.059	1.03 ± 0.08
PCB 92		13.8 ± 3.6	20.5 ± 2.7					
2,4'-DDE					1.04 ± 0.29	1.32 ± 0.41		
PCB 101	58.3 ± 3.2		63.2 ± 3.1	34.6 ± 2.6		40.6 ± 3.2	0.835 ± 0.7	0.999 ± 0.464
cis-chlordane				32.5 ± 1.8		37.7 ± 2.7	0.254 ± 0.024	0.395 ± 0.067
PCB 99	204 ± 4		224 ± 16	25.6 ± 2.3		33.9 ± 4.1	3.93 ± 0.17	4.35 ± 0.42
trans-nonachlor		318 ± 11	361 ± 7	99.6 ± 7.6		124.5 ± 5.8	0.481 ± 0.1	0.618 ± 0.142
PCB 87		18.3 ± 6.1	23.3 ± 2.4	9.4 ± 1.4		14.6 ± 1.7		
diedrin		107 ± 37	133 ± 21	32.5 ± 3.5		38.8 ± 2.9	3.34	3.98 ± 0.69
4,4'-DDE	11510 ± 610		10538 ± 347	373 ± 48		437 ± 38	69.5 ± 6.3	82.3 ± 8.6
PCB 110	34.2 ± 3.9		43.6 ± 2.8	22.8 ± 2.0		29.3 ± 3.6		
2,4'-DDD				2.20 ± 0.25		2.90 ± 0.21		
PCB 77				0.327 ± 0.025		0.465 ± 0.068		
PCB 151	37.8 ± 4.2		46.9 ± 4.6					
PCB 107					8.86 ± 0.20	9.86 ± 1.05		
PCB 149	71.7 ± 3.3		82.6 ± 6.4	26.3 ± 1.3		37.6 ± 0.9	0.403 ± 0.1	0.652 ± 0.098
PCB 118	168 ± 8		187 ± 6	52.1 ± 1.0		61.8 ± 8.0	6.53 ± 1.1	7.97 ± 2.1
cis-nonachlor				59.1 ± 3.6		65.7 ± 1.7	1.94 ± 0.48	2.25 ± 0.87
PBDE 28		11.9 ± 6.7	17.2 ± 2.8[h]					
PCB 114		16.2 ± 3.6	23.6 ± 3.9					
2,4-DDT					22.3 ± 3.2	48.3 ± 5.9[j]		
4,4-DDD				17.7 ± 2.8				

a, PCB congener numbers based on IUPAC system; **b**, certified and reference values are means ± an expanded uncertainty about the mean (approx. 95% confidence); **c**, measured values are means ± 95% confidence of 5 replications of each SRM analysed in this study; **d**, PCB 132 + 153; **e**, PCB 180 + 193; **f**, PCBs 28 + 31; **g**, PCB 95 only; **h**, PBDE 32 + 28; **i**, PCB 138 + 158; **j**, 2,4'-DDT + 4,4'-DDD

Table 3.8. (Cont'd.)

	SRM 1589a (human serum)			SRM 1946 (fish tissue)			QC04-ERM1 (bird egg)	
Compound[a]	Certified[b]	Reference[b]	Measured[c]	Certified[b]	Reference[b]	Measured[c]	Reference[b]	Measured[c]
PCB 146		95 ± 13	112 ± 10	30.1 ± 3.5		35.9 ± 4.1	2.47 ± 0.022	3.01 ± 0.36
PCB 132	936 ± 45[d]		987 ± 26[d]		5.83 ± 0.76	188 ± 9[d]	11.0 ± 0.26[d]	14.2 ± 0.54[d]
PCB 153				170 ± 9				
PCB 105	39.8 ± 3.5		47.8 ± 3.5	19.9 ± 0.9		22.0 ± 0.9	1.84 ± 0.47	2.28 ± 0.62
PCB 137		34.3 ± 6.4	50.0 ± 3.9					
PCB 130		20.4 ± 3.1	25.1 ± 1.6				0.298 ± 0.018	0.344 ± 0.056
PCB 163		123 ± 23	136 ± 7		31.8 ± 0.8	43.7 ± 2.6	2.00 ± 0.16	2.55 ± 0.35
PCB 138	537 ± 19		669 ± 12[i]	115 ± 13		161 ± 17[i]	5.76 ± 1.5	7.38 ± 1.9[i]
PCB 158		15.3 ± 5.4			7.66 ± 0.88		0.317 ± 0.063	
4,4'-DDT	100 ± 7		116 ± 15	37.2 ± 3.5		50.4 ± 3.5		
PCB 178	59.9 ± 7.0		67.7 ± 13.4				0.429 ± 0.02	0.671 ± 0.015
PCB 126				0.380 ± 0.017		0.495 ± 0.064		
PCB 187	267 ± 27		307 ± 8	55.2 ± 2.1		69.9 ± 1.1	2.94 ± 0.09	3.68 ± 0.76
PCB 183	90.0 ± 7.5		96.9 ± 9.6	21.9 ± 2.5		24.8 ± 4.2	0.838 ± 0.094	1.35 ± 0.23
PCB 128	10.8 ± 1.4		15.4 ± 1.9	22.8 ± 1.9		27.8 ± 3.9	0.809 ± 0.077	0.939 ± 0.103
PCB 167	25.9 ± 2.6		38.4 ± 2.6				0.468 ± 0.037	0.684 ± 0.028
PCB 174	19.6 ± 2.4		22.2 ± 1.9		9.3 ± 1.3	10.7 ± 1.6		
PCB 177	67.1 ± 6.1		77.3 ± 3.8				0.395 ± 0.092	0.562 ± 0.11
PCB 202		61 ± 23	69 ± 11					
PCB 156	74 ± 12		104 ± 10	9.52 ± 0.51		10.5 ± 0.9	0.557 ± 0.12	0.752 ± 0.115
PCB 201		7.8 ± 4.3	16.8 ± 3.7		2.83 ± 0.13	3.60 ± 0.23		
PCB 157		18.9 ± 6.3	30.1 ± 9.8				0.21 ± 0.014	0.371 ± 0.017

a, PCB congener numbers based on IUPAC system; **b**, certified and reference values are means ± an expanded uncertainty about the mean (approx. 95% confidence); **c**, measured values are means ± 95% confidence of 5 replications of each SRM analysed in this study; **d**, PCB 132 + 153; **e**, PCB 180 + 193; **f**, PCBs 28 + 31; **g**, PCB 95 only; **h**, PBDE 32 + 28; **i**, PCB 138 + 158; **j**, 2,4'-DDT + 4,4'-DDD

Table 3.8. (Cont'd.)

	SRM 1589a (human serum)			SRM 1946 (fish tissue)			QC04-ERM1 (bird egg)	
Compound[a]	Certified[b]	Reference[b]	Measured[c]	Certified[b]	Reference[b]	Measured[c]	Reference[b]	Measured[c]
PCB 172	33.5 ± 1.8		45.7 ± 3.5				0.371 ± 0.028	0.462 ± 0.028
PCB 180	523 ± 67[e]		726 ± 19[e]	74.4 ± 4.0		95 ± 6[e]	2.28 ± 0.18[e]	3.26 ± 1.1[e]
PCB 193					5.78 ± 0.72			
PCB 191		8.4 ± 1.4	13.9 ± 2.7					
PBDE 47	172 ± 10		227 ± 17					
PCB 169				0.106 ± 0.014		0.142 ± 0.031		
PCB 170	211 ± 16		230 ± 21	25.2 ± 2.2		29.7 ± 3.3	1.47 ± 0.72	1.89 ± 0.39
PCB 199	170 ± 30		194 ± 25					
mirex		112 ± 35	95 ± 17	6.47 ± 0.77		7.35 ± 1.17	1.58 ± 0.16	1.91 ± 0.12
PCB 196+203		154 ± 31	168 ± 22					
PCB 189		7.8 ± 1.9	14.3 ± 0.9					
PCB 208		41.4 ± 7.8	55.0 ± 4.6					
PCB 195	31.3 ± 2.4		37.3 ± 4.7	5.30 ± 0.45		6.31 ± 1.21		
PCB 207		9.4 ± 1.6	14.2 ± 1.8					
PCB 194	129 ± 5		144 ± 9	13.0 ± 1.3		14.6 ± 2.1		
PBDE 100	25.0 ± 3.2		36.3 ± 4.4					
PBDE 99	39.9 ± 5.2		42.4 ± 6.7					
PCB 206	62.9 ± 2.3		68.0 ± 3.5	5.40 ± 0.43		6.58 ± 0.78		
PBDE 154		62.8 ± 7.5	83.9 ± 9.8					
PBDE 153		18.5 ± 4.3	28.7 ± 5.1					
PBDE 138		62.8 ± 7.5	47.1 ± 8.6					

a, PCB congener numbers based on IUPAC system; **b**, certified and reference values are means ± an expanded uncertainty about the mean (approx. 95% confidence); **c**, measured values are means ± 95% confidence of 5 replications of each SRM analysed in this study; **d**, PCB 132 + 153; **e**, PCB 180 + 193; **f**, PCBs 28 + 31; **g**, PCB 95 only; **h**, PBDE 32 + 28; **i**, PCB 138 + 158; **j**, 2,4'-DDT + 4,4'-DDD

3.4. Advantages of GC-MS/MS for measuring POPs in *Chelonia mydas*

The GC-MS/MS method developed for the analysis of POPs in *C. mydas* blood, eggs and tissue has a number of advantages over previous methods. This method can measure the concentration of 125 different POP compounds (23 OCPs, 83 PCBs and 19 PBDEs) with a single sample injection. This is considerably more time and cost efficient than previous methods requiring multiple injections into GC-ECD and GC-MS (both EI and NCI modes) to analyse the same number and type of compounds. Furthermore, the level of detection (5-35 pg g^{-1}) is superior to many past methods and is particularly important for the analysis of *C. mydas* that have low concentrations compared to other sea turtle species due to their lower trophic level as sub-adults and adults.

Recoveries were generally above 60%, with only a gradual decrease with increasing chlorination of PCBs. However, any losses in recovery were negated by the use of structurally similar mass-labelled internal standard compounds in the calculation of the concentration of native compounds. It is assumed that the internal standard compounds will act identically to the native compounds throughout the method and therefore compensate for any loss of the native compounds in the calculations. This is supported by the fact that the method met the certified values of SRMs with equal precision for both the low and high recovered compounds. The GC-MS/MS method also reports to within 70% of the certified values and within 60% of reference values for blood, tissue and egg SRMs and reference materials, and this remains consistent between batches. Furthermore, the intra-batch repeatability of this method is excellent with coefficients of variation of pooled samples all < 20%, and generally < 5%.

The GC-MS/MS method was relatively easy to set up and can be adapted to any GC-MS equipped with a PTV and MS/MS capabilities. Furthermore, the changes in methodology between different tissue types were minimal, involving only minor changes to sample preparation. A single method is therefore now available for analysing POPs in the different tissues of sea turtles. The versatility to analyse POPs in different tissue types also indicates that this method may be easily adapted for contamination screening in other marine animals. Furthermore, it is expected that only minor adjustments to this method would be required for analysis of POPs in marine sediments and vegetation. This method, therefore, not only advances capabilities for

analysing POPs in sea turtles, but also is a significant contribution to contaminant analysis in the marine environment, in general.

The most time consuming step in the development of this method was the determination of the CID voltages for each compound and the construction of the MRM parameters. The MRM method was confined to keeping ≤ four excitation amplitude and CID voltage experiments to each time window and required good separation between the last compound of a window and the first compound of the next window. Due to the large number of compounds analysed, using this method, there were therefore a few areas of the chromatogram where compounds at the end of one window were very close to the compounds at the beginning of the next. These areas are susceptible to the occasional shift in the compound retention times, which can result in peaks falling outside the time window and an inability to integrate the area under the curve. In these situations, time windows can generally be manipulated to ensure compounds of higher interest are preferentially maintained. In these limited cases, one or two less important compounds could be lost from the analysis.

In summary, this GC-MS/MS method is relatively easy to set-up and provides an accurate and repeatable way of measuring a 125 POP compounds at a limit of detection of 5-35 pg g^{-1} in *Chelonia mydas* blood, egg and internal tissues.

Chapter 4 - Blood and carapace sampling as a non-lethal method for predicting internal tissue contamination in the green sea turtle, *Chelonia mydas*

4.1 Introduction

Chelonia mydas spend the majority of their lives foraging in shallow coastal seagrass areas and reefs (Mendonca and Ehrhart 1982; Balazs 1985; Green 1993). These habitats can potentially expose *C. mydas* to chemical pollution. Following the first 3-6 years of life drifting on surface oceanic currents, feeding primarily on pelagic zooplankton, *C. mydas* take up residence in coastal areas (Bjorndal 1985; Limpus and Miller 1993; Musick and Limpus 1997; Reich et al. 2007). At this stage, they convert to an almost exclusively herbivorous diet of seagrass and macroalgae (Mortimer 1982; Bjorndal 1985, 1997). In many cases, these foraging grounds are in close proximity to sources of persistent organic pollutants (POPs) and heavy metals, which make their way into the marine environment from industrial, domestic and agricultural sources. These chemicals accumulate in marine animals nearly exclusively through their diet (Langston and Spence 1995) and can have a wide range of harmful effects on their development and function (see Chapter 1). The monitoring of POPs and metals in *C. mydas* therefore provides vital information about the health of individuals and populations and is an important area of sea turtle conservation research.

Heavy metals and POPs have been identified in a number of *C. mydas* populations around the world (McKim and Johnson 1983; Aguirre et al. 1994; Gordon et al. 1998; Godley et al. 1999; McKenzie et al. 1999; Saeki et al. 2000; Sakai et al. 2000b; Anan et al. 2001; Miao et al. 2001; Fujihara et al. 2003; Lam et al. 2004; Gardner et al. 2006; Kampalath et al. 2006; Talavera-Saenz et al. 2007). However, due to the ethical considerations of obtaining tissue samples from live animals, these studies have generally been opportunistic, sampling tissue from deceased and stranded animals. It is of more interest to toxicologists and sea turtle conservationists to obtain information about the contamination of the live animals in a population. There is therefore a current need to develop reliable non-lethal methods for determining chemical contamination in sea turtles.

A non-lethal method for sampling blood from the dorsal cervical sinuses in the neck of sea turtles was developed by Owens and Ruiz (1980) and is now considered a routine procedure. Blood samples have recently been used to investigate organochlorines in loggerhead (*Caretta caretta*) and Kemp's ridley (*Lepidochelys kempii*) sea turtles (Keller et al. 2004a) and mercury and lead in olive ridley (*Lepidochelys olivacea*) and *C. caretta* (Kenyon et al. 2001; Day et al. 2005; Day et al. 2007). However, there is limited information on how a blood sample represents the concentration of chemicals in internal tissues of sea turtles. Day et al (2005) found significant positive correlations between blood mercury levels and mercury concentrations in the muscle and spinal cord of *C. caretta*. However, similar relationships for other metals, tissues and sea turtle species have not been investigated to date.

Carapace scute sampling also has the potential for being a non-lethal method for determining contamination burdens of the major internal organs, particularly for heavy metals. Chelonian sea turtle scutes are hard, highly keratinised plates that protect the animals from the outside environment and predation (Solomon et al. 1986). Heavy metals are known to bind with keratin (Crewther et al. 1965) and studies on seabird feathers have revealed that elements such as mercury maintain a strong association with keratin following prolonged exposure to UV radiation and extreme temperatures (Appelquist et al. 1984). The keratinised carapace scutes could therefore provide a reliable and temporarily robust measure of determining heavy metal concentration in *C. mydas*. A recent study on *C. caretta* utilised a non-lethal method for sampling scutes from the carapace of juveniles and found significant positive correlations between scute mercury levels and mercury concentrations in the liver, muscle, kidney and spinal cord (Day et al. 2005). A further study on *C. caretta* accidentally caught in fishing nets off the coast of Japan found significant positive correlations between carapace concentrations and whole body burdens for zinc, manganese and mercury (Sakai et al. 2000b).

The primary aim of this study was to investigate blood and carapace samples as non-lethal methods for predicting POP and heavy metal contamination in the internal tissues of *C. mydas*. To do this effectively, blood, carapace and tissue samples from the same individual *C. mydas* were required. The Sea World Sea Turtle Rehabilitation

Program on the Gold Coast, Australia, provided a unique opportunity to collect these samples from rehabilitating *C. mydas* and investigate the correlations in chemical contamination between the different tissue types.

4.2 Methods

Source of samples

Between January 2006 and June 2007, blood, carapace and tissue samples were collected from *C. mydas* at the Sea World Sea Turtle Rehabilitation Program (SWSTRP), Gold Coast, Queensland, Australia (27° 57' 37" S, 153° 25' 27" E). Many turtles at the SWSTRP are successfully rehabilitated and returned to the ocean, although a number with more severe ailments either die or are euthanased by veterinary staff at the centre. *C. mydas* are kept at the SWSTRP for as little as a few hours to as long as several years. Blood samples can therefore be taken from turtles once they arrive at the SWSTRP. Furthermore, turtles that die at the SWSTRP can be sampled within a short time of death, resulting in good quality tissue samples that can be matched against blood samples from the same individual. However, during rehabilitation, turtles are fed fish and squid to optimise weight gain and recovery. For *C. mydas*, this means a significant change in diet from their usual seagrass and algae. The effects of this on blood and tissue biochemistry must therefore be considered when investigating chemicals in these turtles.

Rehabilitated turtles

Twenty-eight *C. mydas* were sampled at the SWSTRP over the duration of this study (Table 4.1). Upon arrival at the SWSTRP, the location and date of stranding, curved carapace length (CCL) and the physical condition of each turtle were recorded (see Chapter 2 for methods). Turtles ranged from 33 to 97.5 cm in CCL and had ailments ranging from severe boat strikes to buoyancy problems. Eight turtles recovered completely and were released, while four remained in rehabilitation past the completion of this study. The time spent by these *C. mydas* at the SWSTRP ranged from one day to > 18 months (adult male, No. 2005029, which was still in rehabilitation at the end of this study). The locations of stranding were generally in the Moreton Bay and Gold Coast Broadwater area but one individual came from as far away as the Great Sandy Strait, near Fraser Island. Twenty-two of the 28 (79%)

individuals had buoyancy problems and were often very lethargic and emaciated, seven (25%) had physical injuries ranging from boat strikes to missing limbs and two (7%) had fibropapilloma tumours.

Blood and tissue sampling

Blood samples were taken from 28 *C. mydas* between January 2006 and March 2007 (Table 4.1). Sampling was dependent on availability from ongoing research and analysis for other chapters and was therefore opportunistic in nature. Samples were taken as close as possible to the time of arrival of turtles at the SWSTRP. However, samples were also taken at monthly intervals in cases where no new turtles arrived over this period. On each occasion, new turtles were sampled and turtles that were still there from the previous occasion were re-sampled. Over the sampling period, 11 turtles were sampled three times or more (Table 4.1). All blood samples were taken from the dorsal cervical sinuses (see Chapter 2 for details), according to methods developed by Owens and Ruiz (1980). Samples were put on ice and immediately transported to the laboratory.

Percent hematocrits were measured upon arrival at the laboratory. For each sample, a heparinised plastic clad micro hematocrit tube (Drummond Scientific Co., Broomall, PA, USA) was filled with blood by capillary action and plugged with clay. The tubes were centrifuged at 3000 rpm for 5 minutes (Model 5702; Eppendorf, Hamburg, Germany) and the percentage hematocrit was calculated as the length of the packed red blood cells over the total length of blood in the tube. Samples for metal analysis were kept in the refrigerator (4 $^{\circ}$C) and samples for persistent organic pollutant (POP) analysis were frozen (-20 $^{\circ}$C) until the time of analysis.

Liver, muscle, kidney, carapace and blood samples were taken from 16 *C. mydas* that died between April 2006 and June 2007 (Table 4.1). Samples were generally taken immediately post mortem. However, in cases where this was not possible, the turtle carcass was immediately frozen and kept at -20 $^{\circ}$C until tissue dissections could be performed. During tissue sampling, the turtle was turned upside down on an alcohol cleaned stainless steel bench and the plastron was removed with a hexane rinsed stainless steel scalpel (Swan Morton, Sheffield, England). Using sterile latex powder-

free surgeon's gloves (Semperit, Vienna, Austria), a separate clean scalpel was then used to remove ~ 10 g each of liver, kidney and muscle tissue in duplicate. Post-mortem blood samples were taken from the pulmonary artery directly above the heart using a 21G x 1½" needle with 10 mL syringe. Liver, kidney and muscle samples for POP analysis were wrapped in hexane rinsed aluminium foil and samples for metal analysis were transferred to labelled plastic zip-lock bags. All samples were immediately surrounded with ice in a foam box and transferred to a laboratory freezer where they were kept frozen (-20 °C) until analysis.

Carapace samples (0.5 - 1 g) were taken from the eight posterior marginal scutes according to methods described by Day et al. (2005). The carapace was cleaned with a plastic scrubbing pad, rinsed with deionised water and wiped with 70% ethanol wipes. A 5 mm sterile single-use biopsy punch (Fray, NY, USA) was used to scrape carapace splinters from the radial edge of the carapace where the dorsal and ventral surfaces meet. Carapace samples were transferred to an alcohol-rinsed plastic vial and kept frozen (-20 °C) until analysis.

Table 4.1. Details of the *C. mydas* sampled from the Sea World Sea Turtle Rehabilitation Program, January 2006 to June 2007.

	Rehabilitation							
Turtle ID[a]	Date Arrived	Fate[b]	Date of Fate	Sampling[c]	CCL[d]	Sex[e]	Location of stranding[f]	Turtle Condition
2004014	4/10/2005	D	8/08/2006	B(6), BCT	48	IF	Peel Island, Horeshoe Bay (MB)	carapace damage, fibropapillomas
2005025	12/08/2005	R	21/12/2006	B(7)	38.6	U	Moreton Bay	floating, lethargic
2005027	13/09/2005	R	5/01/2006	B(1)	51	U	Russell Island (MB)	fibropapillomas
2005028	21/09/2005	R	5/01/2006	B(1)	41.8	U	Great Sandy Strait (FI)	floating, flakey carapace
2005029	24/09/2005	S	N/A	B(10)	94.5	AM	Redcliffe (MB)	floating
2005032	4/10/2005	R	5/01/2006	B(1)	97.5	AF	Nth Stradbroke Island (MB)	floating
2005041	31/12/2005	D	20/01/2006	B(2), BCT	75	IM	Moreton Island (MB)	floating, sunken plastron and eyes
2006001	24/01/2006	D	22/05/2006	B(5), BCT	77.5	IM	Dunwich, N. Stradbroke Island (MB)	carapace boat strike
2006004	2/03/2006	D	20/04/2006	B(2), BCT	82.4	IF	Manly Harbour (MB)	floating, left front flipper missing
2006012	13/04/2006	D	13/04/2006	B(1), BCT	45.5	IF	Moreton Bay	floating, lethargic
2006013	7/05/2006	R	29/06/2006	B(2)	45.5	U	Labrador, Broadwater (GC)	carapace boat strike, injested fishing hook
2006014	8/05/2006	D	26/07/2006	B(3), BCT	44.2	IF	Peel Island, Horeshoe Bay (MB)	floating
2006015	27/05/2006	R	1/08/2006	B(3)	73.5	U	Kurringal (MB)	floating
2006016	20/06/2006	D	22/06/2006	B(1), BCT	74.7	IF	Moreton Bay	head boat strike
2006019	15/08/2006	D	20/11/2006	B(4), BCT	45.5	IF	Reeder's Point, Moreton Island (MB)	floating, sunken plastron
2006020	25/08/2006	R	2/11/2006	B(2)	71	U	St Alina Island (MB)	floating
2006021	14/09/2006	D	10/10/2006	B(2), BCT	40.7	IM	The Spit, Main Beach (GC)	floating, sunken eyes
2006023	22/09/2006	R	2/11/2006	B(2)	41	U	Southport Beach (GC)	floating, right front flipper missing
2006024	29/09/2006	D	20/11/2006	B(3), BCT	46.3	IM	Redcliffe (MB)	floating
2006025	29/09/2006	D	19/10/2006	B(2), BCT	56.5	IF	Couran Cove (GC)	floating
2006026	2/10/2006	D	3/10/2006	B(1), BCT	44.5	IM	Moreton Bay	floating
2006027	1/10/2006	D	11/10/2006	B(2), BCT	53	IM	Manly Harbour (MB)	right eye swollen, swims in circles
2006028	5/10/2006	S	N/A	B(5)	96	AF	Nth Stradbroke Island (MB)	floating
2006033	14/11/2006	R	23/03/2007	B(3)	48	U	Cedar St, Wynnum (MB)	floating
2006034	26/11/2006	S	N/A	B(4)	88	AF	Raby Bay (MB)	floating, head and carapace wounds
2007002	2/01/2007	D	1/06/2007	B(2), BCT	33	IF	Cleveland Point (MB)	floating
2007003	2/01/2007	D	13/01/2007	B(1), BCT	39.5	IF	Fisherman's Island (MB)	floating
2007007	9/02/2007	D	6/03/2007	B(2), BCT	77	IF	Manly Harbour (MB)	floating

a, identification number assigned by Sea World Sea Turtle Rehabilitation Program
b, the fate of turtles once at the SWSTRP: D, deceased; R, rehabilitated and released; S, still at the SWSTRP at the end of this study
c, samples taken: B(n), blood samples (number); BCT, blood, carapace and tissue samples taken
d, curved carapace length (cm)
e, sex and maturity: IF, immature female; IM, immature male; AF, adult male; AF, adult female; U, undetermined
f, region of stranding location: MB, Moreton Bay; FI, Fraser Island; GC, Gold Coast

Sex determination

The sex and maturity of *C. mydas* that died at the SWSTRP were identified at the time of tissue dissections according to the gonad structure described by Limpus and Reed (1985). For *C. mydas* that were either released or still at the SWSTRP at the end of this study, sex and maturity were identified by size and secondary sex characteristics (Wibbels 1999). Adult males were identified when the tail protruded > 30 cm past the marginal scutes of the plastron, and adult females were identified as > 85 cm curved carapace length with no tail protrusion. Turtles were classified as unidentified, if they could not be assigned to the above categories. The sex/size distribution included ten immature females, six immature males, three adult females and a single adult male. The sex of the remaining eight sub-adult turtles could not be identified.

Chemical analysis

All blood and tissue samples were analysed for POPs and metals using methods outlined in Chapters 2 and 3. Carapace samples were only analysed for metals. Briefly, POPs were analysed using GC-MS/MS following accelerated solvent extraction in dichloromethane, gel permeation chromatography and Florisil™ column clean-up (Chapter 3). Percent lipids were determined gravimetrically for each sample following extraction. Metals were analysed using ICP-MS and CV-AAS following acid digestions (Chapter 2). Standard reference materials were run for each type of analysis and pooled samples were run for quality control.

Statistical analysis

For the calculation of mean concentrations of POPs and metals in the different tissues, individuals with multiple blood samples over time were assigned a mean blood concentration for each element. Furthermore, all values below the limit of detection (LOD) for all tissue types were assigned a value of half the LOD. This simple substitution method produces the least amount of bias while not requiring the use of complex iteration software (Helsel 1990). However, as these substituted values were not true concentrations, values below the LOD were excluded from the analysis of blood and carapace as predictors of tissue concentrations.

To investigate the use of blood and carapace as non-lethal predictors of tissue contamination, blood, carapace, liver, muscle and kidney samples taken at the time of

death were used. In cases where post-mortem blood samples could not be taken, the most recent blood sample to the time of death was used. Regression analysis (α = 0.05) was performed separately for each POP and metal compound, between blood and tissues and carapace and tissues.

To investigate differences in the relationship between blood or carapace and the different tissue types (liver, muscle and kidney) for each compound, the slopes and elevations of the regressions were analysed. A custom analysis of covariance (ANCOVA) was initially created to test for homogeneity of slopes. This involved an initial test of interaction between the covariate (blood or carapace concentration) and the factor (tissue type). If this interaction was not significant ($P > 0.05$), slopes were considered to be equal and a one-factorial ANCOVA was performed to determine if the elevations were statistically different. Least significant difference (LSD) post hoc analysis was used in cases of one-factorial ANCOVA significance to determine which regressions were different in elevation.

Percent hematocrit can indicate a number of factors that may influence chemical concentrations in the blood such as dehydration, malnutrition and chronic disease (Frye 1991). To investigate the influence of percent hematocrit on the blood-tissue correlations, blood concentrations were divided by the percent hematocrit value. Regressions were then performed between this hematocrit standardised concentration and tissue concentrations for each POP and metal compound.

The effects of size and sex on chemical contamination were investigated using three concentrations calculated from the initial blood samples: 1) ΣPOP blood concentration, 2) Σ essential metal blood concentration, and 3) Σ toxic metal blood concentration. Initial blood samples represented the first samples taken from each *C. mydas*, generally taken within a few days of arrival at the SWSTRP. The use of the initial blood concentrations in size and sex comparisons therefore allowed investigation of these factors with minimal influence from the change in diet during rehabilitation. To investigate evidence of maternal offloading of chemicals (see Chapter 6), analyses of co-variance (ANCOVAs) were used to compare the differences in the three summed blood concentrations between adult females and the remaining *C. mydas* (covariate = CCL). To investigate differences in chemical

accumulation between sexes before maternal offloading could have an influence, ANCOVAs (covariate = CCL) were used to compare the differences in the three summed blood concentrations between all males and sub-adult females.

To investigate the effect of size on chemical accumulation, adult females were considered separately. This was done to reduce any influence of maternal offloading. Three separate regression analyses were performed between CCL and ΣPOP, Σ essential metal and Σ toxic metal blood concentrations for all male and non-adult female *C. mydas*. However, there were no regressions performed for adult females as only three were identified (Table 4.1).

For all regression and ANCOVA analyses, the assumptions of normality and homogeneity were met by interpretation of residuals plotted against the dependent variables and Levene's test of equality of error variances. To test the assumption of homogeneity of slopes, custom ANCOVAs were constructed to include the interaction between the covariate (CCL) with the factor. There were not enough turtles with sufficient multiple blood samples over time to warrant statistical analysis of changes in concentration during rehabilitation. Casual observations of these results were however discussed in relation to other findings.

4.3 Results

Tissue-specific distribution of POPs and metals

A complex mixture of POP compounds was detected in the *C. mydas* blood and tissue samples analysed in this study (Table 4.2). The mean blood concentrations were similar to tissue samples and the concentration range of each compound in blood was generally large. However, only 21% of the 125 POP compounds analysed were above the limit of detection in blood samples. Furthermore, a number of these compounds were not present in all blood samples. These factors limited the range and the number of compounds that could be analysed for the relationships between blood and tissue concentrations.

Liver samples generally had the highest concentrations of POPs, followed by kidney and muscle. Compounds that were detected in the tissue samples generally had large concentration ranges. The liver also had the largest number of POP compounds

detectable (37%), compared to 33% and 26% for kidney and muscle, respectively. As with the blood samples, a number of POP compounds were not detected in all the samples of each tissue type. This further reduced the range and data points in the analysis of the relationship between blood and tissue concentrations.

All eight metals analysed were detected in the blood of all 28 *C. mydas* (Table 4.3). Blood metal concentrations were generally higher than any other tissue and there was a large concentration range for most elements. This indicated that the relationships between blood and tissues could be investigated over a large range in concentrations.

In carapace samples, cobalt, lead and mercury were only detected in 12.5%, 43.8% and 68.8% of animals, respectively. Carapace samples generally had the lowest concentrations and the smallest range for most elements. The low concentrations of carapace samples reduced the range over which the relationship between carapace and tissues could be tested. Furthermore, the small number of carapace samples with elements above the limit of detection reduced the number of samples that could be used in analysing the relationship between carapace and tissue concentrations.

A number of elements were not detected in some of the tissue samples. In muscle samples, cobalt and lead were not detected at all and mercury was found in only four of 16 turtles (25%). In liver samples, lead and mercury were detected in 43.8% and 75% of turtles, respectively. In kidney samples, lead and mercury were detected in 12.5% and 81.3% of turtles, respectively. Similar to the blood samples, there was a large range in concentrations for most elements in muscle, liver and kidney.

The percent lipids extracted was highest in the liver (2.37 ± 0.44%), followed by the kidney (1.25 ± 0.21%), blood (0.72 ± 0.07%) and muscle (0.46 ± 0.04%). To standardise for lipid content, POP concentrations were presented in ng g^{-1} lipid for the analyses of the correlations between blood and tissue concentrations.

Adult female *C. mydas* had significantly lower ΣPOP concentrations in their initial blood samples than the remaining *C. mydas* ($P = 0.01$). However, there was no difference in blood concentrations of Σ essential or Σ toxic metals between adult

females and the remaining *C. mydas* ($P > 0.05$). Furthermore, when adult females were removed from the analysis, there were no differences in the three summed blood contamination variables between the sexes ($P > 0.05$). For all *C. mydas*, excluding the adult females, there were no significant correlations between CCL and Σ essential metal blood concentration ($P = 0.10$), Σ toxic metal blood concentration ($P = 0.06$) or Σ POP blood concentration ($P = 0.40$).

Table 4.2. Concentration (mean ± SE, range) of POPs in the blood, carapace and tissues (pg g^{-1} wet mass) collected from *Chelonia mydas* at the Sea World Sea Turtle Rehabilitation Program, January 2006 to March 2007. LOD - limit of detection.

	Muscle		Liver		Kidney		Blood	
Compound	**Mean ± SE**	**Range (n[a])**	**Mean ± SE**	**Range (n[a])**	**Mean ± SE**	**Range (n[a])**	**Mean ± SE**	**Range (n[b])**
PCB 1	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 8	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 18	9.5 ± 0.5	7.5 - 12.4	10.3 ± 0.5	8.4 - 12.7	11.2 ± 0.5	8.7 - 13.8	29.0 ± 3.2	15.7 - 50.6
PCB 28+31	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 29	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 44	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 45	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 49	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 50	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 52	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 56	6.4 ± 0.9	<LOD - 11.7 (13)	9.3 ± 0.8	6.0 - 14.2	9.5 ± 0.8	6.6 - 14.0	<LOD	-
PCB 63	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 66	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 70	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 74	<LOD	-	12.6 ± 2.9	<LOD - 26.1 (13)	9.7 ± 1.9	<LOD - 18.5 (13)	<LOD	-
PCB 77	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 79	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 82	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 87	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 92	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 95+121	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 99	15.7 ± 3.4	6.9 - 41.7	69.4 ± 20.9	21.4 - 225.8	33.6 ± 9.9	12.6 - 113.1	49.8 ± 7.6	22.2 - 100.9
PCB 101	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 104	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 105	8.6 ± 1.8	<LOD - 19.2 (14)	33.7 ± 14.6	6.7 - 151.8	15.6 ± 4.3	5.6 - 46.5	24.1 ± 5.5	<LOD - 67.1 (23)
PCB 106	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 107	5.8 ± 0.9	<LOD - 8.7 (13)	9.3 ± 1.1	5.7 - 16.3	7.2 ± 1.1	<LOD - 12.8 (14)	23.7 ± 2.2	16.4 - 38.5
PCB 110	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 112	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 114	<LOD	-	<LOD	-	<LOD	-	<LOD	-

a, number of samples above the limit of detection indicated in parentheses if < 16
b, number of samples above the limit of detection indicated in parentheses if < 28

Table 4.2. (Cont'd.)

	Muscle		Liver		Kidney		Blood	
Compound	**Mean ± SE**	**Range (n[a])**	**Mean ± SE**	**Range (n[a])**	**Mean ± SE**	**Range (n[a])**	**Mean ± SE**	**Range (n[b])**
PCB 118	13.8 ± 2.9	5.8 - 31.2	77.3 ± 34.2	9.9 - 330.9	29.4 ± 9.6	7.2 - 91.2	33.0 ± 4.5	17.0 - 62.2
PCB 119	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 126	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 127	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 128	10.1 ± 1.7	5.1 - 20.9	41.0 ± 1317	13.3 - 114.3	19.0 ± 5.1	7.9 - 55.8	28.2 ± 3.4	<LOD - 50.2 (26)
PCB 130	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 132+153	53.8 ± 14.8	13.8 - 137.0	322.0 ± 103.2	66.1 - 912.1	130.1 ± 39.5	29.1 - 386.7	125.3 ± 31.0	36.1 - 362.5
PCB 137	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 138+158	31.3 ± 9.2	9.9 - 100.7	157.3 ± 40.2	49.9 - 416.7	69.4 ± 14.7	24.1 - 154.6	109.6 ± 33.1	21.4 - 378.6
PCB 146	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 149	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 151	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 154	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 156	<LOD	-	29.0 ± 9.6	7.5 - 89.6	12.0 ± 3.4	<LOD - 34.9 (14)	<LOD	-
PCB 157	<LOD	-	9.1 ± 1.9	<LOD - 23.3 (15)	<LOD	-	<LOD	-
PCB 159	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 163	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 165	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 166	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 167	<LOD	-	20.5 ± 6.2	<LOD - 59.0 (13)	<LOD	-	<LOD	-
PCB 169	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 170	12.9 ± 2.0	7.0 - 25.5	51.1 ± 16.0	16.5 - 146.2	23.6 ± 6.0	10.2 - 63.7	30.6 ± 6.4	<LOD - 73.0 (23)
PCB 172	<LOD	-	9.0 ± 1.3	5.3 - 17.8	6.0 ± 1.0	<LOD - 10.9 (13)	<LOD	-
PCB 174	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 175	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 176	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 177	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 178	<LOD	-	14.1 ± 2.3	6.3 - 27.2	7.0 ± 1.5	<LOD - 92.2 (10)	14.5 ± 2.0	<LOD - 30.0 (21)
PCB 180+193	33.2 ± 4.7	18.1 - 58.6	129.4 ± 29.1	41.9 - 321.2	57.5 ± 9.3	27.5 - 107.6	99.6 ± 16.8	<LOD - 212.8 (26)
PCB 183	8.9 ± 1.4	<LOD - 15.9 (15)	27.2 ± 6.5	11.0 - 72.3	15.1 ± 2.9	6.9 - 33.1	32.2 ± 4.5	18.4 - 67.5
PCB 185	<LOD	-	<LOD	-	<LOD	-	<LOD	-

a, number of samples above the limit of detection indicated in parentheses if < 16
b, number of samples above the limit of detection indicated in parentheses if < 28

Table 4.2. (Cont'd.)

	Muscle		Liver		Kidney		Blood	
Compound	**Mean ± SE**	**Range (n[a])**	**Mean ± SE**	**Range (n[a])**	**Mean ± SE**	**Range (n[a])**	**Mean ± SE**	**Range (n[b])**
PCB 187	11.0 ± 1.1	6.2 - 16.0	28.6 ± 4.8	10.7 - 54.6	16.3 ± 1.8	9.2 - 23.3	39.2 ± 5.5	<LOD - 75.5 (26)
PCB 188	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 189	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 191	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 194	<LOD	-	19.5 ± 8.5	<LOD - 79.2 (13)	<LOD	-	<LOD	-
PCB 195	7.6 ± 0.9	<LOD - 11.1 (15)	9.4 ± 1.4	<LOD - 17.7 (15)	9.5 ± 1.1	6.3 - 16.9	<LOD	-
PCB 196+203	20.6 ± 1.7	12.8 - 26.5	25.5 ± 2.7	16.4 - 40.7	23.6 ± 2.41	15.9 - 39.9	56.9 ± 12.8	<LOD - 129.3 (21)
PCB 197	10.9 ± 1.3	<LOD - 14.7 (15)	13.6 ± 1.4	8.9 - 21.5	11.0 ± 2.0	<LOD - 20.2 (14)	<LOD	-
PCB 199	<LOD	-	15.0 ± 6.5	<LOD - 53.9 (10)	10.1 ± 3.5	<LOD - 33.7 (11)	<LOD	-
PCB 200	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 201	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 202	<LOD	-	11.4 ± 1.9	<LOD - 20.0 (15)	<LOD	-	<LOD	-
PCB 205	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 206	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 207	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PCB 208	<LOD	-	<LOD	-	<LOD	-	<LOD	-
ΣPCBs	260.2 ± 45.3	129.5 - 494.9	1152.7 ± 309.2	411.1 - 3082.3	526.2 ± 113.7	227.5 - 1239.9	694.5 ± 130.8	217.3 - 1701.4
4,4'-DDE	<LOD	-	35.5 ± 13.7	<LOD - 114.4 (11)	<LOD	-	<LOD	-
4,4'-DDT	<LOD	-	<LOD	-	<LOD	-	<LOD	-
2,4-DDT + 4,4-DDD	<LOD	-	<LOD	-	<LOD	-	<LOD	-
2,4'-DDD	<LOD	-	<LOD	-	<LOD	-	<LOD	-
2,4'-DDE	<LOD	-	<LOD	-	<LOD	-	<LOD	-
oxychlordane	<LOD	-	41.8 ± 14.1	<LOD - 151.5 (15)	15.1 ± 7.3	<LOD - 70.3 (11)	<LOD	-
trans-chlordane	<LOD	-	<LOD	-	<LOD	-	<LOD	-
cis-chlordane	<LOD	-	<LOD	-	<LOD	-	<LOD	-
trans-nonachlor	<LOD	-	81.1 ± 35.2	6.2 - 367.8	24.0 ± 10.4	<LOD - 107.5 (15)	<LOD	-
cis-nonachlor	<LOD	-	15.1 ± 1.2	8.2 - 20.8	12.6 ± 1.6	<LOD - 20.3 (14)	<LOD	-
dieldrin	55.7 ± 9.9	17.3 - 119.8	387.1 ± 71.6	76.6 - 777.0	161.5 ± 22.6	47.1 - 274.4	<LOD	-
endrin	<LOD	-	<LOD	-	<LOD	-	<LOD	-
aldrin	<LOD	-	<LOD	-	<LOD	-	<LOD	-

a, number of samples above the limit of detection indicated in parentheses if < 16
b, number of samples above the limit of detection indicated in parentheses if < 28

Table 4.2. (Cont'd.)

	Muscle		Liver		Kidney		Blood	
Compound	**Mean ± SE**	**Range (n[a])**	**Mean ± SE**	**Range (n[a])**	**Mean ± SE**	**Range (n[a])**	**Mean ± SE**	**Range (n[b])**
heptachlor	<LOD	-	<LOD	-	<LOD	-	<LOD	-
heptachlor epoxide	9.8 ± 2.3	<LOD - 26.2 (14)	169.2 ± 40.3	21.8 - 410.9	64.5 ± 17.9	12.8 - 182.8	12.9 ± 1.7	<LOD - 22.8 (23)
endosulfan I	<LOD	-	<LOD	-	<LOD	-	<LOD	-
HCB	4.8 ± 1.0	<LOD - 11.4 (10)	40.4 ± 12.0	<LOD - 120.0 (15)	15.0 ± 4.3	<LOD - 43.5 (14)	<LOD	-
mirex	<LOD	-	73.51 ± 35.28	5.92 - 357.39	20.7 ± 7.7	<LOD - 81.3 (14)	49.4 ± 11.7	<LOD - 126.8 (26)
octachlorosytrene	<LOD	-	<LOD	-	<LOD	-	<LOD	-
pentachlorobenzene	9.8 ± 1.0	<LOD - 13.1 (11)	13.2 ± 1.4	8.0 - 22.1	12.9 ± 1.3	9.5 - 19.9	<LOD	-
alpha-HCH	14.5 ± 4.7	<LOD - 39.4 (10)	24.7 ± 11.8	<LOD - 92.2 (10)	20.0 ± 8.0	<LOD - 71.4 (11)	48.0 ± 12.1	<LOD - 104.3 (16)
beta-HCH	15.4 ± 3.2	<LOD - 30.5 (14)	106.5 ± 55.9	15.1 - 568.1	36.8 ± 18.0	<LOD - 174.6 (13)	38.6 ± 9.8	<LOD - 96.90(19)
gamma-HCH	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PBDE 17+25	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PBDE 30	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PBDE 33+28	6.9 ± 1.0	<LOD - 11.0 (14)	7.9 ± 0.9	<LOD - 10.8 (15)	9.9 ± 0.9	6.5 - 14.1	<LOD	-
PBDE 47	18.6 ± 3.1	7.5 - 39.9	21.9 ± 2.6	9.9 - 38.4	23.3 ± 3.2	10.1 - 43.4	13.3 ± 0.9	<LOD - 18.3 (26)
PBDE 49	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PBDE 66	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PBDE 71	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PBDE 75	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PBDE 85	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PBDE 99	22.8 ± 3.0	11.6 - 38.3	56.8 ± 14.6	15.1 - 140.7	34.9 ± 5.4	13.9 - 68.8	23.0 ± 3.8	<LOD - 41.0 (26)
PBDE 100	12.8 ± 2.1	<LOD - 23.2 (14)	22.9 ± 5.5	<LOD - 64.5 (15)	18.1 ± 2.2	<LOD - 28.3 (15)	25.3 ± 6.1	<LOD - 60.1 (19)
PBDE 116	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PBDE 119	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PBDE 138	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PBDE 153	<LOD	-	<LOD	-	<LOD	-	<LOD	-
PBDE 154	8.1 ± 0.5	5.4 - 10.2	10.6 ± 1.0	6.9 - 15.3	10.1 0.7	6.7 - 13.2	19.0 ± 2.1	<LOD - 29.9 (24)
PBDE 155	<LOD	-	<LOD	-	<LOD	-	<LOD	-
ΣPBDEs	69.1 ± 7.4	39.2 - 105.0	120.1 ± 21.3	54.4 - 236.0	96.3 ± 10.2	45.9 - 142.0	80.7 ± 8.3	45.0 - 127.0
ΣPOPs	1047.0 ± 85.0	792.7 - 1482.6	2948.5 ± 450.0	1404.4 - 5431.4	1643.1 ± 183.3	1071.5 - 2827.5	945.0 ± 139.6	375.2 - 1960.4
% lipids	0.46 ± 0.04	0.31 - 0.69	2.37 ± 0.44	1.15 - 4.84	1.25 ± 0.21	0.68 - 2.58	0.72 ± 0.07	0.41 - 1.02

a, number of samples above the limit of detection indicated in parentheses if < 16
b, number of samples above the limit of detection indicated in parentheses if < 28

Table 4.3. Concentration (mean ± SE, range) of essential and toxic elements in the blood (μg L^{-1}), carapace and tissues (μg g^{-1} wet mass) collected from *Chelonia mydas* at the Sea World Sea Turtle Rehabilitation Program, January 2006 to June 2007. LOD - limit of detection.

Tissue	Element	Essential Elements			Element	Toxic Metals		
		Mean ± SE	Range	n >LOD		Mean ± SE	Range	n >LOD
Muscle	**Co**	< LOD		0	**As**	5.25 ± 1.47	0.32 - 14.01	16
	Cu	0.34 ± 0.07	0.15 - 1.06	16	**Cd**	0.08 ± 0.01	0.05 - 0.19	16
	Zn	12.36 ± 2.83	5.95 - 38.94	16	**Hg**	0.02 ± 0.004	<LOD - 0.05	4
	Se	1.17 ± 0.31	0.19 - 3.60	16	**Pb**	< LOD		0
Liver	**Co**	0.61 ± 0.23	0.08 - 3.18	16	**As**	3.19 ± 0.79	0.63 - 9.71	16
	Cu	90.63 ± 11.64	38.22 - 153.40	16	**Cd**	13.54 ± 2.40	4.33 - 32.46	16
	Zn	36.30 ± 2.16	20.73 - 47.24	16	**Hg**	0.19 ± 0.04	<LOD - 0.54	12
	Se	3.95 ± 1.08	0.52 - 10.40	16	**Pb**	0.09 ± 0.02	<LOD - 0.28	7
Kidney	**Co**	1.50 ± 0.47	0.13 - 5.50	16	**As**	2.74 ± 0.89	0.12 - 9.27	16
	Cu	2.57 ± 0.24	1.40 - 4.55	16	**Cd**	45.98 ± 9.06	12.70 - 101.25	16
	Zn	28.75 ± 2.23	17.90 - 48.19	16	**Hg**	0.06 ± 0.02	<LOD - 0.20	13
	Se	1.67 ± 0.49	0.29 - 5.11	16	**Pb**	0.03 ± 0.01	<LOD - 0.12	2
Carapace	**Co**	0.04 ± 0.01	<LOD - 0.17	2	**As**	0.41 ± 0.08	0.08 - 1.17	16
	Cu	3.59 ± 1.81	0.75 - 24.74	16	**Cd**	0.16 ± 0.03	0.05 - 0.38	16
	Zn	77.12 ± 11.44	33.34 - 136.99	16	**Hg**	0.06 ± 0.02	<LOD - 0.20	11
	Se	0.57 ± 0.11	0.12 - 1.32	16	**Pb**	0.15 ± 0.06	<LOD - 0.72	7
Blood	**Co**	31.78 ± 5.84	4.18 - 95.71	28	**As**	2719.51 ± 883	91.37 - 16465.49	28
	Cu	957.06 ± 84.33	361.22 - 1880.44	28	**Cd**	31.76 ± 8.17	1.11 - 140.07	28
	Zn	7639.54 ± 526.4	3310.80 - 12263.4	28	**Hg**	1.88 ± 0.34	0.31 - 7.50	28
	Se	2202.53 ± 630.02	55.47 - 10111.4	28	**Pb**	20.78 ± 3.62	3.95 - 65.13	28

Relationships between blood and tissue contamination

The relationships between blood and tissue concentrations were statistically significant for many POP compounds. Blood levels of nine PCB congeners (99, 105, 118, 128, 138+158, 132+153, 170, 183 and 187) were significantly correlated with their respective liver, kidney and muscle concentrations (Figure 4.1; $R^2 > 0.66$, $P < 0.01$). Blood levels of three PBDEs (47, 99 and 154) were significantly correlated to their respective liver, kidney and muscle concentrations, although PBDE 47 only for liver (Figure 4.2; $R^2 > 0.54$, $P < 0.01$). Similarly, blood levels of three organochlorine pesticides (β-HCH, heptachlor epoxide and mirex) were significantly correlated to their respective liver, kidney and muscle concentrations, although mirex only for liver (Figure 4.3; $R^2 > 0.79$, $P < 0.01$).

The ANCOVAs that tested homogeneity of the blood-tissue regression slopes produced variable results for the different POP compounds. The blood-tissue regression slopes for liver, kidney and muscle were statistically parallel for all PCB compounds, except PCB 105 and PCB 138+158 (custom ANCOVA: $P > 0.05$). Furthermore, the elevation of these slopes showed similar patterns between compounds. The elevations of the regressions for all three tissue types were the same in PCB 132+153 and PCB 170 (ANCOVA: $P > 0.05$). However, in the remaining seven PCBs, the elevations of the blood-muscle regression were always statistically higher than liver and kidney (ANCOVA: $P > 0.05$; LSD: $P < 0.05$). The blood-tissue regression slopes for the liver, kidney and muscle were statistically parallel for PBDEs 99 and 154 (custom ANCOVA: $P > 0.05$). However, the elevations were statistically separated with muscle higher than kidney, which was higher than liver (ANCOVA: $P < 0.05$; LSD: $P < 0.05$). In contrast, the blood-tissue regression slopes for liver, kidney and muscle were statistically different for the OCPs (custom ANCOVA: $P < 0.05$). However, the blood-tissue relationships between the different tissues were consistent for both OCPs with the gradient for muscle steeper than that for kidney, which was steeper than that for liver.

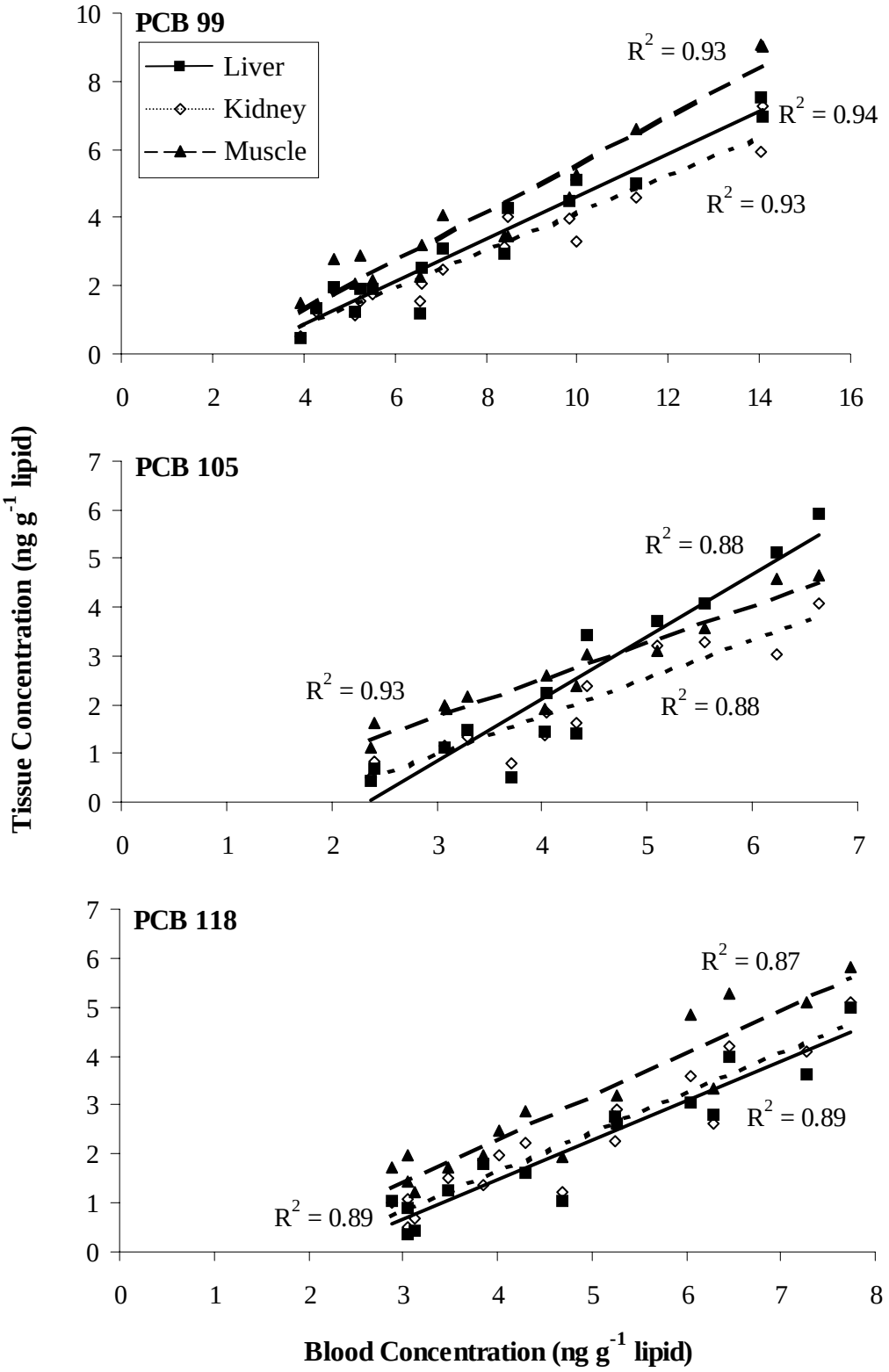

Figure 4.1a. Relationships between the blood concentration of PCBs 99, 105 and 118 with concentrations in the liver, kidney and muscle of *Chelonia mydas* from the Sea World Sea Turtle Rehabilitation Program, Queensland, Australia. The best-fitting lines are given for all significant regressions ($P < 0.05$).

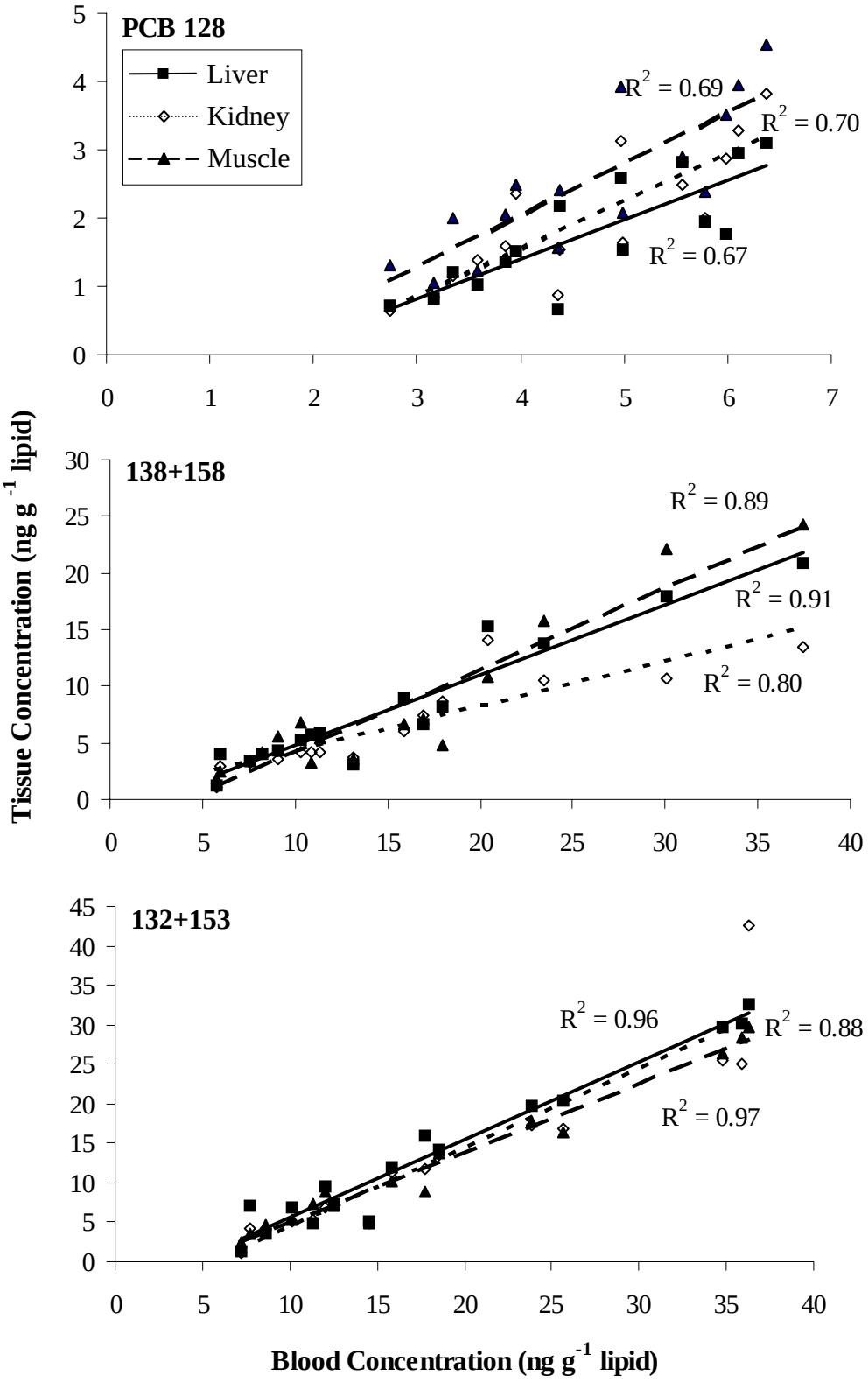

Figure 4.1b. Relationships between the blood concentration of PCBs 128, 138+158 and 132+153 with concentrations in the liver, kidney and muscle of *Chelonia mydas* from the Sea World Sea Turtle Rehabilitation Program, Queensland, Australia. The best-fitting lines are given for all significant regressions ($P < 0.05$).

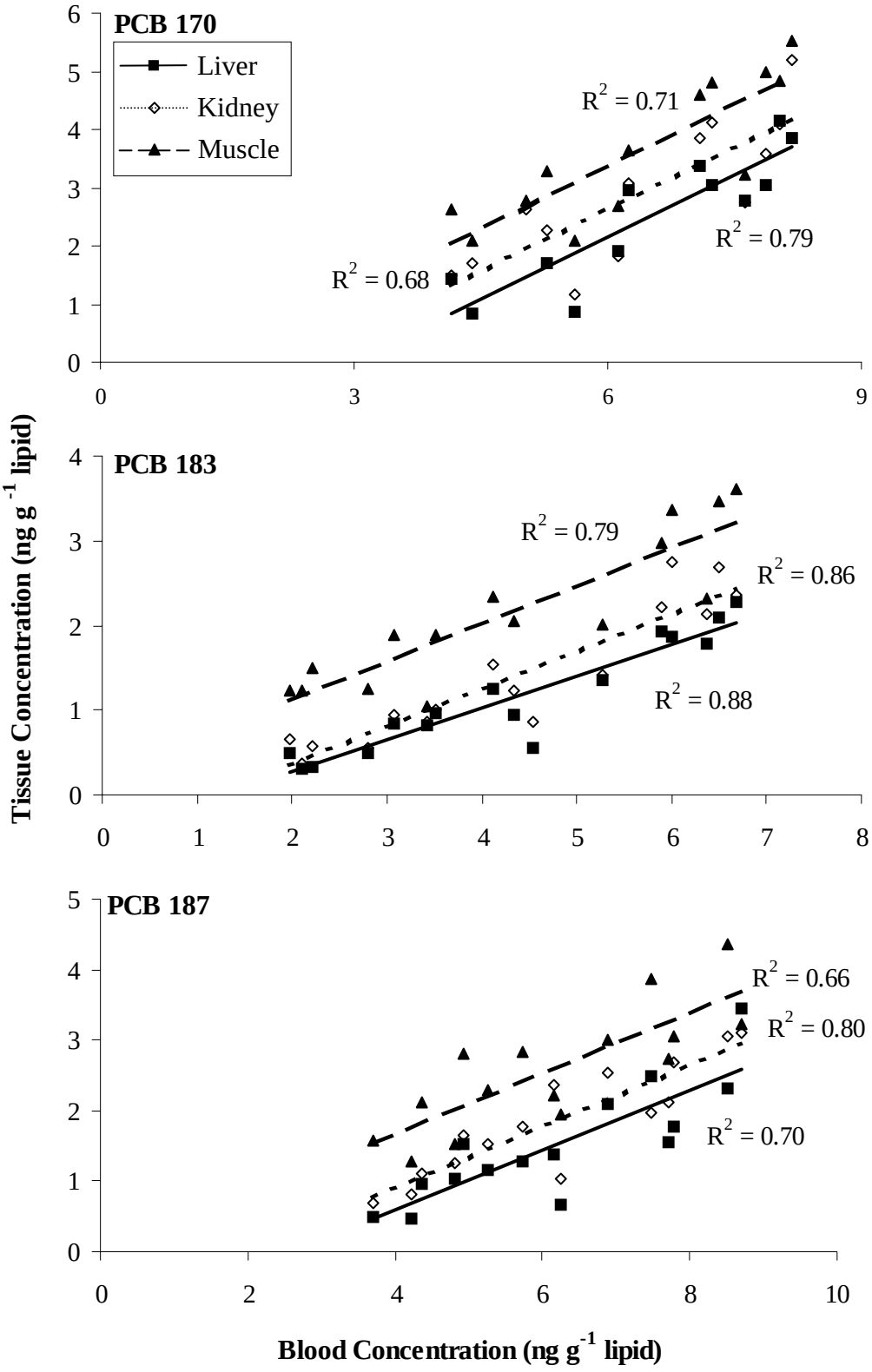

Figure 4.1c. Relationships between the blood concentration of PCBs 170, 183 and 187 with concentrations in the liver, kidney and muscle of *Chelonia mydas* from the Sea World Sea Turtle Rehabilitation Program, Queensland, Australia. The best-fitting lines are given for all significant regressions ($P < 0.05$).

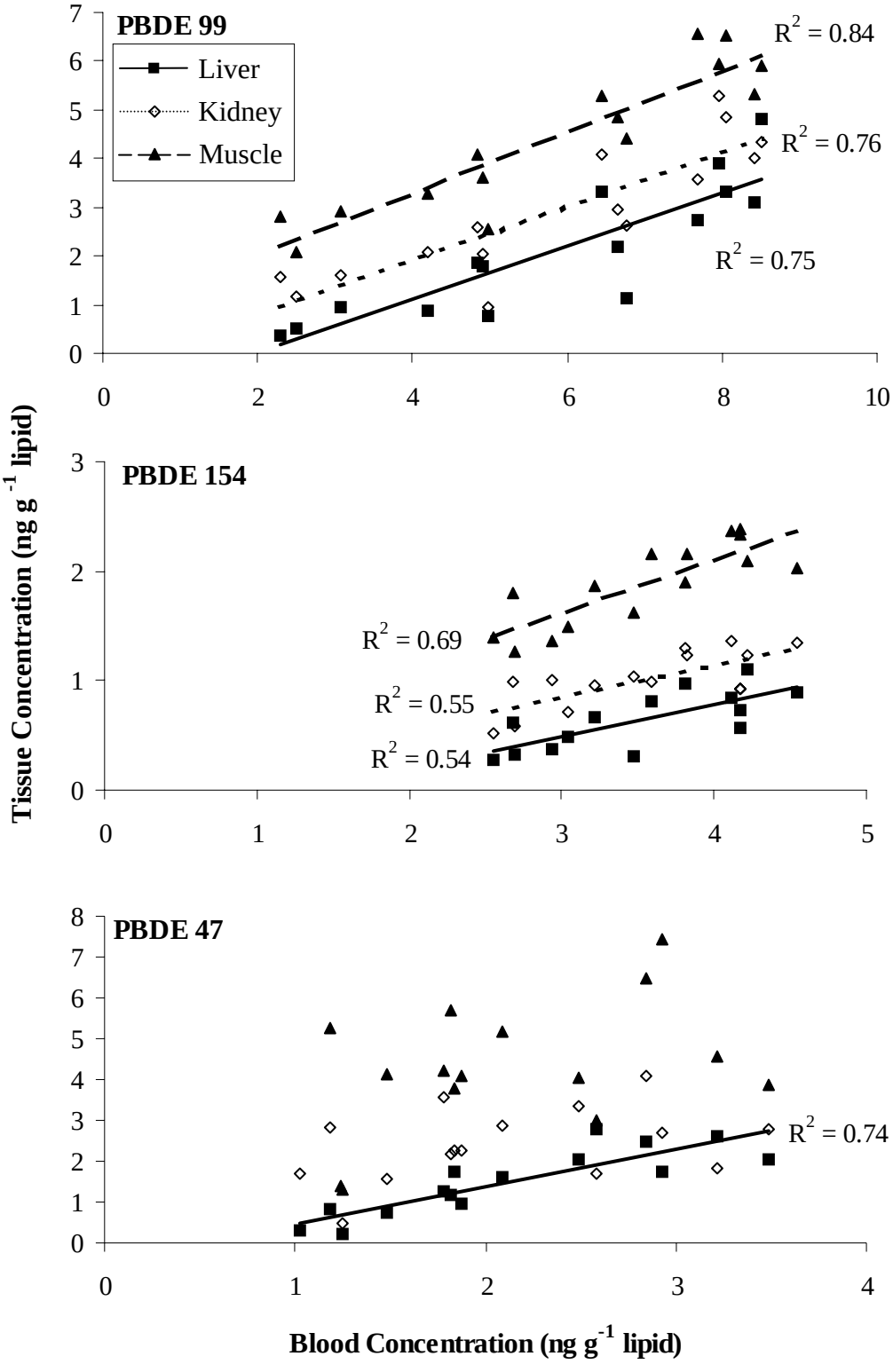

Figure 4.2. Relationships between the blood concentration of PBDEs 99, 154 and 47 with concentrations in the liver, kidney and muscle of *Chelonia mydas* from the Sea World Sea Turtle Rehabilitation Program, Queensland, Australia. The best-fitting lines are given for all significant regressions ($P < 0.05$). For PBDE 47, only the regression between blood and liver was significant.

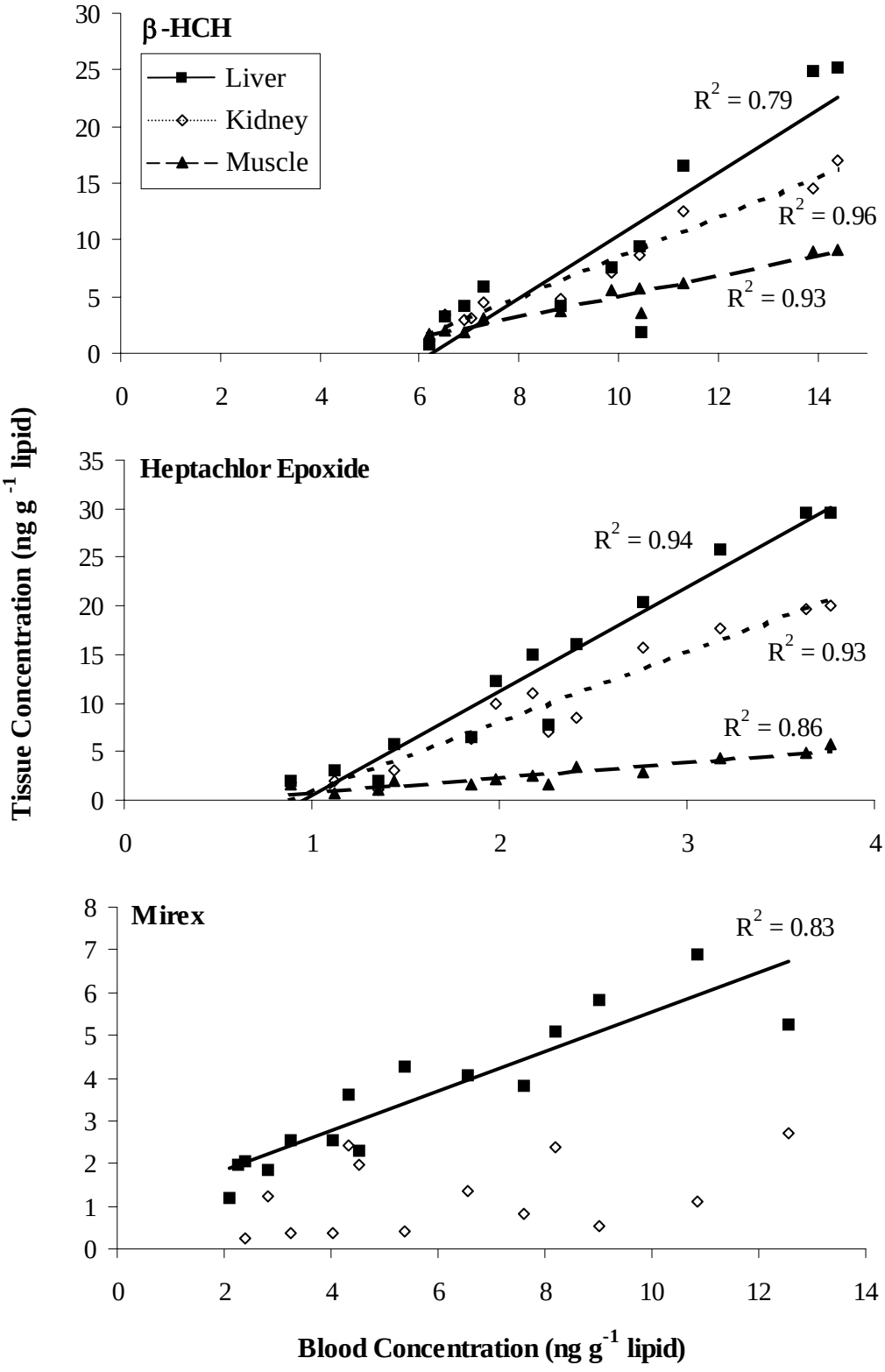

Figure 4.3. Relationships between the blood concentration of β-HCH, heptachlor epoxide and mirex with concentrations in the liver, kidney and muscle of *Chelonia mydas* from the Sea World Sea Turtle Rehabilitation Program, Queensland, Australia. The best-fitting lines are given for all significant regressions ($P < 0.05$). For mirex, only the regression between blood and liver was significant. Mirex was not detected in muscle.

The correlations between blood and tissue metal concentrations varied considerably between elements and tissue types. For essential elements, blood levels of selenium showed significant correlation with selenium concentrations in all tissue types (Figure 4.4a; $R^2 > 0.88$, $P < 0.005$). Blood levels of cobalt were significantly correlated with cobalt concentrations in the liver and kidney (Figure 4.4a: $R^2 > 0.80$, $P < 0.03$). However, the blood levels of copper or zinc were not significantly correlated with the concentrations of the respective metals, in any tissue type ($R^2 < 0.26$, $P > 0.25$).

For the toxic metals, blood levels of arsenic, cadmium and mercury each showed significant positive correlations with the corresponding element concentrations in the liver and kidney (Figure 4.4b; $R^2 > 0.62$, $P < 0.01$). However, only blood levels of arsenic showed a significant correlation to arsenic concentration in the muscle ($R^2 = 0.62$, $P < 0.03$). Lead was not detected in kidney or muscle and only in four of the liver samples. Consequently, blood levels of lead did not have significant relationships with lead concentrations of any of the tissues types ($P > 0.05$).

The ANCOVAs that tested for homogeneity of the blood-tissue regression slopes for various tissue types were significant for all metals (ANCOVA: $P < 0.05$). This indicated that the regression slopes for liver, kidney and muscle were statistically different from each other for each metal. Furthermore, the slopes of the different blood-tissues regressions were not consistent between the metals. For example, the blood-muscle regression was steepest for cadmium and the flattest for arsenic and mercury.

The mean (± SE) blood percent hematocrit was 26.2 ± 1.7% and values ranged from 3.5% to 72.8%. Percent hematocrits were significantly correlated with blood concentrations for Zn, As, Se, Cd, Hg and Pb (linear regressions: $P < 0.003$), although correlation coefficients were low (R^2: 0.16 - 0.52). There were no significant correlations between hematocrits and any of the POP compound concentrations (linear regressions: $P > 0.05$). When blood metal concentrations were divided by the hematocrit values, the relationships between blood and tissue concentrations changed (Table 4.4). However, the changes were not consistent, with some regressions and correlation coefficients increasing, while others decreased. For this reason, the

regressions between blood and tissue concentrations were analysed and presented as raw metal blood concentrations.

Table 4.4. Significance values (P) and correlation coefficients (R^2) of the regressions between blood and tissue metal concentrations. Blood values represent the raw blood concentrations ($\mu g\ L^{-1}$) and blood/hematocrit values are blood concentrations divided by percent hematocrits.

		Blood		Blood/Hematocrit	
Element	**Tissue**	**P**	**R^2**	**P**	**R^2**
Co	Kidney	**0.007**	0.80	**< 0.0005**	0.91
	Liver	**0.03**	0.82	**< 0.0005**	0.93
	Muscle	< LOD		< LOD	
Cu	Kidney	0.78	0.01	0.40	0.1
	Liver	0.38	0.11	0.16	0.26
	Muscle	0.72	0.02	**0.008**	0.66
Zn	Kidney	0.25	0.18	0.27	0.17
	Liver	0.58	0.05	0.22	0.21
	Muscle	0.25	0.26	0.68	0.04
As	Kidney	**< 0.0005**	0.97	**< 0.0005**	0.99
	Liver	**0.001**	0.89	**< 0.0005**	0.92
	Muscle	**0.03**	0.62	**0.006**	0.75
Se	Kidney	**< 0.0005**	0.89	**< 0.0005**	0.99
	Liver	**< 0.0005**	0.95	**< 0.0005**	0.98
	Muscle	**0.001**	0.88	**< 0.0005**	0.95
Cd	Kidney	**< 0.0005**	0.96	**0.001**	0.79
	Liver	**0.007**	0.76	0.2	0.23
	Muscle	0.81	0.01	0.86	0.01
Hg	Kidney	**0.02**	0.89	0.05	0.77
	Liver	**0.01**	0.89	**0.04**	0.92
	Muscle	0.27	0.54	0.25	0.56
Pb	Kidney	< LOD		< LOD	
	Liver	0.18	0.68	0.11	0.79
	Muscle	< LOD		< LOD	

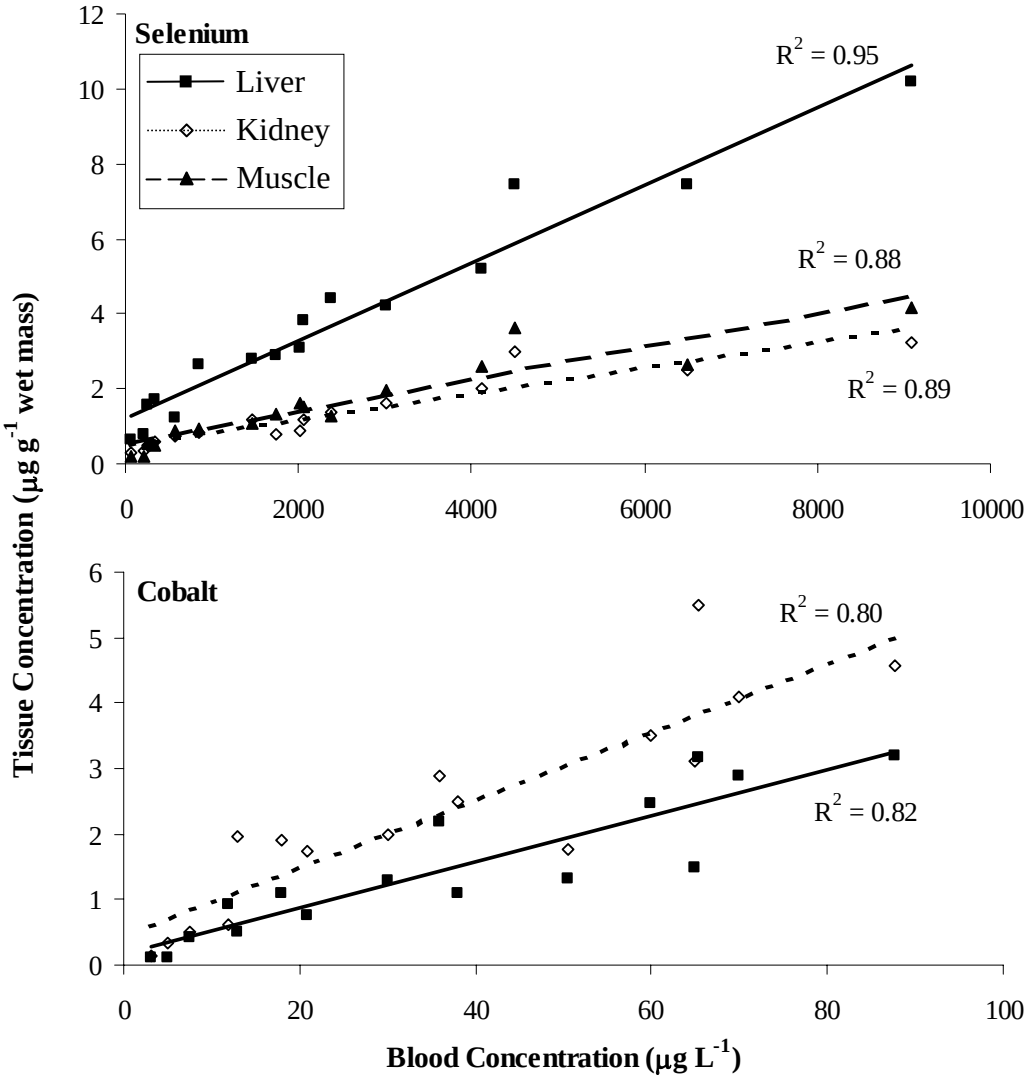

Figure 4.4a. Relationships between the blood concentration of selenium and cobalt with concentrations in the liver, kidney and muscle of *Chelonia mydas* from the Sea World Sea Turtle Rehabilitation Program, Queensland, Australia. All concentrations of cobalt in the muscle were < LOD. The best-fitting lines are given for all significant regressions ($P < 0.05$).

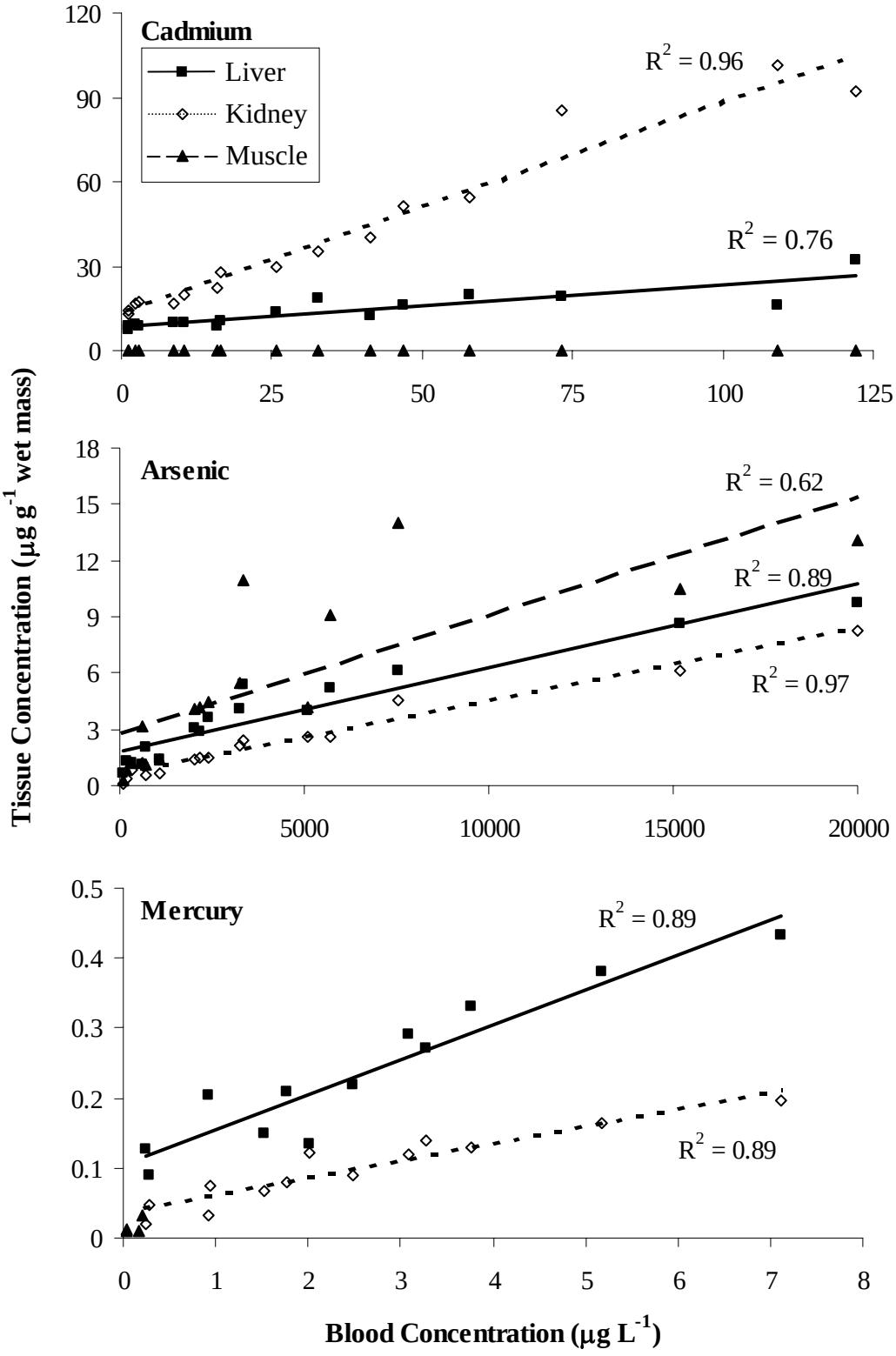

Figure 4.4b. Relationships between the blood concentration of cadmium, arsenic or mercury with concentrations in the liver, kidney and muscle of *Chelonia mydas* from the Sea World Sea Turtle Rehabilitation Program, Queensland, Australia. The best-fitting lines are given for all significant regressions ($P < 0.05$).

Relationships between carapace and tissue metal concentrations

The relationships between carapace and tissue concentrations of metals were less predictive and meaningful than those between blood and tissues. For the essential metals, only selenium levels in the carapace were significantly correlated to selenium concentrations in the liver, kidney and muscle (Figure 4.5a; $R^2 > 0.79$, $P < 0.003$). For the toxic metals, arsenic levels in the carapace showed significant correlations with arsenic concentrations in the liver, kidney and muscle (Figure 4.5b; $R^2 > 0.64$, $P < 0.02$). Mercury levels in the carapace were correlated to mercury concentrations in the liver and kidney (Figure 4.5b; $R^2 > 0.49$, $P < 0.05$). Cadmium levels in the carapace were correlated to cadmium concentration in the kidney only (Figure 4.5b; $R^2 = 0.86$, $P = 0.002$). Lead showed signs of a correlation between carapace and tissue concentrations. However, lead was not detected in a large number of carapace and tissue samples.

The ANCOVAs that tested for homogeneity of the carapace-tissue regression slopes were significant for all metals (custom ANCOVA: $P < 0.05$). This indicated that the regression slopes for liver, kidney and muscle were statistically different within each metal. Furthermore, the slopes of the different carapace-tissues regressions were not consistent between the metals. For example, the carapace-liver regression was steeper than muscle for selenium, but flatter than muscle for arsenic.

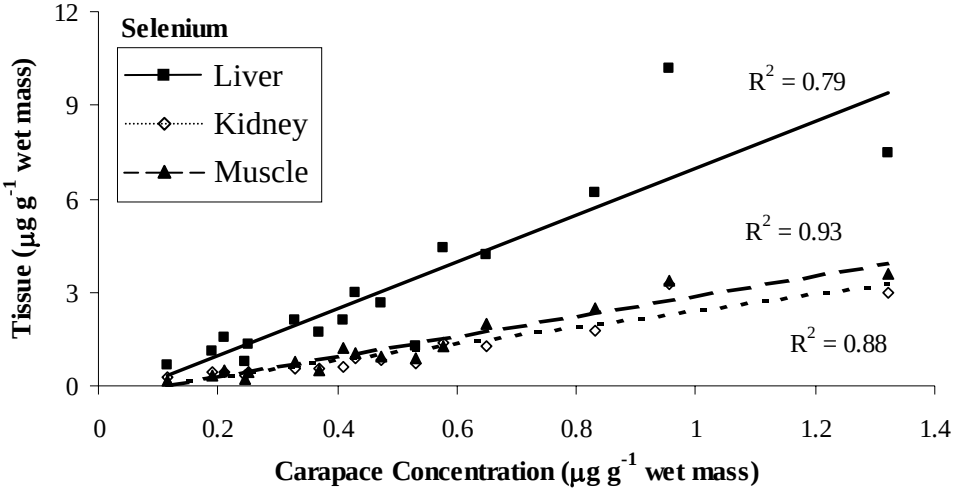

Figure 4.5a. Relationships between the carapace concentration of selenium with concentrations in the liver, kidney and muscle of *Chelonia mydas* from the Sea World Sea Turtle Rehabilitation Program, Queensland, Australia. The best-fitting lines are given for all significant regressions ($P < 0.05$).

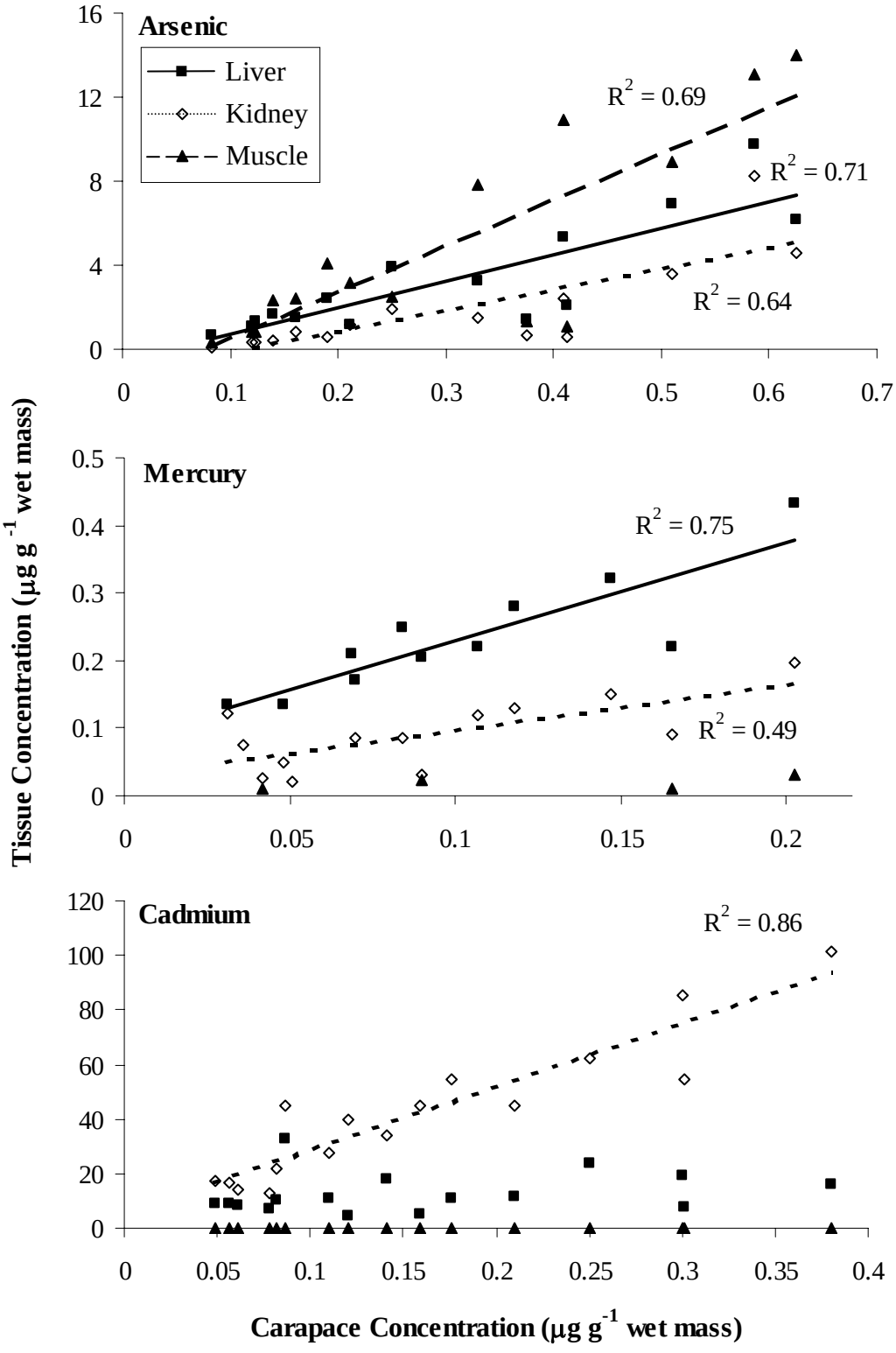

Figure 4.5b. Relationships between the carapace concentration of arsenic and cadmium with concentrations in the liver, kidney and muscle of *Chelonia mydas* from the Sea World Sea Turtle Rehabilitation Program, Queensland, Australia. All regressions were significant ($P < 0.05$). The best-fitting lines are given for all significant regressions ($P < 0.05$).

4.4 Discussion

Chemical contamination of rehabilitating turtles

The concentrations of PCBs, chlordanes and DDTs in the liver and muscle were 5-60 times lower than *C. mydas* in Florida, Hawaii, the Mediterranean Sea and the Atlantic Ocean (see Table 1.3 (Chapter 1): McKim and Johnson 1983; McKenzie et al. 1999; Miao et al. 2001). However, dieldrin concentrations in the liver were comparable to *C. mydas* studied in the Mediterranean Sea and Atlantic Ocean (McKenzie et al. 1999). The concentrations of the essential element copper were generally similar to other studies (see Table 1.5 - Chapter 1). This was expected as essential metals are involved in a number of important physiological and homoeostatic processes. These elements are therefore generally maintained within narrow concentration ranges by regulatory mechanisms (Bury et al. 2003). However, zinc concentrations were consistently lower (up to 6 times) than wild *C. mydas* populations sampled in Hong Kong, Mexico and Japan (see Table 1.5 (Chapter 1): Anan et al. 2001; Lam et al. 2004; Gardner et al. 2006; Talavera-Saenz et al. 2007). In addition, selenium concentrations were about five times higher than populations sampled in Hawaii and Southeast Queensland (see Table 1.5 (Chapter 1): Aguirre et al. 1994; Gordon et al. 1998). However, selenium concentrations are largely dependent on dietary intake with very little internal regulation (Reilly 1993). The concentrations of toxic metals in this study also differed considerably from previous research on *C. mydas*. Most interestingly, the tissue concentrations of arsenic and cadmium were up to 10 times higher than *C. mydas* studied in the same area (Moreton Bay) in 1990/91 (see Table 1.5 (Chapter 1): Gordon et al. 1998).

The differences in POP and toxic metal concentrations between previous studies could indicate chemical contamination of the *C. mydas* feeding areas. As described in Chapter 5, individuals from different feeding areas have significantly different chemical profiles, reflecting the contamination of their foraging habitats. This would indicate that POP contamination in Moreton Bay is generally lower than in other areas where *C. mydas* has been studied. Furthermore, the higher concentrations of cadmium and arsenic compared to *C. mydas* sampled in Moreton Bay in 1990/91 (Gordon et al. 1998) could indicate an increase in toxic metal contamination in this area over the last 17 years. This is supported by increases in the concentrations of toxic metals in sediment samples from Moreton Bay over this period. The concentrations of cadmium

and arsenic in marine and sand flat sediments around the time of the study by Gordon et al. (1998) were 0.01 - 0.8 mg kg^{-1} and 0.1 - 2.4 mg kg^{-1}, respectively (Saenger et al. 1991; French 1992). However, more recently, the concentrations of cadmium and arsenic in sediments of Moreton Bay have increased to 0.43 - 1.69 mg kg^{-1} and 0.2 - 16.9 mg kg^{-1}, respectively (Burton et al. 2005; Cox and Preda 2005).

When discussing the chemical contamination of a feeding *C. mydas* population, it is important to consider the subtle concentration differences within each foraging area. Although the *C. mydas* in this study came from within a small geographic region, there are still possibly site-specific differences in sediment and seagrass contamination within this area. This is supported by recent studies in Moreton Bay that found a large range in toxic metal concentrations in sediments collected from sites separated by only a few kilometres (Burton et al. 2005; Cox and Preda 2005). Furthermore, the specific feeding grounds for the *C. mydas* in this study are unknown. These individuals have potentially been foraging in habitats far removed from the Moreton Bay area and have drifted into this area once they had become debilitated.

There are also a number of internal processes that could contribute to the differences in POP and metal concentrations observed in this study. As previously mentioned, the *C. mydas* in this study required rehabilitation from a number of unidentified ailments. The stress of these conditions could therefore influence a number of internal processes. This is supported by the low levels of zinc observed in this study, an essential element normally regulated within narrow limits by homeostatic processes (Law et al. 1991). In addition, the cellular response to toxic metal contamination involves the production of metallothioneins that bind to the metals and reduce their bioavailability (Sanders et al. 1996). This process can also be disrupted by stress and disease, therefore allowing toxic metal concentrations to increase (Sanders et al. 1996). Furthermore, there are a number of complex interactions between essential and toxic metals (Goyer 1997). For example, the transcription of metallothionein in response to metal toxicity is mediated by zinc (Palmiter 1994). The lower zinc concentrations in this study could therefore reduce the ability to respond to toxic metal contamination.

Whether the POP and metal concentrations observed in this study are a reflection of the environment or disruption to internal processes, these chemicals may be contributing to debilitation of these turtles. Zinc deficiency can affect the structure and function of proteins involved in growth, reproduction, development, vision and immune function (Watanabe et al. 1997). Excess selenium has been associated with poisoning symptoms, such as respiratory, epidermal and mental problems (Reilly 1993). Furthermore, POPs and toxic metals have been associated with many diseases and physiological dysfunction (see Chapter 1). However, the chemical concentration ranges observed in this study were generally large. There were therefore a number of *C. mydas* at the SWSTRP with relatively low concentrations of toxic chemicals. Furthermore, numerous other factors may be contributing to the debilitation of these turtles. Therefore, direct links between contamination and *C. mydas* health cannot be made from the results of the present study, and warrants further investigation.

There is another important fact to consider in the interpretation of the accumulation of POPs and heavy metals in this study. When *C. mydas* arrive at the SWSTRP their diet is immediately changed from seagrass and algae to fish and squid. This is designed to enhance weight gain and recovery, but may also contribute to contamination and alter aspects of blood and tissue biochemistry. Furthermore, concentrations observed in this study may not represent the steady state of these chemicals in *C. mydas.* The stress and change in diet during rehabilitation may disrupt the processes involved in sequestration and distribution of these chemicals in *C. mydas*. The concentrations of POPs and metals observed in this study therefore do not completely reflect wild populations of *C. mydas*. However, rehabilitating *C. mydas* at the SWSTRP provide a platform on which to evaluate the correlations in chemical concentrations between blood, carapace and tissues.

Tissue-specific distribution of POPs and metals

The tissue distribution of POPs (on a wet mass basis) was generally highest in the liver, followed by the kidney and muscle. This was expected due to the lipophilic nature of POPs, causing them to preferentially accumulate in the lipid-rich tissues (Baird and Cann 2005). Interestingly, concentrations of POPs in the blood were often higher than tissue concentrations. This could be related to lipid content in the blood being higher than muscle and more than two-fold higher than blood concentrations in

loggerheads (*C. caretta*) and Kemp's ridleys (*Lepidochelys kempii*) of comparable size (Keller et al. 2004a). This was not expected as blood generally has lower lipid content than tissues. However, when sea turtles are debilitated, they generally become aphagic and may mobilise lipid stored in their body for energy. This was supported by the absence or severe depletion of carapace fat in the *C. mydas* that were dissected in the present study. As carapace fat usually has the highest concentration of POPs in *C. mydas* (McKenzie et al. 1999; Miao et al. 2001) it is probable that the mobilisation of fat stores into the bloodstream during debilitation and rehabilitation would also mobilise POP compounds and elevate blood concentrations. Alternatively, or additively, these compounds were elevated in the blood samples a result of ingesting these compounds during rehabilitation. However, for the individuals that were sampled on multiple occasions, there were no consistent increases in metal or POP blood concentrations over time. This indicates that the fish and squid being fed to these rehabilitating *C. mydas* are unlikely to have a strong influence on blood contamination.

The tissue distribution of metals was complex and variable, although the trends were similar to previous studies on metals in *C. mydas* (Aguirre et al. 1994; Gordon et al. 1998; Godley et al. 1999; Saeki et al. 2000; Sakai et al. 2000b; Anan et al. 2001; Fujihara et al. 2003; Lam et al. 2004; Gardner et al. 2006; Kampalath et al. 2006; Talavera-Saenz et al. 2007). Metal concentrations were generally lowest in the muscle, except for arsenic, which was highest in muscle. The concentrations of the essential metals zinc, copper and selenium were higher in the liver compared to kidney and cobalt concentrations were higher in the kidney than in the liver. Cadmium, lead and arsenic concentrations were higher in the kidney compared to the liver, while mercury concentrations were highest in the liver.

The reason for the variable distribution of metals in the different tissue types could be associated with biochemical processes. The essential elements distributed to different tissue types according to their requirement in metabolic processes (Foulkes 1996). The toxic metals, however, have no known function in the body and their tissue-specific distribution would be related to their bioavailability in the different tissues. This is determined by factors that are variable between different tissue types, such as

cell membrane structure and efficiency of metallothionein production (Foulkes 1996; Nieboer and Fletcher 1996; Sanders et al. 1996).

Relationships between blood and tissue contamination

The significant correlations observed between blood and tissue concentrations for the majority of POPs and heavy metals indicates that blood samples are a good predictor of the chemical contamination of liver, muscle and kidney. Furthermore, these correlations have been determined over large concentration ranges for most compounds. The strong relationships between blood and tissue contamination existed despite the large variation in the size, sex, condition and duration of rehabilitation of the *C. mydas* sampled in this study. This indicated that blood was a very useful non-lethal method for predicting the POP and metal concentrations in the internal tissues of *C. mydas* under a wide range of biological and environmental conditions.

The slopes and elevations of these blood-tissue correlations should also be considered when proposing the use of blood samples as a predictor of tissue contamination. The relationships between blood and tissue for POPs showed very consistent relationships between blood and tissue concentrations. When blood and tissue concentrations were standardised for lipid content (ie. ng g^{-1} of lipid), the relationships between blood and liver, kidney and muscle had similar slopes and elevations. This further implies that lipophilic POPs bind with lipids and are distributed to tissues in proportions dependent on their lipid content. However, the relationships between blood and tissue concentrations for metals were much more variable than for POPs, indicating that the use of blood to predict general metal contamination is more tissue and element specific.

There are a number of factors that could contribute to the range and variability of the correlations between blood and tissue concentrations. These include factors that would affect all sea turtle populations, such as age, sex and health of individuals. However, there are also more rehabilitation specific factors such as time between feeding and sampling and the stage of recovery.

Many POPs and metals are very resistant to breakdown and, consequently, bioaccumulate in marine vertebrates over time (Baird and Cann 2005). Chemical

contamination in sea turtles would therefore increase with time spent in contaminated foraging areas. For this reason, adult turtles would be expected to have higher chemical concentrations than juveniles and sub-adults feeding in the same foraging areas. However, over a specific time period, smaller individuals feeding in the same areas would accumulate higher concentrations relative to their body mass. Adult males and females also migrate away from the foraging area every 2-8 years for breeding (Miller 1997). This can take several months and therefore reduces exposure to contamination in their foraging area over this period. Furthermore, as previously mentioned, individual health and differences in chemical contamination within a foraging area may also contribute to variation in chemical concentrations between turtles. The size specific contamination of sea turtles in a foraging ground is therefore more complex than a simple increase with age. This is supported by the non-significant correlations between CCL and initial blood concentrations observed in this study.

The sex and reproductive activities of *C. mydas* may also influence their chemical concentrations. Sexually mature females can offload POPs during egg production and oviposition processes (see Chapter 6). Therefore adult females would be expected to have lower contaminant concentrations than males and non-adult females feeding in the same location. This is supported in the present study by the POP concentrations in the initial blood samples of adult females being significantly lower than in non-adult females and males. However, there were no differences in essential or toxic metal concentrations between adult females and the remaining *C. mydas*. Furthermore, when adult females were removed from the analysis, there was no influence of sex on the concentrations of POPs or heavy metals. This indicates that sex is a particularly important factor to consider when analysing POPs in a *C. mydas* population that contains adult females.

For a number of metals, blood concentrations were significantly correlated with percent hematocrit. Furthermore, when blood metal concentrations were adjusted for hematocrit, some of the correlation coefficients of the blood-tissue relationships increased. Adjusting blood concentrations for hematocrit effectively increases the influence of erythrocyte concentrations. In humans, plasma metal concentrations reflect more recent dietary intake, while the erythrocyte levels represent longer term

exposure (Thomson and Robinson 1980; Chang 1996). Therefore, the correlation coefficients that increased when adjusted for hematocrit indicated that blood samples could also predict longer-term exposure to these elements. Percent hematocrit should therefore be considered when using blood samples to predict tissue concentrations of metals. Within a healthy population, percent hematocrit is generally less variable (Grumbles et al. 1990; Bolten and Bjorndal 1992). However, it may still be an important consideration, particularly when comparing populations of different health. For POPs, there was no correlation between blood concentrations and percent hematocrit. This is most likely due to the fact that POPs are found in highest concentrations in the plasma and therefore less affected by the proportion of erythrocytes in the sample (Keller et al. 2004b). The influence of percent hematocrit on the blood-tissue correlations was therefore not further investigated.

More specific to the chemical contamination of rehabilitating *C. mydas* are factors such as time between feeding and sampling and stage of recovery. Chemicals ingested with food are passed through the digestive tract and into the bloodstream where they are transported to the different tissues (Langston and Spence 1995). There is therefore a time lapse between ingestion and storage in the tissues when blood concentrations of these chemicals may be elevated. This must be considered when using blood to estimate contamination as samples taken soon after the animal has eaten may provide overestimates of POP and metal concentrations in the tissues. Furthermore, the stage of recovery may influence blood contamination levels. As individuals begin to recover and regenerate lipid supplies, the lipophilic POPs could be removed from the blood for storage within the lipid. Also, homeostatic processes and cell responses to chemical contamination may improve with recovery. However, as mentioned previously, there were no consistent increases in POP or metal concentrations over time for *C. mydas* sampled on multiple occasions. This indicated that the contamination of fish and squid fed to rehabilitating *C. mydas* was negligible and unlikely to have a strong influence on blood concentrations.

Despite the significant correlations observed in many POPs and metals, there were a number of compounds that did not have significant correlations between blood and tissue. In most cases, this was due to too few sample numbers above the limit of detection and/or narrow concentrations ranges. However, there was also high degree

of variation in blood concentrations of POPs and metals observed over time. In the few turtles where three or more blood samples were taken over the duration of the study, there was up to 8-fold concentration differences between successive sampling. However, the variation was haphazard with no observable trends. Further research into the variations of blood concentrations over time is required to further validate blood as a predictor of tissue contamination.

Relationships between carapace and tissue metal contamination

Significant correlations between carapace and tissue metal concentrations were only found for the essential metal selenium and the toxic metals arsenic, mercury and cadmium. Furthermore, the correlation coefficients were generally lower than for the blood-tissue regressions. Due to the high levels of keratin, which binds strongly and permanently with metals (Crewther et al. 1965), carapace samples were expected to represent a more stable and long-term measure of metal contamination. In comparison to the results of the present study, a study on *C. caretta* on the east coast of the United States using the same carapace sampling methods, found more significant and meaningful correlations between concentrations of mercury in the carapace and tissues (Day et al. 2005). However, these correlations were driven by a single sample with high concentrations in blood and tissue. Furthermore, these *C. carretta* were captured in their feeding grounds, and had presumably been exposed to relatively constant mercury contamination throughout their coastal feeding life-stage.

The aforementioned factors that may have influenced the blood-tissue correlations would generally impact over a short time period. These factors would therefore not be reflected in the carapace samples. Also, the *C. mydas* in this study had been exposed to a change in diet during rehabilitation. This may have introduced metals that had time to accumulate in tissue samples but not in the carapace. Furthermore, as the carapace keratin is laid down over time, the sampling method may have taken a cross section of these layers (Solomon et al. 1986). This could incorporate variability associated with past exposure patterns to these elements. There is therefore much more variation associated with using carapace samples to represent real time metal concentrations of the internal tissues, particularly in a situation of a changing diet.

4.5 Conclusions

This study provides background levels of chemical contamination in rehabilitating *Chelonia mydas* in Southeast Queensland. Concentrations of POPs and metals differed significantly from other *C. mydas* populations, although accumulation showed similar tissue-specific patterns. This was also the first study of its kind to investigate the use of blood and carapace as non-lethal samples for predicting chemical contamination in the liver, kidney and muscle tissues of sea turtles for such a wide range of POP and metal compounds. Blood was a very good predictor of internal organ tissue contamination with significant positive correlations observed for many important POP and heavy metal compounds. These correlations were very consistent between tissue types for the POP compounds, although the tissue-specific correlations for metals were more complex. Carapace samples were also significantly correlated with tissue concentrations for a number of metals. However, correlation coefficients were lower than for the blood-tissue relationships.

The results of this study therefore indicate that blood and carapace samples are good predictors of POP and metal contamination in the liver, kidney and muscle of *C. mydas*. Although investigations into these relationships in healthy animals may be warranted, the results of this study indicate that researchers around the world can now take blood and carapace samples from wild *C. mydas* populations with the confidence that these samples will represent internal tissue contamination. This will reduce the need for more invasive methods, such as tissue biopsies, and will allow a much more efficient and thorough assessment of the health of sea turtle individuals and populations.

Chapter 5 - Satellite telemetry and egg contaminant analysis: identifying conservation issues for a major C*helonia mydas* breeding population in Peninsular Malaysia

5.1 Introduction

The chemical contamination and habitat utilisation of *Chelonia mydas* populations are important issues in their conservation. Chemicals such as persistent organic pollutants (POPs) and heavy metals have been reported in a number of sea turtle populations (see Chapter 1), and due to their wide range of harmful effects, they are a current conservation concern. However, to fully understand the extent and variation of chemical contamination in a breeding *C. mydas* population, information on the turtles' movement within and between nesting, breeding and foraging habitats is required. Migration routes and areas utilised during foraging are particularly important habitats in terms of the accumulation of chemicals by sea turtles. Understanding the location and use of these habitats is therefore critical to chemical contamination research in sea turtles.

Chelonia mydas hatchlings spend the initial few years of life in open ocean pelagic habitats, feeding on an omnivorous diet of gelatinous zooplankton and the sargassum rafts with which they associate for protection (Bjorndal 1985; Limpus and Miller 1993; Musick and Limpus 1997; Zug and Glor 1998; Reich et al. 2007). Between the ages of three and six years (25-35 cm straight carapace length), *C. mydas* take up residence in neritic habitats, such as coastal seagrass meadows and reefs (Mendonca and Ehrhart 1982; Balazs 1985; Green 1993; Musick and Limpus 1997; Zug and Glor 1998; Bjorndal et al. 2005). At this stage, *C. mydas* convert to an almost exclusively herbivorous diet of seagrass and macroalgae (Mortimer 1982; Bjorndal 1985, 1997). Sub-adult *C. mydas* remain in these foraging areas until they become sexually mature and are ready to begin migration to breeding grounds, which are in their natal areas (Meylan et al. 1990; Bowen et al. 1992). During their reproductive years, *C. mydas* show strong fidelity to these foraging and breeding sites, which can be up to thousands of kilometres apart (Carr 1964; Carr and Carr 1972; Limpus et al. 1992; Lohmann et al. 1997; Lohmann et al. 1999). *Chelonia mydas* from a single foraging

area may migrate to a number of different breeding areas, and a breeding population is often populated by individuals from a wide range of foraging areas (Balazs 1994; Balazs et al. 1994; Liew et al. 1995; Cheng 2000; Godley et al. 2002; Seminoff et al. 2008). Therefore, threats to *C. mydas* in a foraging area have the potential to affect a number of different breeding populations and a single breeding population can be influenced by threats in a number of different foraging areas.

The strong fidelity to natal rookeries results in genetically distinct populations being established around major breeding areas (Bowen et al. 1992; Norman et al. 1994; Fitzsimmons et al. 1996). This implies that each breeding *C. mydas* population must be treated as an independent management unit when considering threats and conservation. However, effective management of a breeding population also requires knowledge of the threats at the different foraging grounds and along the migration routes. This is complicated by the fact that foraging areas can be long distances from the breeding areas and can often be under the control of other countries with varying conservation ethics, regulations and resources. Satellite telemetry has been used to track the movement of *C. mydas* in breeding and foraging habitats and along migration routes (Balazs 1994; Balazs et al. 1994; Liew et al. 1995; Luschi et al. 1998; Balazs and Ellis 2000; Cheng 2000; Godley et al. 2002; Troeng et al. 2005; Hatase et al. 2006). These studies provide important information on *C. mydas* behaviour and can be used to more adequately manage the conservation of *C. mydas* populations through identification of critical habitats.

The identification of foraging grounds through satellite telemetry is particularly important in understanding the chemical contamination of a breeding sea turtle population. Toxic chemicals such as POPs and heavy metals can have a wide range of harmful effects on the development and function of marine animals (see Chapter 1). Measuring these chemicals in *C. mydas* populations is therefore an important area of conservation research. Heavy metals and POPs make their way into the sediments and seagrasses of coastal areas from a variety of agricultural, domestic and industrial sources (Connell et al. 1999) and accumulate in marine vertebrates nearly exclusively through their diet (Langston and Spence 1995). As *C. mydas* are generally aphagic during migration and breeding (Bjorndal 1985), chemical contamination will occur nearly exclusively in their foraging areas. Chemical contamination of *C. mydas*

nesting populations will therefore be dependent on the extent of contamination in each foraging ground, which can be diverse in location, ranging from estuaries adjacent to coastal urban development and agricultural areas to relatively pristine offshore reef areas (Limpus and Walther 1980; Mendonca and Ehrhart 1982; Balazs 1985; Limpus et al. 1992; Green 1993). Establishing the location of the foraging areas of a breeding population is therefore the first step in addressing the source of chemical contamination in breeding *C. mydas* populations.

The accumulation of chemicals in *C. mydas* from different feeding grounds could also provide information about the foraging distribution of a breeding population. As conservative pollutants, POPs and heavy metals are particularly resistant to breakdown (Connell et al. 1999). It could therefore be expected that animals of similar ages feeding in the same foraging ground might accumulate the same chemical compounds at similar concentrations. This would produce distinct "chemical profiles" which would presumably be similar for same aged individuals feeding in the same area. Satellite telemetry is relatively expensive and it is often not possible to identify the foraging grounds of all individuals of a single breeding population using these methods. Chemical profiles based on conservative pollutants could therefore provide a more cost and time effective means for obtaining information about foraging ground variability within a nesting population, with the advantage of also assessing the extent of the chemical contamination of this population.

The Ma'Daerah Sea Turtle Sanctuary, on the east of Peninsular Malaysia, supports a large breeding population of *C. mydas*. This population has declined by > 80% since the 1950s, primarily due to the collection of eggs for human consumption, nesting beach development and the accidental capture of turtles in fishing gear (Ibrahim 1994). The current management of this population involves the use of hatcheries on the nesting beaches to protect the eggs from collection and a "no trawl zone" which extends 5.5 nautical miles offshore from the nesting sites (*Fisheries Act*, 1985). However, there is currently no information on the chemical contamination of this population or the important areas used during breeding, inter-nesting, migration and foraging. This study aimed to use satellite telemetry to identify the important habitats of this population. Furthermore, this study will use egg samples to assess the chemical contamination of *C. mydas* individuals from different foraging grounds and evaluate

the potential of using chemical profiles to estimate foraging ground variability of *C. mydas* nesting populations.

5.2 Methods

Satellite transmitter attachment

Between August 20 and September 9, 2005, satellite transmitters were attached to three female *C. mydas* nesting at the Ma'Daerah Turtle Sanctuary (4° 32' 17" N, 103° 28' 14" E) and one adult male captured in the cooling tanks of the YTL Power station (4° 35' 26" N, 103° 27' 7" E) in Paka, Terengganu, Peninsular Malaysia (Table 5.1). At the time of transmitter attachment, morphological measurements were taken (see Chapter 2) and nesting records for the season were consulted to determine any previous nesting activity of the tracked turtles. Of the three nesting females, two had nested previously (A and C) and one had no nests recorded for this season (B). Although Turtle A had laid only one previous nest, recorded on June 25, 2005, the 11 to 15 day inter-nesting interval for this population (Kamarruddin Ibrahim, pers. comm.) suggests that this individual laid a total of five clutches before the date of transmitter attachment (see Table 5.1). Similarly, Turtle C had most likely laid two more clutches between the five clutches recorded prior to the date of transmitter attachment (see Table 5.1). Although there was no record of Turtle B nesting prior to satellite attachment, it is possible that this individual had previously nested undetected in the area this season.

Table 5.1. Tag, morphology and nesting details of the *Chelonia mydas* that were satellite tagged in August/September, 2005 at the Ma'Daerah Turtle Sanctuary, Terengganu Malaysia.

	Turtle A	**Turtle B**	**Turtle C**	**Turtle D**
Satellite ID	42515	59980	59979	42737
Date captured	20/8/05	20/8/05	28/8/05	9/9/05
Flipper tag	IF2736 / IF2927	IF2734 / IF2935	IF2728 / IF2929	N/A
Sex	Female	Female	Female	Male
CCL (cm)	100	95	105	90.5
CCW (cm)	94.7	90	95	77.7
Mass (kg)	115	110	124	75
Clutch size	98	124	95	N/A
Previous nesting	25/6/05 (104)	No record	3/6/05 (90)	N/A
Date (# eggs)	~ 6/7/05[a]		15/6/05 (91)	
	~ 18/7/05[a]		~ 28/6/05[a]	
	~ 29/7/05[a]		10/7/05 (95)	
	~ 9/8/05[a]		22/7/05 (97)	
			4/8/05 (98)	
			~ 16/8/05[a]	

a, nesting dates estimated based on recorded nests and the 11-14 day inter-nesting period for this population of *C. mydas*.

Kiwi Sat (0.5 W) Platform Terminal Transmitters (PTTs) powered by two lithium C cells (Sirtrack, New Zealand) were attached to the carapace using methods modified from Balazs et al. (1996). During attachment, turtles were kept in a 1.5 x 1.5 m fibreglass tub, which allowed minimal movement of the turtle. Medium-grain sandpaper was used to clear the two anterior vertebral scutes and surrounding costal scutes of algae, barnacles and other fouling organisms. This area was then scrubbed, rinsed with freshwater and dried before finally cleaning with an acetone dampened cloth. The PTT was placed along the spine of the carapace over the first and second vertebral scutes and the outline was traced with a permanent marker. The PTT was removed and 110 g of a two-part waterproof epoxy putty (Knead It Aqua; Selleys, Australia) was kneaded and rolled into a 1 cm diameter length and pressed onto the carapace around the outline of the PTT. Approximately 28 g of two-part waterproof epoxy (S-31; ITW Devcon, Japan) was then applied to the area of attachment forming a reservoir within the putty construction. The PTT was then placed in the reservoir and the putty was pressed against the sides of the PTT forming a tight seal. Approximately 45 drops of catalyst were added to a polyester surfboard resin and

stirred for 15 seconds. Making sure not to cover the saltwater switches and the magnetic activation switch, the resin was liberally applied to the PTT and surrounding carapace. Four strips of fibreglass cloth were laid over the PTT, completely wetting each layer with resin before application of the next layer. The fibreglass was completely dried within 5 hours of application (Figure 5.1), after which the transmitters were activated and the turtles released.

Figure 5.1. Photograph of satellite transmitter attached to the carapace of a nesting female *Chelonia mydas*.

Tracking and mapping

Location information for each turtle was obtained from the Argos satellite system, a joint venture between the Centre National d'Etudes Spatiales (CNES, the French space agency), the National Aeronautics and Space Administration (NASA, USA) and the National Oceanic and Atmospheric Administration (NOAA, USA) (Argos 1996). Position fixes were received in seven classes (3, 2, 1, 0, A, B, Z) and classes 0, B and Z were removed from the data set due to their unreliability (Hays et al. 2001a). Route maps and movement within nesting and foraging grounds were generated using the Satellite Tracking and Analysis Tool (Coyne and Godley 2005) and Maptool

(SEATURTLE.ORG 2002). A speed filter of 5 km h^{-1} between successive points was applied to eliminate any biologically unreasonable data. Minimum convex polygons, joining the outermost points of an individuals foraging or inter-nesting range, were generated with the Animal Movement extension for ArcView 3.2 (ESRI, CA, USA). As well as providing location data, these transmitters also recorded sea surface temperature (± 1°C). Mean temperatures were calculated for breeding, migration and foraging habitats of each turtle.

Egg collection and contaminant analysis

A sample of 10 eggs was randomly collected from a single clutch of each of the three nesting females equipped with satellite transmitters. Eggs were collected at the time of oviposition and care was taken not to contaminate the samples with sand from the nest environment. The eggs were individually wrapped in hexane rinsed aluminium foil, sealed in a zip-lock bag and immediately frozen and kept at -20 °C until transport back to Australia. The frozen eggs were transported in an insulated box surrounded by ice packs as carry-on luggage on a commercial flight from Kuala Lumpur to Brisbane. Upon arrival in Australia, the eggs were still frozen solid and were immediately taken to the Organics Section, Queensland Health Scientific Services, in Coopers Plains where they were kept frozen (-20 °C) until analysis.

All egg samples were analysed for POPs and metals using methods outlined in Chapters 2 and 3. Briefly, POPs were analysed using GC-MS/MS following accelerated solvent extraction in dichloromethane, gel permeation chromatography and Florisil™ column clean-up. Percent lipids were determined gravimetrically for each sample following extraction (Chapter 3). Metals were analysed using ICP-MS and CV-AAS following acid digestions (Chapter 2). Standard reference materials were run for each type of analysis and pooled samples were run for quality control.

Statistical analyses

Mean POP and metal concentrations (± SE) for each clutch were calculated with all values below the level of detection assigned a value half the limit of detection. This produced the least amount of bias while not requiring the use of complex iteration

software (Helsel 1990). However, if all eggs of a single clutch were below the limit of detection, the mean was not calculated.

Similar POP compounds were grouped into: 1) polychlorinated biphenyls (PCBs), 2) chlordanes, 3) hexachlorocyclohexanes (HCHs), 4) dichlorodiphenyltrichloroethane and its metabolites (DDTs), 5) polybrominated diphenyl ethers (PBDEs), and 6) the remaining organochlorine pesticides (OCPs), and the groups were summed for each egg (Table 5.2). One-factor ANOVAs were used to test for differences in concentration between clutches for each POP group and metal element. In cases of significant difference, LSD post-hoc tests were performed to indicate which clutches were different. The assumptions of normality and homogeneity were met by interpretation of residuals plotted against the dependent variables and Levene's test of equality of error variances.

Table 5.2. Compounds assigned to the major POP groups.

POP Group	POP Compounds
PCBs	83 PCB congeners
OCPs	Pentachlorobenzene, heptachlor, aldrin, octachlorostyrene, heptachlor epoxide, endosulfan I, mirex dieldrin, endrin
Chlordanes	Oxychlordane, *trans/cis*-chlordane, *trans/cis*-nonachlor
HCHs	α, β and γ - HCH
DDTs	2,4'-DDE, 4,4'-DDE, 2,4'-DDD, 2,4'-DDT, 4,4'-DDD, 4,4'-DDT
PBDEs	19 PBDE congeners

To investigate differences in contamination profiles between clutches, two separate Bray-Curtis similarity matrices with no data transformation were constructed for POPs and metals using Primer v5 (PRIMER-E, UK). Each POP compound and metal element was entered as a separate variable and each egg was analysed as an individual sample. To investigate similarity in the chemical composition of the three clutches, analysis of similarity (ANOSIM) was performed on each of the matrices and non-metric multi dimensional scaling (*n*MDS) plots were constructed (PRIMER-E, UK).

5.3 Results

Satellite tracking

Location data from the satellite transmitters identified inter-nesting movement, post-nesting migration and movement within home foraging grounds (Table 5.3). Two of the turtles (B and D) remained within 30 km of the nesting beach after satellite deployment for 41 and 11 days, respectively, before beginning post-nesting/breeding migration to foraging areas (Figure 5.2). The nesting female (B) occupied an area of 78.5 km^2 within 6.5 km of the shore and 9 km north and south of the Ma'Daerah Sea Turtle Sanctuary. During this period, she laid three more clutches at Ma'Daerah (Table 5.3). The adult male (D) occupied an area of 398 km^2 within 30 km of the coast and 14 km north and south of Ma'Daerah. However, the range of the male rarely overlapped with the female, generally remaining further than 8 km from the coastline in an area of 320 km^2 (Figure 5.2).

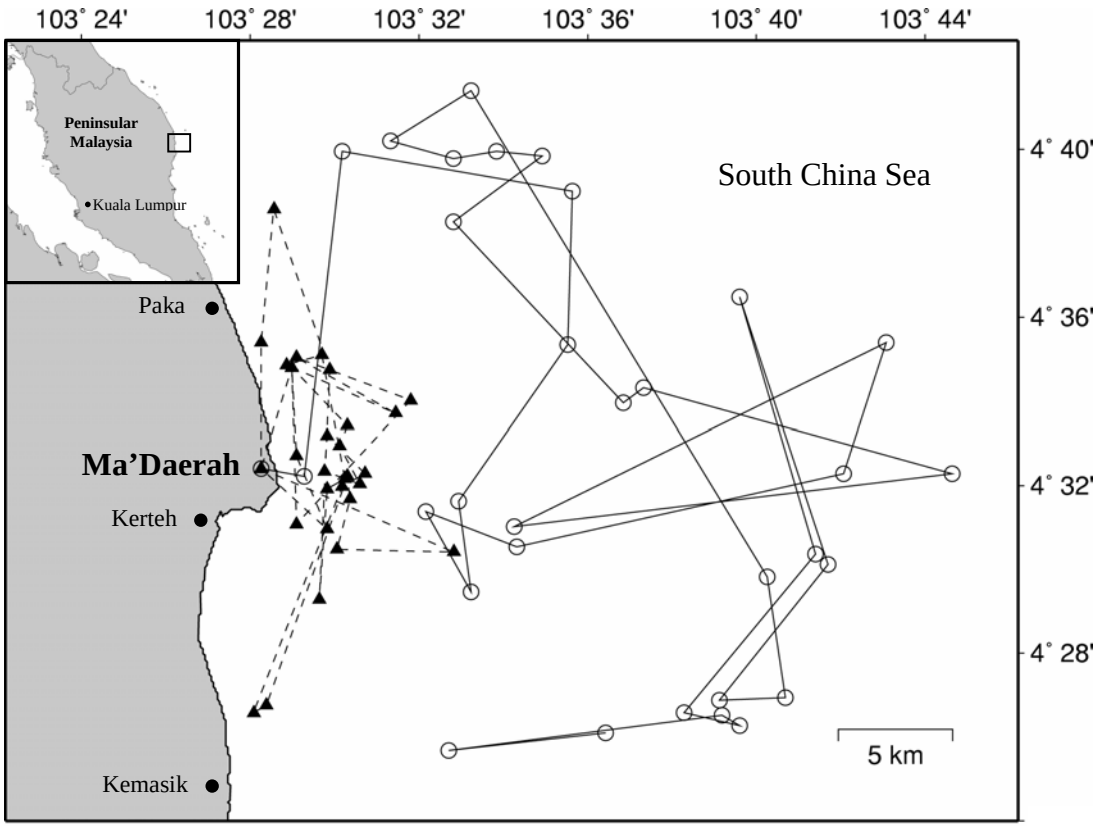

Figure 5.2. Breeding habitat used by an adult male (○) and a nesting female (▲) *Chelonia mydas* near the Ma'Daerah Sea Turtle Sanctuary, Terengganu, Malaysia. Inset map: Peninsular Malaysia. Map created using Maptool (SEATURTLE.ORG 2002).

Table 5.3. Inter-nesting, post-nesting and foraging ground movement details of three female (A, B and C) and 1 male (D) *Chelonia mydas* released from the Ma'Daerah Sea Turtle Sanctuary, Terengganu, Malaysia.

	Turtle A	Turtle B	Turtle C	Turtle D
Breeding/Nesting:				
Nesting date (# eggs)	Nil	4/9/05 (118) 18/9/05 (93) 30/9/05 (96)	Nil	N/A
Days in breeding area	N/A	41	N/A	11
Area occupied[a] (km^2)	N/A	78.5	N/A	398
Mean SST[b] (SD)	N/A	29.2 (0.8)	N/A	28.5 (0.4)
Locations/day[d]	N/A	0.8	N/A	2.9
Post-breeding Migration:				
Distance travelled (km)	1955	1330	1590	310
Days travelled	45	31	28	15
Average speed[c] ($km\ hr^{-1}$)	1.81	1.79	2.37	0.86
Final destination	Sabah, Borneo Malaysia	Thousand Islands, Indonesia	Ly Son, Vietnam	Pemanggil Island, Peninsular Malaysia
Mean SST[b] (SD)	29.8 (0.8)	30.3 (0.5)	29.2 (0.8)	29.1 (0.4)
Locations/day[d]	1.4	1.4	4.6	1.9
Foraging:				
Days foraging	N/A	16	35	43
Area occupied[a] (km^2)	N/A	91	24	3575
Mean SST[b] (SD)	N/A	30 (-)	27.3 (0.6)	29.3 (0.4)
Locations/day[d]	N/A	0.6	1.6	1.8

a, minimum convex polygons, joining the outermost points of an individuals range
b, sea surface temperature ($^{\circ}C$)
c, calculated as the total distance divided by number of days travelled
d, mean number of Argos location hits (classes 3, 2, 1, A) received per day

Following inter-nesting/breeding activity turtles B and D began post-breeding migration, and the remaining two individuals (A and C) began migration immediately after satellite attachment. Each turtle migrated towards a different foraging ground within the Southeast Asian region (Figure 5.3) and the distance, duration and speed of each migration were calculated (Table 5.3). Turtle A began migration in a north-easterly direction before turning east, then southeast towards the southern coast of Sarawak in Borneo Malaysia. Turtle A then followed the coast in a north-easterly direction towards Sabah, until the PTT stopped transmitting, presumably before the foraging ground had been reached. Turtle B migrated south along the coast of Peninsular Malaysia and east Sumatra (Indonesia) to the Thousand Islands (Indonesia), a group of ~ 100 islands stretching 45 km north from Jakarta into the

Java Sea. Turtle C migrated in a north-easterly direction before making a sharp turn west towards the Vietnam coast. Turtle C then followed the Vietnam coastline north to the Quang Ngai province before moving ~ 25 km offshore to the Dao Ly Son Marine Protected Area. Turtle D migrated south-east to the Pemanggil Island Marine Park, just off the east coast of Peninsular Malaysia.

Movement of turtles B, C and D in their foraging grounds was collected for 16, 35 and 43 days respectively (Figures 5.4 to 5.6). Turtle B foraged within a 91 km^2 area in the Thousand Islands among Kolok, Congkat, Panjang and Pedamaran Islands (Figure 5.4); turtle C foraged within a 24 km^2 area in the waters of Ly Son Island (Figure 5.5); and turtle D (male) foraged within a 3575 km^2 area encompassing the marine parks of Pemanggil, Tioman, Aur, Tinggi and Sibu islands. Turtle D migrated at a considerably slower speed than the females (Table 5.3) and there was a distinct loop in the migration path about halfway along migration (Figure 5.3).

POP contamination

The number of POP compounds identified in clutches from *C. mydas* A, B and C were 29, 44 and 62, respectively (Table 5.4). The means of the different POP groups varied significantly between the three nesting females (Figure 5.7; ANOVA: $P < 0.05$; LSD: $P < 0.05$). Furthermore, there was a significant difference in the POP concentration profiles between clutches (ANOSIM: $R = 1$, $P = 0.001$). This is confirmed by the *n*MDS plot (stress = 0.01) that showed all clutches to be very well grouped and separated from each other (Figure 5.8).

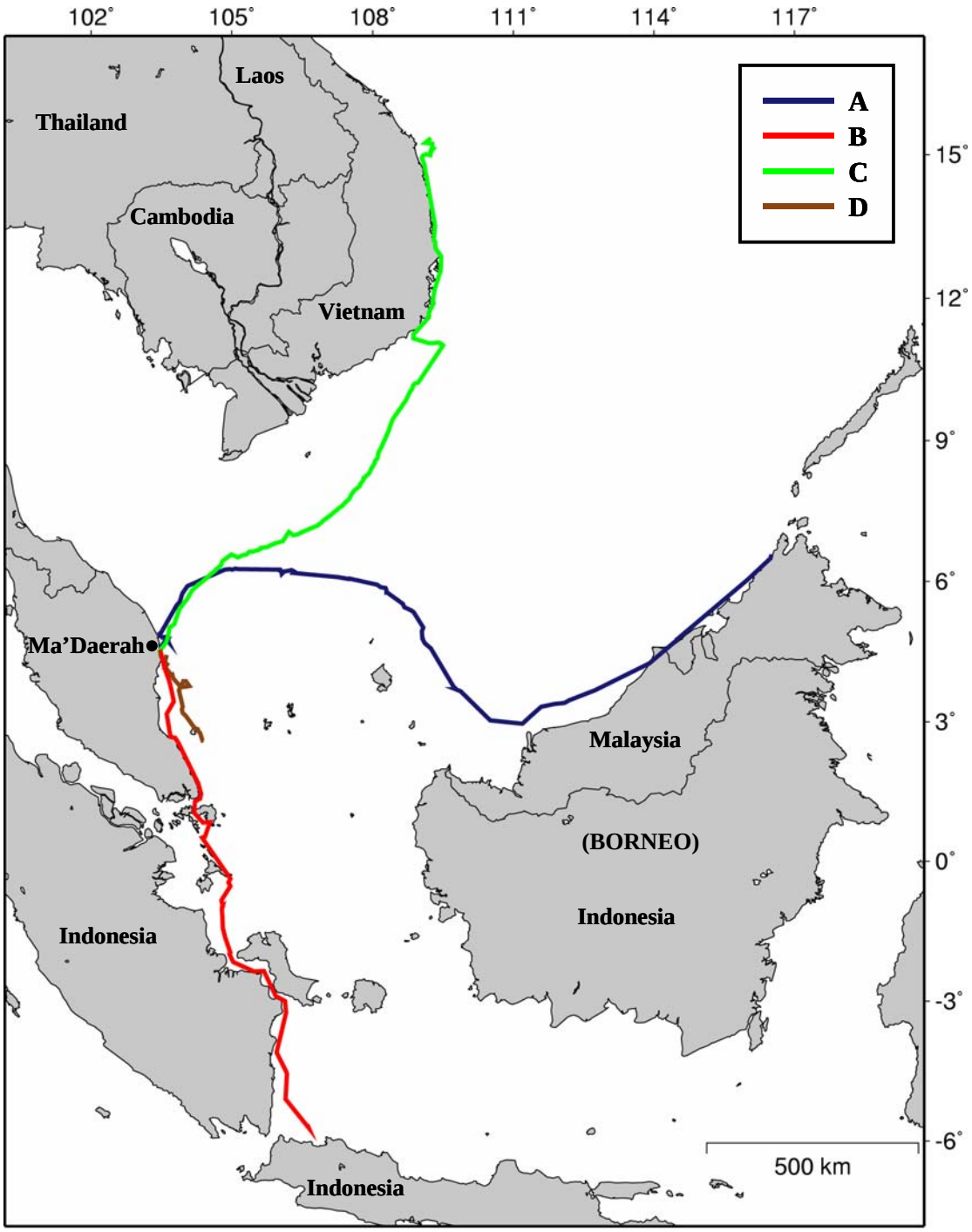

Figure 5.3. The post-breeding migration of three female (A, B and C) and one male (D) *Chelonia mydas* released from the Ma'Daerah Sea Turtle Sanctuary, Terengganu, Malaysia. Map created using Maptool (SEATURTLE.ORG 2002).

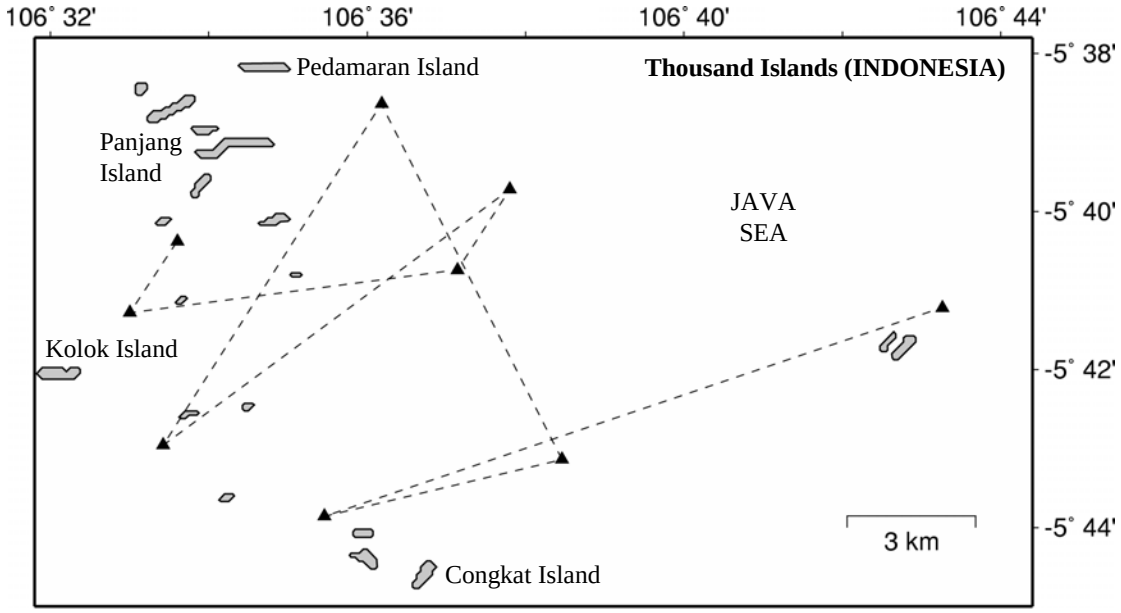

Figure 5.4. Satellite position data of an adult female *Chelonia mydas* (B) within a foraging ground in the Thousand Islands (Indonesia), following post-nesting migration from the Ma'Daerah Sea Turtle Sanctuary, Terengganu, Malaysia. General area corresponds to the end of the migration route of turtle B (see Figure 5.3). Map created using Maptool (SEATURTLE.ORG 2002).

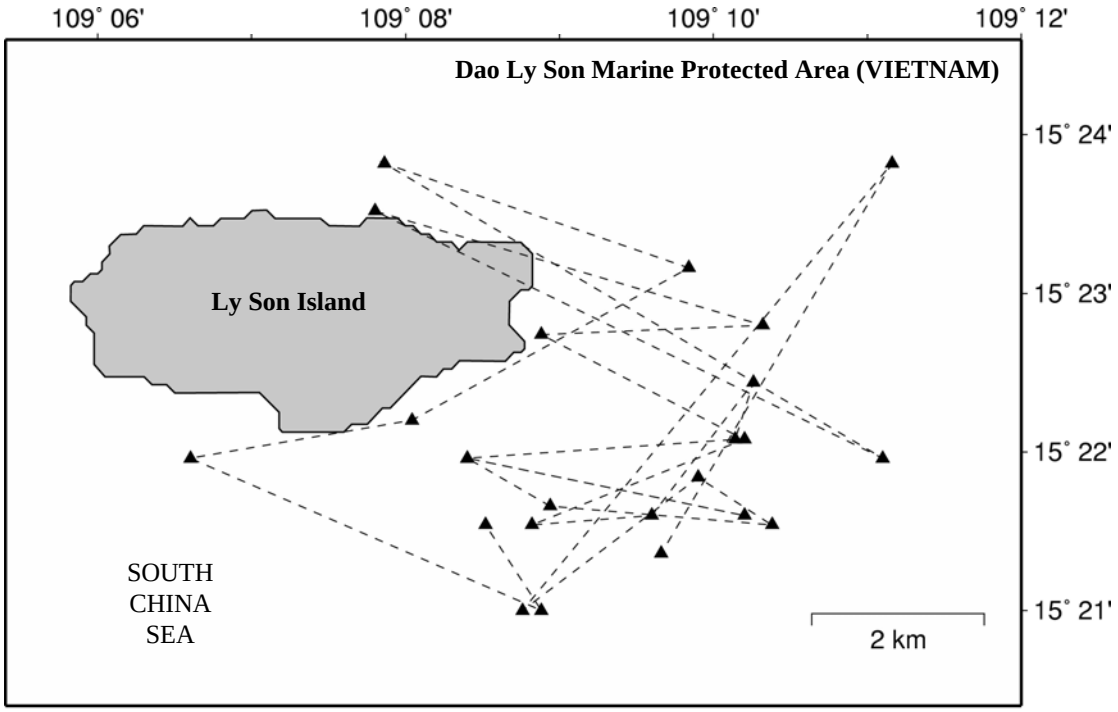

Figure 5.5. Satellite position data of an adult female *Chelonia mydas* (C) within a foraging ground near Ly Son Island (Vietnam), following post-nesting migration from the Ma'Daerah Sea Turtle Sanctuary, Terengganu, Malaysia. General area corresponds to the end of the migration route of turtle C (see Figure 5.3). Map created using Maptool (SEATURTLE.ORG 2002).

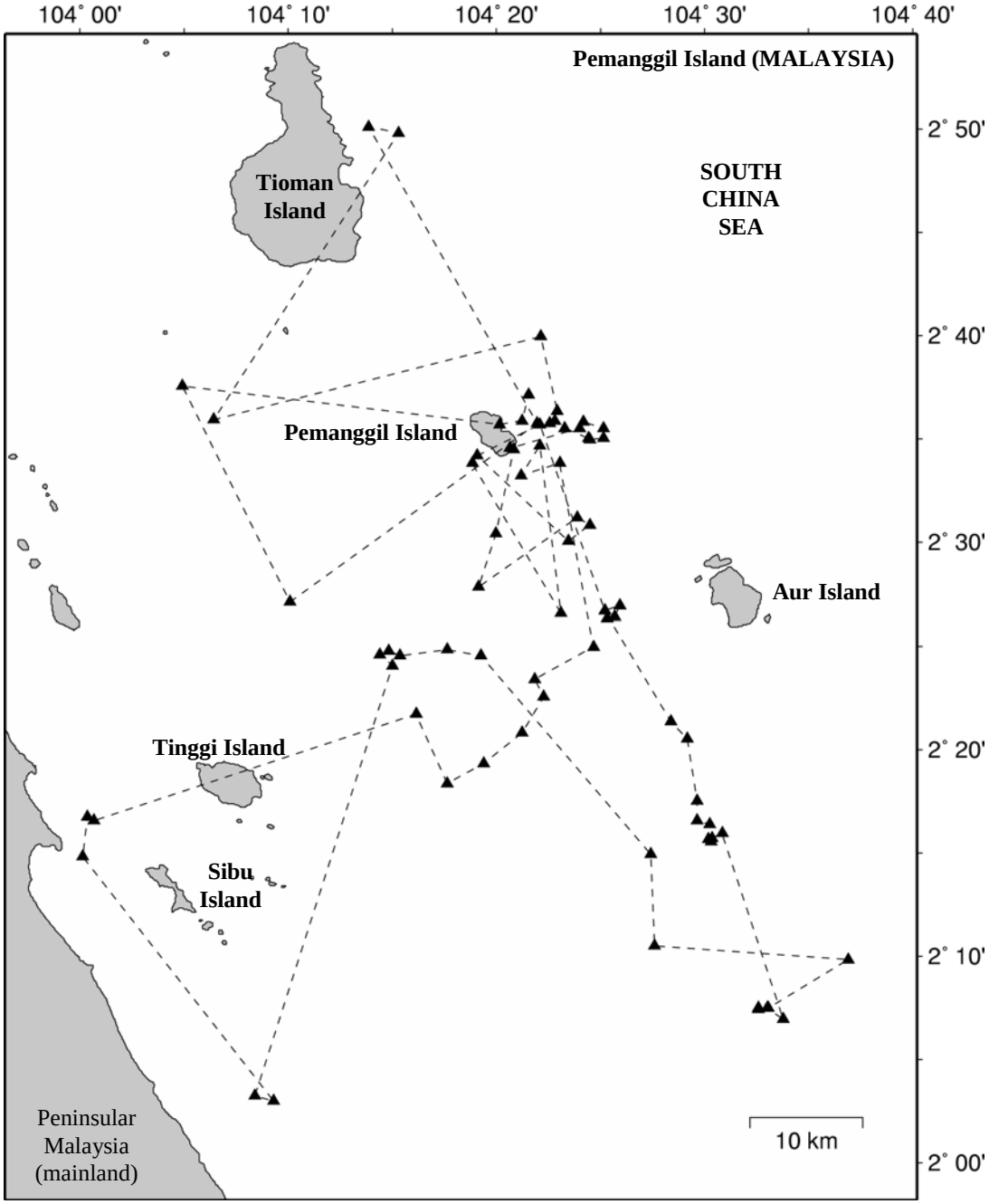

Figure 5.6. Satellite position data of an adult male *Chelonia mydas* (D) within a foraging ground near Pemanggil Island (Malaysia), following migration from the Ma'Daerah Sea Turtle Sanctuary, Terengganu, Malaysia. General area corresponds to the end of the migration route of turtle D (see Figure 5.3). Map created using Maptool (SEATURTLE.ORG 2002).

Table 5.4. Mean (± SE) concentrations of all POP compounds (pg g^{-1} wet mass) in the eggs of *C. mydas* nesting at the Ma'Daerah Turtle Sanctuary, Terengganu Malaysia, 2005.

Compound	Turtle A	Turtle B	Turtle C
PCB 1	<LOD	<LOD	<LOD
PCB 8	<LOD	<LOD	<LOD
PCB 18	12.5 ± 0.4	16.8 ± 1.2	13.8 ± 0.9
PCB 28+31	<LOD	13.9 ± 0.9	8.2 ± 0.5
PCB 29	<LOD	<LOD	<LOD
PCB 44	<LOD	<LOD	<LOD
PCB 45	<LOD	<LOD	<LOD
PCB 49	<LOD	<LOD	<LOD
PCB 50	<LOD	<LOD	<LOD
PCB 52	<LOD	11.0 ± 0.8	<LOD
PCB 56	10.9 ± 0.3	12.6 ± 0.3	15.0 ± 0.5
PCB 63	<LOD	<LOD	<LOD
PCB 66	<LOD	<LOD	20.9 ± 0.5
PCB 70	<LOD	<LOD	<LOD
PCB 74	<LOD	16.8 ± 0.5	21.9 ± 0.6
PCB 77	<LOD	<LOD	<LOD
PCB 79	<LOD	<LOD	<LOD
PCB 82	<LOD	<LOD	<LOD
PCB 87	<LOD	<LOD	<LOD
PCB 92	<LOD	<LOD	<LOD
PCB 95+121	<LOD	<LOD	<LOD
PCB 99	12.8 ± 0.3	16.4 ± 0.3	25.8 ± 0.3
PCB 101	<LOD	<LOD	<LOD
PCB 104	<LOD	<LOD	<LOD
PCB 105	<LOD	12.6 ± 0.3	18.8 ± 0.3
PCB 106	<LOD	<LOD	<LOD
PCB 107	9.4 ± 0.2	9.7 ± 0.2	10.7 ± 0.2
PCB 110	<LOD	<LOD	<LOD
PCB 112	<LOD	<LOD	<LOD
PCB 114	<LOD	<LOD	10.4 ± 0.2
PCB 118	11.3 ± 0.3	17.5 ± 0.3	24.5 ± 0.4
PCB 119	<LOD	<LOD	<LOD
PCB 126	<LOD	<LOD	<LOD
PCB 127	<LOD	<LOD	<LOD
PCB 128	<LOD	13.4 ± 0.3	20.7 ± 0.3
PCB 130	<LOD	<LOD	<LOD
PCB 132+153	21.1 ± 0.4	45.3 ± 0.7	113.1 ± 1.1
PCB 137	<LOD	<LOD	6.4 ± 0.1
PCB 138+158	<LOD	35.6 ± 0.5	99.2 ± 0.6
PCB 146	<LOD	<LOD	12.6 ± 0.2
PCB 149	<LOD	<LOD	<LOD
PCB 151	<LOD	<LOD	<LOD
PCB 154	<LOD	<LOD	<LOD
PCB 156	<LOD	10.2 ± 0.2	14.6 ± 0.3
PCB 157	<LOD	<LOD	9.7 ± 0.3

Table 5.4. (Contd.)

Compound	Turtle A	Turtle B	Turtle C
PCB 159	<LOD	<LOD	<LOD
PCB 163	<LOD	<LOD	13.8 ± 0.3
PCB 165	<LOD	<LOD	<LOD
PCB 166	<LOD	<LOD	<LOD
PCB 167	<LOD	<LOD	22.7 ± 0.5
PCB 169	<LOD	<LOD	<LOD
PCB 170	12.6 ± 0.3	17.5 ± 0.4	30.6 ± 0.3
PCB 172	<LOD	7.7 ± 0.2	9.1 ± 0.2
PCB 174	<LOD	<LOD	<LOD
PCB 175	<LOD	<LOD	<LOD
PCB 176	<LOD	<LOD	<LOD
PCB 177	9.8 ± 0.2	10.1 ± 0.3	11.6 ± 0.2
PCB 178	<LOD	<LOD	8.2 ± 0.2
PCB 180+193	<LOD	43.7 ± 1.0	85.6 ± 1.0
PCB 183	10.0 ± 0.2	12.4 ± 0.2	21.5 ± 0.3
PCB 185	<LOD	<LOD	<LOD
PCB 187	13.3 ± 0.3	14.2 ± 0.4	20.8 ± 0.3
PCB 188	<LOD	<LOD	<LOD
PCB 189	<LOD	<LOD	<LOD
PCB 191	<LOD	<LOD	<LOD
PCB 194	<LOD	<LOD	9.5 ± 0.1
PCB 195	<LOD	<LOD	12.7 ± 0.2
PCB 196+203	29.6 ± 0.5	29.9 ± 0.8	32.7 ± 0.6
PCB 197	<LOD	16.7 ± 0.4	17.1 ± 0.4
PCB 199	<LOD	13.0 ± 0.3	20.2 ± 0.3
PCB 200	<LOD	<LOD	<LOD
PCB 201	<LOD	<LOD	<LOD
PCB 202	<LOD	<LOD	14.4 ± 0.3
PCB 205	<LOD	<LOD	8.2 ± 0.1
PCB 206	<LOD	<LOD	<LOD
PCB 207	<LOD	<LOD	<LOD
PCB 208	<LOD	<LOD	<LOD
ΣPCBs	153.3 ± 2.5	397.1 ± 7.8	784.6 ± 9.1
4,4'-DDE	<LOD	<LOD	3263.1 ± 51.7
4,4'-DDT	<LOD	<LOD	334.1 ± 7.4
2,4-DDT + 4,4-DDD	<LOD	<LOD	27.2 ± 3.2
2,4'-DDD	<LOD	<LOD	<LOD
2,4'-DDE	<LOD	<LOD	<LOD
oxychlordane	<LOD	<LOD	15.5 ± 0.9
trans-chlordane	13.9 ± 0.6	16.0 ± 0.5	17.4 ± 0.6
cis-chlordane	<LOD	<LOD	<LOD
trans-nonachlor	<LOD	<LOD	25.3 ± 0.4
cis-nonachlor	16.9 ± 0.4	16.3 ± 0.5	16.9 ± 0.3

Table 5.4. (Contd.)

Compound	Turtle A	Turtle B	Turtle C
dieldrin	54.7 ± 3.3	49.4 ± 2.1	76.5 ± 1.9
endrin	<LOD	<LOD	<LOD
aldrin	<LOD	<LOD	<LOD
heptachlor	<LOD	<LOD	<LOD
heptachlor epoxide	23.9 ± 0.6	10.7 ± 0.8	24.2 ± 0.8
endosulfan I	198.2 ± 6.7	174.2 ± 5.6	198.0 ± 4.7
hexachlorobenzene	16.5 ± 1.2	15.5 ± 1.3	38.5 ± 2.8
mirex	<LOD	9.4 ± 0.2	13.5 ± 0.3
octachlorosytrene	<LOD	<LOD	<LOD
pentachlorobenzene	<LOD	<LOD	<LOD
alpha-HCH	<LOD	<LOD	<LOD
beta-HCH	64.8 ± 1.4	31.1 ± 0.8	80.1 ± 1.4
gamma-HCH	<LOD	128.9 ± 6.8	<LOD
PBDE 17+25	<LOD	<LOD	<LOD
PBDE 30	89.4 ± 6.4	71.0 ± 4.9	74.2 ± 4.8
PBDE 33+28	12.5 ± 0.2	12.4 ± 0.3	12.3 ± 0.2
PBDE 47	63.6 ± 3.3	12.2 ± 0.7	23.6 ± 1.0
PBDE 49	<LOD	<LOD	<LOD
PBDE 66	<LOD	<LOD	<LOD
PBDE 71	<LOD	<LOD	<LOD
PBDE 75	<LOD	<LOD	<LOD
PBDE 85	49.8 ± 1.8	<LOD	<LOD
PBDE 99	49.5 ± 2.7	20.3 ± 1.3	36.5 ± 1.0
PBDE 100	21.4 ± 0.9	18.2 ± 0.5	33.1 ± 0.9
PBDE 116	<LOD	<LOD	<LOD
PBDE 119	<LOD	<LOD	11.1 ± 1.3
PBDE 138	<LOD	<LOD	<LOD
PBDE 153	37.0 ± 2.1	29.8 ± 1.0	48.8 ± 2.9
PBDE 154	12.5 ± 0.7	11.0 ± 0.3	21.5 ± 0.7
PBDE 155	<LOD	<LOD	8.9 ± 0.4
ΣPBDEs	246.4 ± 9.1	104.2 ± 3.1	197.0 ± 6.2
ΣPOPs	772.0 ± 16.3	937.2 ± 20.0	5073.4 ± 57.5
Percent lipids (%)	8.4 ± 0.1	8.7 ± 0.1	9.1 ± 0.1

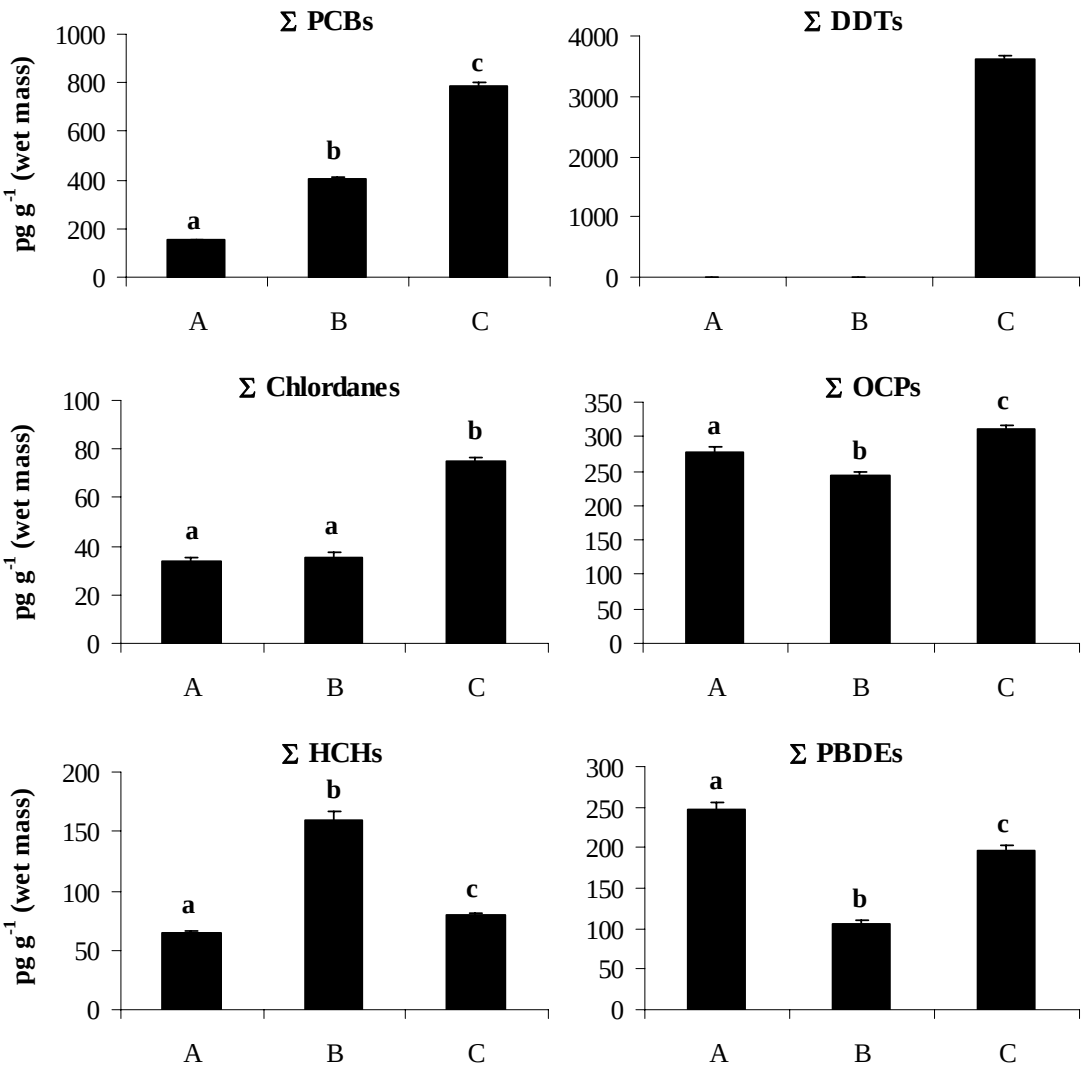

Figure 5.7. Mean (+SE) concentration of the six major POP compound groups in the clutches of nesting female *Chelonia mydas* foraging in 3 different areas: A, Borneo Malaysia; B, Indonesia; C, Vietnam. Values with different letters are significantly different ($P < 0.05$).

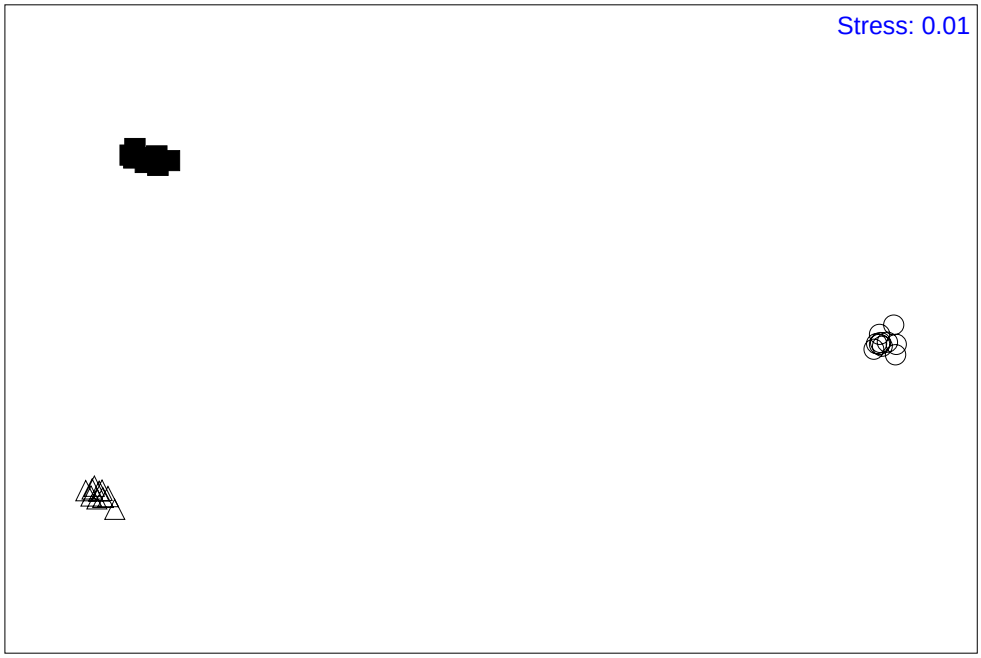

Figure 5.8. *n*MDS plot of egg POP concentrations for the three satellite tracked *Chelonia mydas* females foraging in different areas: A, Borneo Malaysia (+); B, Indonesia (!); C, Vietnam ()).

Metal contamination

The mean concentration of zinc, selenium and arsenic varied significantly (ANOVA: $P < 0.05$; LSD: $P < 0.05$) between clutches from the three nesting female *C. mydas* (Table 5.5, Figure 5.9). Furthermore, there was a significant difference in the heavy metal concentration profiles between clutches (ANOSIM: $R = 0.566$, $P = 0.001$). Clutches A and C (ANOSIM: $R = 0.908$, $P = 0.001$) and clutches C and B (ANOSIM: $R = 0.703$, $P = 0.001$) were well separated. However clutches A and B (ANOSIM: $R = 0.140$, $P = 0.049$) were less separated although still significantly different. This is depicted by the *n*MDS plot (stress = 0.01), which shows clutch C to be well separated from clutches A and B, while clutches A and B are closer although still relatively well separated (Figure 5.10).

Table 5.5. Mean (± SE) concentrations of heavy metals (μg g^{-1} wet mass) in the eggs of *C. mydas* nesting at the Ma'Daerah Turtle Sanctuary, Terengganu Malaysia, 2005.

Element	Turtle A	Turtle B	Turtle C
Copper	0.470 ± 0.012	0.461 ± 0.031	0.443 ± 0.018
Zinc	9.166 ± 0.248	9.461 ± 0.722	14.923 ± 0.838
Selenium	0.217 ± 0.006	0.353 ± 0.017	0.493 ± 0.014
Arsenic	< LOD	0.045 ± 0.005	0.200 ± 0.009
Cadmium	0.006 ± 0.001	< LOD	0.006 ± 0.001
Lead	0.076 ± 0.029	0.114 ± 0.031	0.123 ± 0.029

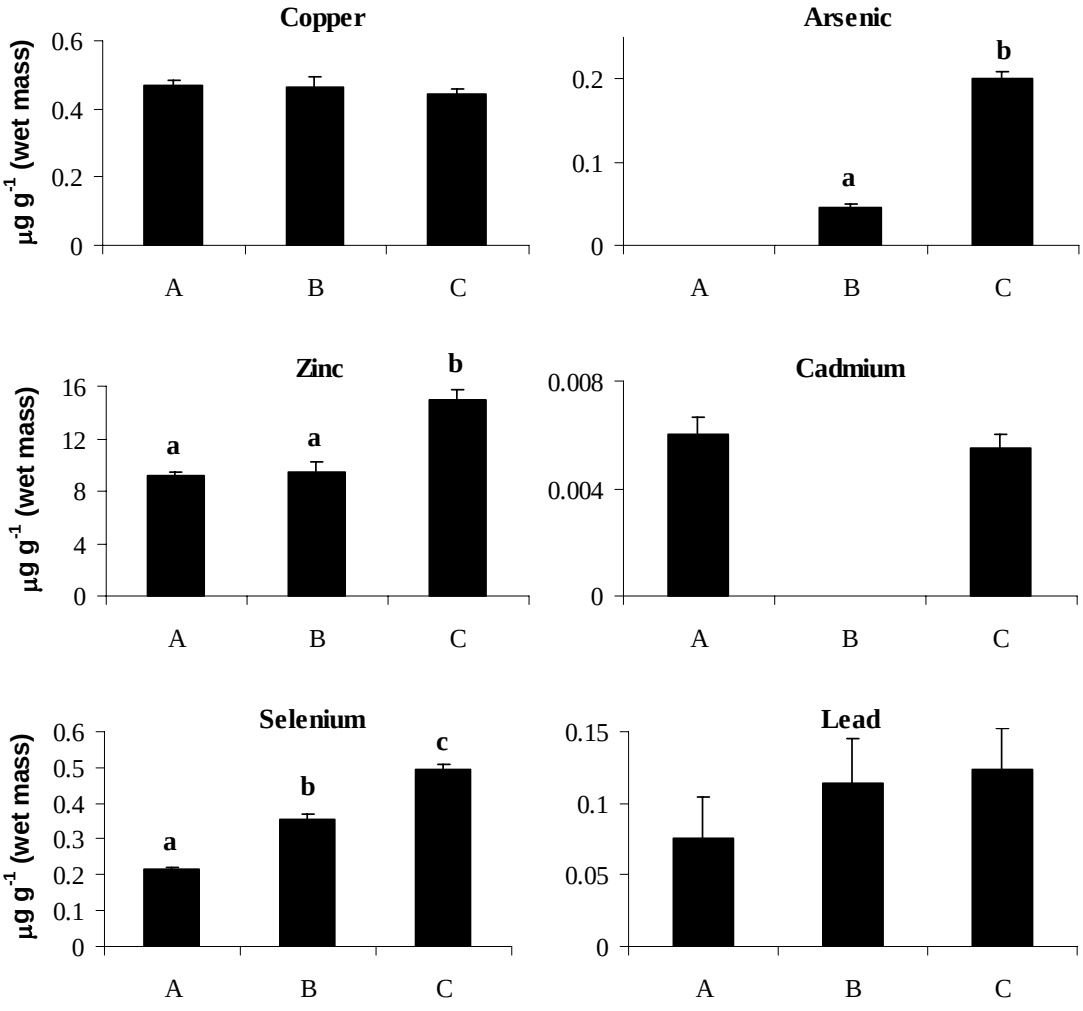

Figure 5.9. Mean (+SE) concentration of essential and toxic metals in the clutches of nesting female *Chelonia mydas* foraging in 3 different areas: A, Borneo Malaysia; B, Indonesia; C, Vietnam. Values with different letters are significantly different ($P < 0.05$).

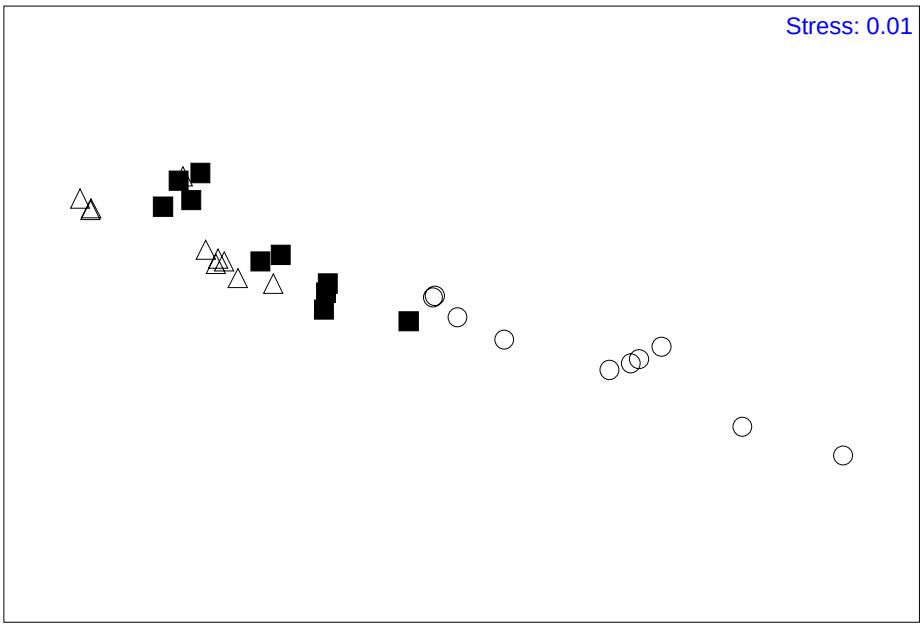

Figure 5.10. *n*MDS plot of egg metal concentrations for the three satellite tracked *Chelonia mydas* females foraging in different areas: A, Borneo Malaysia (+); B, Indonesia (!); C, Vietnam ( )).

5.4 Discussion

Range of breeding turtles around Ma'Daerah

Satellite telemetry of *Chelonia mydas* at the Ma'Daerah Sea Turtle Sanctuary has identified some important habitats used by this population in Peninsular Malaysia and the surrounding Southeast Asian region. The habitats used by the male (D) and one of the females (B) during the nesting season covered areas of 398 km^2 and 78 km^2 respectively, and extended 30 km offshore from the Ma'Daerah Turtle Sanctuary (see Figure 5.2). Much of this area lies outside the current "no trawl" zone, which extends 5.5 nautical miles (~ 10 km) offshore (*Fisheries Act*, 1985). Furthermore, with only two individuals tracked, these areas and ranges should be considered a conservative estimate of the habitat used by this *C. mydas* population during the breeding season. It is therefore likely that these *C. mydas* would be exposed to threats such as incidental capture in fishing gear and boat strikes during this period. These results can be used to recommend the extent of offshore protection required for adequate management of the Ma'Daerah *C. mydas* breeding population, which is currently being assessed by the Malaysian government as a gazetted sanctuary for sea turtles.

The movement of the male *C. mydas* in the area adjacent to the Ma'Daerah nesting beach could also indicate habitat used by this population for courtship and breeding. Although the male was not observed mating after release, its curved carapace length (90.5 cm) was within the range of adult males observed breeding in other *C. mydas* populations (Limpus 1993b; Balazs and Ellis 2000). However, male and female *C. mydas* generally aggregate for breeding prior to the beginning of nesting, with males returning to foraging grounds once nesting has commenced (Booth and Peters 1972; Limpus 1993b). It is rare for males to be still in the breeding area at this late stage of the nesting season, which generally begins in late April for this *C. mydas* population (Kamarruddin Ibrahim, pers. comm.). However, its size and subsequent 310 km migration to a known foraging area indicated that this male was most probably in the area for breeding, potentially with the females that had not yet begun nesting. If the area occupied by this adult male is a breeding area for *C. mydas*, it could also be important for other rookeries in the area, as a single breeding area can support a number of nearby nesting areas (Limpus 1993b).

The range of the adult male (D) and the nesting female (B) during the breeding/nesting period rarely overlapped and the female remained in a much smaller area, relatively close to the nesting beach. The difference in area covered could be indicative of breeding males actively searching for mates during this period and females remaining close to the nesting beach between clutches to conserve energy. Furthermore, the separation of breeding and nesting areas is consistent with Southern Great Barrier Reef *C. mydas* populations (Limpus 1993b). This would presumably reduce harassment of females in the inter-nesting area by adult males and would increase the likelihood of males encountering females receptive to mating in the breeding area.

Post-breeding migrations to foraging areas

Post-breeding movement of male and female *C. mydas* has identified important migration routes from the Ma'Daerah Turtle Sanctuary to foraging grounds in Southeast Asia. The four individuals tracked in this study went to four different foraging areas: Borneo Malaysia (A), Indonesia (B), Vietnam (C) and Peninsular Malaysia (D). The dispersal from breeding areas to foraging grounds differs between

C. mydas populations. Individuals of some breeding populations follow similar migration routes to adjacent foraging areas (Balazs et al. 1994; Luschi et al. 1998; Hatase et al. 2006), while in other populations, individuals migrate a wide range of distances and directions to different foraging areas (Balazs 1994; Liew et al. 1995; Cheng 2000; Godley et al. 2002; Seminoff et al. 2008).

Migration speed and distance travelled from the Ma'Daerah rookery to foraging grounds was also quite variable among individuals. The distance covered by the three females (A, B and C) ranged from 1330 to 1955 km over a period of 28 to 45 days, at speeds ranging from 1.81 to 2.37 km h^{-1}. The distance, duration and speed of these migrations were similar to those observed in nesting female *C. mydas* of other satellite tracking studies (Table 5.6). The male turtle (D) migrated a relatively shorter distance (310 km), which was within the range of male *C. mydas* post-breeding migration in other populations (Limpus 1993b; Hays et al. 2001b). However, in contrast to the faster, more direct routes taken by the nesting females, the male moved at a slower average speed of (0.86 km h^{-1}) and there was a distinct loop about halfway through migration (see Figure 5.3). This was unexpected behaviour as male *C. mydas* are known to migrate at comparable speeds to females following breeding (Balazs and Ellis 2000; Hays et al. 2001b).

Table 5.6. Summary of the distance (km), duration (days) and speed (km h^{-1}) of *Chelonia mydas* post-nesting migrations, determined by satellite tracking studies.

Nesting site	n	Distance	Days	Speed	Reference
Ascension Island	5	1792 - 2346	33 - 47	1.9 - 2.8	Luschi et al (1998); Hays et al. (2001c)
Ascension Island[c]	2	N/A	N/A	2.2 - 2.3	Hays et al. (2001b)
Japan	4	966 - 1962	28 - 42	1.4 - 2.1	Hatase et al. (2006)
Taiwan[a]	8	193 - 1909	7 - 57	1.2 - 2.8	Cheng (2000)
Hawaii/Samoa	5	1100 - 1750	26 - 45	1.6 - 1.9	Balazs et al. (1994)
Hawaii	3	830 - 1260	22 - 26	1.6 - 2.0	Balazs (1994)
Hawaii[d]	3	1050 - 1200	23 - 30	1.7 - 1.9	Balazs and Ellis (2000)
Costa Rica	10	410 - 1470	11 - 27	0.9 - 2.5	Troeng et al. (2005)
Cyprus[a,b]	6	322 - 2199	8 - 43	1.6 - 2.1	Godley et al. (2002)
Peninsular Malaysia[a]	5	669 - 1744	13 - 30	1.7 - 2.6	Liew et al. (1995)
Galapagos Islands[b]	12	1217 - 1912	26 – 52	1.1 - 2.2	Seminoff et al. (2008)
Peninsular Malaysia	4	310 - 1955	15 - 45	0.9 - 2.4	Present study

a, not all migrations completed to foraging areas
b, overall interesting, migration and foraging movement
c, male *C. mydas* only
d, includes male *C. mydas*
N/A, not available

The management and conservation of a breeding *C. mydas* population face considerable challenges when individuals migrate to a large number of different areas under the control of other countries. The migration routes and foraging grounds observed in the present study have highlighted areas of concern for the conservation of this Peninsular Malaysian *C. mydas* breeding population. The Thousand Islands (foraging ground for turtle B) lie directly to the north of the Indonesian capital city, Jakarta, and are under threat from sediment and land-based pollution generated by a human population of > 20 million (UNESCO 1997). Similarly, Ly Son Island (foraging ground of turtle C) is 25 km off the central Vietnamese coast, and although a Marine Protected Area, the marine environment is under threat from destructive fishing practices and agricultural activities on the island and adjacent mainland (ADB 1999). Furthermore, a similar study in 1993 and 1994 on *C. mydas* nesting at the nearby Redang Island rookery (~ 150 km away) indicated there are at least three other

foraging grounds that would be within the migration range of the Ma'Daerah breeding population and outside the direct control of Peninsular Malaysian authorities (Liew et al. 1995). A larger scale satellite telemetry study would therefore be required to identify further foraging sites and highlight the areas and countries in the region that Peninsular Malaysian authorities would need to collaborate with to more effectively manage this *C. mydas* population.

Range of turtles in foraging areas

Satellite telemetry has also identified the habitat utilisation of *C. mydas* individuals within their foraging grounds following migration. As with inter-nesting/breeding movement, the male (D) occupied a much larger area during foraging (3575 km^2) than the two females (24 km^2 and 91 km^2). Although the area covered by turtle C (24 km^2) was within the range of the largest *C. mydas* foraging ranges previously recorded (Seminoff et al. 2002), turtle B and the adult male (D) of this study had unusually large foraging ranges (Table 5.7). Previous studies found the home ranges of foraging *C. mydas* to be associated with small abundant patches of macroalgae and seagrass food resources. Furthermore, the small areas used during feeding have been attributed to maximising efficiency of energy expenditure and maintaining familiarity of surroundings to assist in avoidance of predators and environmental extremes (Makowski et al. 2006). However, comparison to these previous studies should be made with caution due to the considerable differences in turtle size, duration of tracking and physical characteristics of the foraging areas.

Table 5.7. Summary of foraging home ranges of *Chelonia mydas* in previous studies.

Site	Turtles	Mean SCL (cm)	Foraging Area Range (km^2)	Reference
Mosquito Lagoon, FL, USA	9	< 65	0.48 - 5.06	Mendoca (1983)
Kaneohe Bay, Hawaii	12	51.3	2.62 ± 0.96[a]	Brill et al. (1995)
South Padre Island, TX, USA	9	34.5	0.22 - 3.11	Renaud et al. (1995)
Repulse Bay, Australia	10	105.4	0.84 - 8.50	Whiting and Miller (1998)
Gulf of California, Mexico	12	66.7	5.84 - 39.08	Seminoff et al. (2002)
Palm Beach, FL, USA	6	36.7	0.69 - 5.05	Makowski et al. (2006)

a, mean (± SD) movement of individuals from the point of release (km).
SCL, straight carapace length

A study on the *C. mydas* foraging population in the Gulf of Mexico suggested that the larger home ranges observed were due to the large area of this site and scattered distribution of food resources (Seminoff et al. 2002). These factors could account for the large foraging areas observed in the present study. Furthermore, foraging areas could be influenced by the quality of seagrass habitats in these areas. Lower density seagrass meadows and high degree of patchiness would require more extensive movement within a foraging area. Turtle B was found to forage in the waters off the Thousand Islands in Indonesia that cover an area of ~ 750 km^2 and the adult male (D) foraged around a large group of islands just off the east coast of Peninsular Malaysia covering an area of 4800 km^2. Although the specific location and quality of the macroalgae and seagrass meadows are not known in these locations, it is likely that these island congregations would provide shallow coastal areas with suitable foraging habitat, separated by deeper, less productive waters. These extensive ranges of well-separated food resources could account for the relatively large areas covered during foraging by the *C. mydas* in this study. Furthermore, none of the literature to date has tracked the movement of adult male *C. mydas* in a foraging area. With reduced threat from predation due to their size and without the energetic demands of preparing for

and performing nesting (Kwan 1994; Hamann et al. 2002), adult males may be able to travel further in search of higher quality food sources.

Chemical contamination of the eggs

The concentrations of the major POP and toxic metal compounds found in the *C. mydas* eggs of this study are comparable to previous research (Clark and Krynitsky 1980; Podreka et al. 1998; Godley et al. 1999; McKenzie et al. 1999; Lam et al. 2006). Furthermore, the concentrations observed in this study are similar to concentrations calculated to pose considerable risk to sea turtle embryonic development (see Chapter 7). There were also a large number of compounds reported in the *C. mydas* egg of the present study, with a total of 29, 44 and 62 individual POP compounds reported in clutches from turtles A, B and C, respectively. This further raises the question of the effects of such a large number of chemicals on the development of *C. mydas* eggs, despite each individual compound being present at relatively low concentrations. This is an important area for future research and will be investigated, in part, in Chapter 7 of this thesis.

The migration of the *C. mydas* to different foraging areas in the present study also has implications for the variation in chemical contamination of this nesting population. The nesting female that migrated to Vietnam (C) had the largest number of POP compounds present in its eggs. Furthermore, this turtle had significantly higher levels of arsenic, PCBs, chlordanes and OCPs, and was the only turtle to have DDT and its metabolites present in its eggs. The turtle that migrated toward Borneo Malaysia (A) had the lowest number of toxic compounds, although the highest concentrations of PBDEs. The turtle that migrated to Indonesia had the highest levels of HCHs present in its eggs. Investigation of the major chemical groups separately identifies areas of Southeast Asia where these different types of chemicals are accumulating. Furthermore, the variations observed in the chemical contamination of these *C. mydas* clutches indicate that the foraging areas are being subjected to different levels of contamination. The trends in these results were expected as the foraging grounds in Vietnam and Indonesia are in close proximity to large cities and agricultural areas, while Borneo Malaysia is more remote from human development and its impacts.

Analysis of similarity of the chemical contamination of these clutches revealed that each clutch had a distinct contamination profile, determined by both the presence and concentration of particular compounds. This was supported by the *n*MDS plots that illustrated clear separation of the clutches, with minimal within-clutch variation. This was particularly evident in the POP analysis, whereas the separation of metals between clutches was less distinct and the variation within each clutch was greater. These were expected results as eggs have previously been found to be poor predictors of toxic metals in sea turtles (Sakai et al. 1995) and there is evidence of maternal transfer of POPs to eggs of nesting female *C. mydas* (see Chapter 6). Furthermore, due to the fact that these chemicals are most likely accumulating in sea turtles through feeding, these results provide a basis for the use of POP contamination profiles in comparing the foraging locations of individuals within a nesting population.

The use of chemical profiles potentially presents a more cost effective alternative to satellite tracking for investigating the foraging ground location of sea turtles. Furthermore, chemical profiles may provide a more reliable alternative to carbon and nitrogen stable isotope signatures, which have been extensively used to investigate animal movement (Peterson and Fry 1987; Rubenstein and Hobson 2004). Stable isotope signatures are not very temporally stable due to the continual metabolism of carbon and nitrogen by the animals. However, they are more effective in investigating the trophic source and status of animals, including sea turtles (Godley et al. 1998; Wallace et al. 2006). Studies on *C. mydas* have identified strong stable isotope correlations between tissue and diet (Seminoff et al. 2006), and have distinguished both neritic herbivores and oceanic planktivores from a single nesting population (Hatase et al. 2006). However, minimal variation in $\delta^{13}C$ and $\delta^{15}N$ has been found between *C. mydas* from different neritic foraging grounds (Hatase et al. 2006). This is due to the small geographic variation in carbon and nitrogen signatures for the different producers. Stable isotope signatures therefore provide limited distinction between *C. mydas* feeding on the same food source in different areas. More conservative substances, such as POPs and metals, would be less influenced by metabolism and would have higher geographic variation. These chemicals could therefore be used to more effectively investigate the foraging locations of sea turtles.

The POP contamination profiles observed in this study could provide the basis for distinguishing the foraging grounds of individuals of a breeding population of *C. mydas.* Due to the small within-clutch variation and very good separation, it could be predicted with relative confidence that turtles with similar contamination profiles to those observed in this study would be from the same foraging areas. However, there is no replication of *C. mydas* from the same foraging area in the present study. There are a number of other factors that could influence the chemical profiles of individual *C. mydas* and it is unclear how much separation in these profiles would indicate a completely different foraging area. For example, individuals in the same foraging area could have different contamination profiles due to duration of exposure (ie. age). The health of individuals may also alter their contamination profiles. However, nesting female *C. mydas* could be considered to be relatively healthy. It is expected that they would not begin breeding migration until they had reached a level of physical condition that would enable successful completion of a nesting season.

There may also be variation in contamination profiles among clutches for an individual *C. mydas* over a nesting season. There is evidence that POPs are transferred from nesting females to eggs during vitellogenesis and oviposition (see Chapter 6). However, it is unclear whether individual *C. mydas* transfer equal amounts of these chemicals to all clutches over a nesting season. Further research into the variation in contamination among an individual's clutches over a nesting season is therefore warranted before the use of chemical profiles to predict foraging locations can be completely validated. Furthermore, the relatively small areas used by *C. mydas* during foraging would expose individuals to localised chemical contamination, which could vary within a single foraging area. However, due to the large number of chemicals analysed and the ANOSIM based on both the presence and concentration of POP compounds, many of these subtle differences are likely to only increase the between-clutch variation slightly. Contamination profiles similar to those observed in this study would therefore suggest foraging in these three identified foraging grounds, and individuals with significantly different contamination profiles are likely to be from foraging areas not identified in this study. This concept will be investigated further in Chapter 6 where the contamination profiles of a further 11 individuals from this Ma'Daerah *C. mydas* population will be analysed.

5.5 Conclusions

Through satellite telemetry, this study has identified several important inter-nesting, breeding, migration and foraging habitats used by the Ma'Daerah *C. mydas* breeding population. There is evidence that during the breeding season, mating and inter-nesting habitats do not overlap and after breeding and nesting, the four tracked turtles migrated to four different foraging areas. This has significant conservation implications for the Ma'Daerah *C. mydas* population and has identified the local and regional areas where Malaysian sea turtle managers must focus their efforts. Given the long distances between breeding and foraging grounds and the regional spread of foraging areas for this breeding population, a network of protected areas is required for effective management.

Analysis of egg samples collected from the satellite-tracked females has also provided unique data suggesting the possibility of distinguishing foraging areas by toxin profiles. The migration to different foraging grounds was related to a large degree of variation in chemical contamination in the eggs of these *C. mydas* individuals. At several locations, a complex mixture of a large number of chemical compounds at relatively low concentrations has been found in the eggs of this *C. mydas* population, with varying contributions from the different chemical groups. This highlights the potential variability in the chemical contamination of *C. mydas* breeding populations and reinforces the need to determine the location of foraging areas for effective management. Furthermore, there was a significant separation of the chemical profiles of these *C. mydas* clutches. Further research is required to understand whether chemical profiles could be used to determine the range of foraging locations in a *C. mydas* population.

Chapter 6 - Persistent organic pollutants in *Chelonia mydas* eggs: Investigation into maternal transfer, nesting population variation and effects on hatchling development

6.1 Introduction

Persistent organic pollutants (POPs) have been reported in sea turtle eggs from a limited number of studies (Thompson et al. 1974; Clark and Krynitsky 1980, 1985; Podreka et al. 1998; McKenzie et al. 1999; Alam and Brim 2000; Alava et al. 2006). Furthermore, these chemicals have been found to have a number of adverse effects on sea turtles and other oviparous reptiles, particularly on embryonic development (see Chapter 1). Contaminant analysis of sea turtle eggs is therefore an important area of conservation research. In freshwater turtles and other oviparous reptiles, egg contamination is commonly used to predict the chemical contamination of individuals and populations. However, in sea turtles, very little is known about the maternal transfer of POPs to eggs and hatchlings, or the relationship between egg and parent contamination levels. Investigation into the maternal transfer process is therefore required to validate the use of eggs to predict POP contamination in adult female sea turtles. Furthermore, it has recently been found that egg contamination may be significantly different in individuals from different foraging areas (Chapter 5). Egg contamination of a distinct nesting population could therefore provide insight into the variation in pollution level of foraging grounds used by a population. Finally, the contamination of sea turtle eggs can potentially disrupt embryonic development. However, relationships between egg contamination and hatchling developmental abnormalities have not previously been investigated in sea turtles.

Maternal transfer of POPs has been well documented in marine mammals. Due to the lipophilic nature of POPs, marine mammals transfer large amounts of their chemical burden to their offspring during gestation and lactation (Aguilar and Borrell 1994; Lee et al. 1996; Ylitalo et al. 2001). While transplacental transfer is significant in determining the initial contamination of the newborns, a large amount of maternal toxic burden is also accumulated in offspring in the first few months via feeding (Cockcroft et al. 1989; Addison and Stobo 1993). As a result of maternal offloading,

reproductively active female mammals have much lower POP burdens than males and immature females in the same populations (Ylitalo et al. 2001). Furthermore, female POP concentrations can increase again in female marine mammals after they stop producing offspring (Lee et al. 1996).

Evidence of maternal transfer of POPs has also been observed in a limited number of studies on oviparous turtles. Vitellogenic females can concentrate POPs that they acquire from contaminated food sources into the egg yolk during follicle development (Guillette and Crain 1996). In the freshwater snapping turtle (*Chelydra serpentina*), egg concentrations of PCBs, DDTs, mirex, octachlorostyrene and HCB were significantly correlated with concentrations in the liver, muscle and adipose tissue (Herbert et al. 1993; Pagano et al. 1999). In the leatherback sea turtle (*Dermochelys coriacea)* significant correlations were found between blood and egg samples for concentrations of DDTs, PCBs and PBDEs (Stewart et al. 2008). These results indicate that nesting females are incorporating POP contaminants into eggs during vitellogenesis and oviposition. Furthermore, due to the correlations between egg contamination and body burdens in oviparous reptiles, egg samples may be used as a biological screen for POP contaminants in these species.

The use of eggs to estimate POP contamination in oviparous reptile populations is an important area of toxicology research. Blood and carapace samples have previously been found to be good non-lethal predictors of chemical contamination in the internal tissues of *Chelonia mydas* (Chapter 4). Similarly, egg samples could be used as a less invasive method for predicting POP contamination in *C. mydas* populations. However, the extent of maternal transfer would need to be investigated to verify the use of eggs to predict internal chemical contamination in *C. mydas*. Furthermore, egg contamination could also be used to investigate the variability in foraging grounds used by individual *C. mydas* of a nesting population. The eggs of *C. mydas* from different foraging areas have previously been found to have significantly different chemical profiles (Chapter 5). It has therefore been proposed that variation in foraging grounds of a *C. mydas* population may be investigated through variation in the contamination of eggs of individuals.

The accumulation of POPs in sea turtle eggs may also have implications for hatchling development. However, the transfer of POPs from eggs to hatchlings during development has not previously been reported for sea turtles. During growth, sea turtle embryos mobilise lipid reserves from the egg yolk to meet developmental requirements (Miller 1985). It is therefore likely that the lipophilic POP contaminants in the yolk are transferred to the hatchlings during this stage. High incidence of abnormality has been observed in *C. serpentina* hatchlings with high levels of POPs (Bishop et al. 1991; Bishop et al. 1994; Bishop et al. 1998). The abnormalities in hatchlings with high POP concentrations may be due to the disruptive effects of these chemicals on the endocrine system during development (Miller 1985; Guillette and Crain 1996). A high occurrence of scute abnormalities has also been observed in *C. mydas* hatchlings of Peninsular Malaysia (van de Merwe 2002; Schauble et al. 2003; Ibrahim et al. 2004; Schauble et al. 2004), with up to 89% of *C. mydas* hatchlings in a single nest found to have abnormal scute counts (van de Merwe 2002). The transfer of POPs from eggs to embryos in sea turtles may be responsible for these observed developmental abnormalities. However, as aforementioned, a relationship between POP concentrations and hatchling abnormalities has not been previously investigated for *C. mydas* or any other sea turtle populations.

The primary aim of this study was to investigate potential maternal transfer of POPs to eggs and hatchlings in *C. mydas*. This is an important initial step in validating the use of eggs to monitor contamination of *C. mydas* populations. In addition to investigating maternal transfer of POPs, egg concentrations were used to investigate the variation in contamination between individuals of a *C. mydas* nesting population. Furthermore, egg contamination was analysed to estimate possible variation in foraging grounds used by a *C. mydas* nesting population. Finally, the relationships between egg POP concentrations and hatchling development parameters were investigated to provide information on the effects of POP contamination on hatchling development in *C. mydas*.

6.2 Methods

Egg and blood collection

In June and July of 2004, eggs and blood were collected from 11 adult female *C. mydas* nesting at the Ma'Daerah Turtle Sanctuary, Terengganu, Malaysia (Table 6.1).

Three eggs were randomly collected from each clutch at the time of oviposition. Care was taken to minimise egg contact with the sand to avoid external contamination. Each egg was wrapped in hexane rinsed aluminium foil and transferred to a plastic zip-lock bag. The eggs were kept on ice until they could be transferred to a freezer (-20 °C) at the Turtle and Marine Ecosystem Centre, usually within 24 hours of oviposition. Following oviposition, curved carapace length (CCL), curved carapace width (CCW) and the mass of each nesting female were measured (see Chapter 2). The carapace and head scutes were also counted and recorded (Table 6.1).

Table 6.1. Adult female and clutch information on the *Chelonia mydas* nesting at Ma'Daerah used in the investigation of maternal transfer of POPs.

Tag	Scales[a]	CCL (cm)	CCW (cm)	Mass (kg)	Date laid (# eggs)	Nest #[b]	Clutch Sequence[c]
MY4133	Normal	112.5	97.4	129	21/6/04 (132)	221	3
TF3315	Normal	111	96	127	22/6/04 (128)	228	3
No tags	Normal	99	85	101	22/6/04 (123)	229	1
IF2565	Normal	95	87.5	80	23/6/04 (39)	232	6
TF3321 / TF3319	Normal	112	108.8	140	23/6/04 (131)	233	3
TF3351 / MY1551	Normal	103	92.5	105	23/6/04 (103)	234	2
MY1552	Normal	93	86	72	30/6/04 (64)	259	1
MY1602	PP: 3A	99.5	83.5	94	30/6/04 (127)	260	2
MY0297 /MY1502	PO: 4L/5R	88.9	80.8	81	1/7/04 (98)	263	3
IF2720	Normal	113	98.5	135	1/07/04 (143)	264	4
MY1555	Normal	98.4	86.2	89	3/7/04 (77)	274	1

a**,** carapace and head scute counts. A nesting female with the following scute nomenclature was considered "normal": 1 nuchal, 5 vertebrals, 4L/4R costals, 11L/11R marginals, 1L/1R supracaudals, 4L/4R post-occulars (PO), 2 symmetrical post-parietals (PP). A, asymmetrical; L, left side; R, right side (see Chapter 2). Deviations from normal have been recorded in this table.
b**,** hatchery nest number for that clutch
c**,** the sequence number of the clutch used in this study in relation to other clutches laid in the same season by that nesting female.

At the time of oviposition, 2-5 mL of blood was collected from the dorsal cervical sinuses in the neck of the nesting female using a 10 mL syringe with a 21G x 1.25" needle (Owens and Ruiz 1980). The eggs not taken for chemical analysis were transferred to a shaded hatchery within two hours of oviposition and allowed to incubate at a depth of 60 cm. Once the hatchlings emerged, a sample of five individuals was randomly collected from each nest. Blood samples (300 - 500 μL) were taken from the dorsal cervical sinuses in the neck using an insulin syringe and

pooled for each nest. All blood samples were immediately transferred to glass lithium heparin vacutainer tubes and kept on ice until they could be transferred to a freezer (-20 °C) at the Turtle and Marine Ecosystem Centre, usually within 24 hours of collection.

The frozen eggs and blood samples were transported in an insulated box surrounded by ice packs as carry-on luggage on a commercial flight from Kuala Lumpur to Brisbane. Upon arrival in Australia, the samples were still frozen solid and were immediately taken to the Organics Section, Queensland Health Scientific Services, in Coopers Plains where they were kept frozen (-20 °C) until analysis.

Clutch incubation and hatchling morphometrics

The incubation of the clutches in the hatchery was monitored closely around the expected time of emergence. This was estimated from the incubation period of other nests in the hatchery that had emerged previously in the season. Immediately following emergence, hatchling mass, straight carapace length (SCL) and scute patterns were measured and recorded for a random sample of 10 hatchlings from each nest (see Chapter 2). For each hatchling, the mass:SCL ratio was calculated and the abnormality index was recorded based on deviations from a normal scute count (see Chapter 2). The percentage of hatchlings with abnormal scute counts and the mean (± SE) of each of hatchling morphometric parameters were calculated for each nest.

Seven days after emergence, nests were excavated to determine hatching and emergence success. Eggs were classified to have successfully hatched (H) if a complete but empty eggshell was identified. Each intact egg remaining in the nest was opened and classified as either unhatched (UH), if there was evidence of embryonic development, or undeveloped (UD) if there were no signs of development. The number of live and dead hatchlings still in the nest was also recorded. Hatching and emergence success were calculated for each nest (Equations 6.1 and 6.2).

$$\text{Hatching success (\%)} = \left(\frac{H}{H+UD+UH}\right)\times 100 \qquad \text{…… Equation 6.1}$$

$$\text{Emergence success (\%)} = \left[\frac{H-(L+D)}{H+UD+UH}\right]\times 100 \qquad \text{…… Equation 6.2}$$

H = hatched eggs (empty eggshells)
UD = undeveloped eggs
UH = unhatched eggs
L = live hatchlings
D = dead hatchlings

Chemical analysis

All egg samples were prepared and analysed for POPs using methods outlined in Chapter 3. Briefly, POPs were analysed using GC-MS/MS following accelerated solvent extraction in dichloromethane, gel permeation chromatography and Florisil™ column clean-up. Percent lipids were determined gravimetrically for each sample following extraction.

Statistical analysis

The mean (± SE) of each POP compound was calculated for maternal blood, eggs and hatchling blood. All concentrations below the limit of detection (LOD) were assigned a value of half the LOD. This produced the least amount of bias while not requiring the use of complex iteration software (Helsel 1990).

To investigate transfer of POPs from nesting females to eggs, linear regressions were performed between maternal blood and egg POP concentrations. To investigate the transfer of POPs from eggs to hatchlings, linear regressions were performed between egg and hatchling blood POP concentrations. Only the compounds detected in maternal blood, eggs and hatchling blood were used in these analyses. The sum of PCB (ΣPCB) and PBDE (ΣPBDE) concentrations were calculated by summing the individual PCB and PBDE congeners in each egg, respectively. The remaining organochlorine pesticides (OCPs) were investigated individually. The mean ΣPCB, ΣPBDE and OCP concentrations were calculated for each clutch and linear

regressions were performed between mean egg concentration and maternal or hatchling blood concentrations.

In cases where both maternal and hatchling blood were significantly correlated with egg concentrations, analysis of covariance (ANCOVA) was performed to investigate the similarities of the slopes and elevations of the regressions. A custom ANCOVA was initially created to test for homogeneity of slopes. This involved an interaction term in the ANCOVA model between the covariate (egg concentration) and the factor (blood source). If this interaction was not significant ($P > 0.05$), slopes were considered to be equal and a one-factorial ANCOVA was performed to determine if the elevations were statistically different ($P < 0.05$).

To investigate the influence of maternal age on egg contamination a linear regression was performed between the mean sum POP (ΣPOP) concentration for each clutch and the corresponding curved carapace length (CCL) of the nesting female. The mean ΣPOP concentration for each clutch was calculated by summing the concentrations of all POP compounds for each egg and calculating each clutch mean. Furthermore, analysis of variance (ANOVA) was used to investigate the differences in mean ΣPOP concentrations among different clutches. Least significant difference (LSD) post hoc tests were performed to investigate whether individual clutches had significantly different mean ΣPOP concentrations. The influence of the clutch sequence number (ie. clutch number in relation to other clutches from the same female in that nesting season) on egg POP concentrations was also investigated. A regression was performed between the mean ΣPOP concentration of each clutch and clutch sequence number.

To further investigate POP concentration profile differences among clutches a Bray-Curtis similarity matrix with no data transformation was constructed using Primer v5 (PRIMER-E, UK). Each POP compound was entered as a separate variable and each egg was analysed as an individual sample. To investigate similarity in the chemical composition of all 11 clutches, analysis of similarity (ANOSIM) was performed on the matrix and a non-metric multi-dimensional scaling (*n*MDS) plot was constructed (PRIMER-E, UK).

Analysis of similarity (ANOSIM) was also used to investigate whether the clutches of this study had similar POP profiles to clutches from known feeding grounds in Chapter 5. To create a single data set, the POP concentrations of the 30 eggs from three clutches (10 eggs per clutch) laid at Ma'Daerah in 2005 (see Chapter 5) were combined with the POP concentrations of the 32 eggs from 11 clutches laid at Ma'Daerah in 2004 (present study). Each POP compound was entered as a separate variable and each egg was analysed as an individual sample. A Bray-Curtis similarity matrix with no data transformation was constructed using these combined data (PRIMER-E, UK). To investigate similarity in the chemical composition of all 14 clutches, analysis of similarity (ANOSIM) was performed on the matrix and a *n*MDS plot was constructed.

To investigate the effect of POP contamination on hatchling development, regressions were performed between hatchling development parameters and the mean egg ΣPOP concentration for each clutch. Egg concentrations were used in preference to hatchling blood concentrations as more POP compounds were detected in eggs. These compounds could therefore be affecting development despite not being detected in the hatchling blood samples. Linear regressions were performed between mean egg ΣPOP concentration and hatching success, emergence success and the percentage of abnormal hatchlings, and means of hatchling mass, SCL, mass:SCL ratio or abnormality index.

The influence of egg mass on embryonic development was investigated by performing regressions between mean initial egg mass and any hatchling or nest variable that showed significant regression with mean egg ΣPOP concentration.

6.3 Results

The POP concentrations (pg g^{-1} wet mass) were generally highest in hatchling blood, followed by maternal blood and eggs (Table 6.2). However, the number of POP compounds identified in eggs (54) was higher than in the maternal blood (21) and hatchling blood (20). Percent lipid was highest in the eggs (8.9 ± 0.2%), while maternal and hatchling blood had similar percent lipid content (1.5 ± 0.1% and 1.6 ± 0.1%, respectively). To standardise for lipid content in the correlations between blood and egg concentrations, POP concentrations were presented in ng g^{-1} lipid.

Table 6.2. Mean (±SE) concentration and range of POPs (pg g^{-1} wet mass) in eggs and blood from nesting female and hatchling *Chelonia mydas* from the Ma'Daerah Sea Turtle Sanctuary, Peninsular Malaysia.

	Egg		Maternal Blood		Hatchling Blood	
Compound	**Mean (±SE)**	**Range[a]**	**Mean (±SE)**	**Range[a]**	**Mean (±SE)**	**Range[a]**
PCB 1	<LOD	-	<LOD	-	<LOD	-
PCB 8	<LOD	-	<LOD	-	<LOD	-
PCB 18	18.3 ± 1.1	10.8 - 25.1	36.4 ± 5.4	5.0 - 66.7	64.4 ± 9.9	7.5 - 134.9
PCB 28+31	11.9 ± 1.1	7.8 - 19.7	<LOD	-	<LOD	-
PCB 29	<LOD	-	<LOD	-	<LOD	-
PCB 44	14.3 ± 0.6	10.6 - 18.2	37.4 ± 5.0	11.8 - 65.8	60.4 ± 6.3	28.6 - 108.7
PCB 45	<LOD	-	<LOD	-	<LOD	-
PCB 49	<LOD	-	<LOD	-	<LOD	-
PCB 50	<LOD	-	<LOD	-	<LOD	-
PCB 52	11.1 ± 0.6	7.3 - 15.1	<LOD	-	<LOD	-
PCB 56	12.5 ± 0.5	9.1 - 16.3	<LOD	-	<LOD	-
PCB 63	<LOD	-	<LOD	-	<LOD	-
PCB 66	11.1 ± 0.6	7.4 - 15.7	<LOD	-	<LOD	-
PCB 70	17.9 ± 0.8	14.2 - 22.7	26.5 ± 12.3	<LOD - 88.0 (6)	52.4 ± 13.4	<LOD - 102.1 (8)
PCB 74	15.1 ± 1.7	<LOD - 19.3 (10)	<LOD	-	<LOD	-
PCB 77	<LOD	-	<LOD	-	<LOD	-
PCB 79	<LOD	-	<LOD	-	<LOD	-
PCB 82	<LOD	-	<LOD	-	<LOD	-
PCB 87	<LOD	-	<LOD	-	<LOD	-
PCB 92	<LOD	-	<LOD	-	<LOD	-
PCB 95+121	17.6 ± 0.6	15.2 - 21.9	54.0 ± 4.5	34.3 - 78.9	70.5 ± 6.0	35.5 - 109.6
PCB 99	17.4 ± 1.4	13.0 - 27.7	39.4 ± 4.8	22.0 - 64.3	81.4 ± 8.2	50.0 - 124.0
PCB 101	<LOD	-	<LOD	-	<LOD	-
PCB 104	<LOD	-	<LOD	-	<LOD	-
PCB 105	11.8 ± 0.9	9.2 - 19.0	<LOD	-	<LOD	-
PCB 106	<LOD	-	<LOD	-	<LOD	-

a, number of samples above LOD indicated in parentheses if < 11

Table 6.2. (Cont'd.)

	Egg		Maternal Blood		Hatchling Blood	
Compound	**Mean (±SE)**	**Range[a]**	**Mean (±SE)**	**Range[a]**	**Mean (±SE)**	**Range[a]**
PCB 107	9.5 ± 1.1	<LOD - 12.6 (10)	<LOD	-	<LOD	-
PCB 110	<LOD	-	<LOD	-	<LOD	-
PCB 112	<LOD	-	<LOD	-	<LOD	-
PCB 114	9.0 ± 1.0	<LOD - 13.0 (10)	<LOD	-	<LOD	-
PCB 118	16.4 ± 1.3	11.6 - 23.6	33.9 ± 5.8	18.4 - 83.6	58.0 ± 7.1	36.0 - 105.8
PCB 119	<LOD	-	<LOD	-	<LOD	-
PCB 126	<LOD	-	<LOD	-	<LOD	-
PCB 127	<LOD	-	<LOD	-	<LOD	-
PCB 128	14.1 ± 1.2	9.9 - 20.1	28.7 ± 3.8	16.3 - 54.4	35.4 ± 2.5	23.1 - 47.1
PCB 130	<LOD	-	<LOD	-	<LOD	-
PCB 132+153	65.8 ± 13.1	25.8 - 136.4	93.9 ± 24.4	30.6 - 288.6	171.9 ± 43.1	64.7 - 462.0
PCB 137	<LOD	-	<LOD	-	<LOD	-
PCB 138+158	45.6 ± 8.9	17.0 - 94.1	62.9 ± 13.9	25.8 - 174.6	101.3 ± 20.9	46.7 - 226.7
PCB 146	10.5 ± 0.6	8.0 - 13.4	<LOD	-	<LOD	-
PCB 149	<LOD	-	<LOD	-	<LOD	-
PCB 151	<LOD	-	<LOD	-	<LOD	-
PCB 154	<LOD	-	<LOD	-	<LOD	-
PCB 156	9.2 ± 1.2	<LOD - 13.1 (10)	<LOD	-	<LOD	-
PCB 157	<LOD	-	<LOD	-	<LOD	-
PCB 159	<LOD	-	<LOD	-	<LOD	-
PCB 163	12.7 ± 0.6	10.1 - 17.0	<LOD	-	<LOD	-
PCB 165	<LOD	-	<LOD	-	<LOD	-
PCB 166	<LOD	-	<LOD	-	<LOD	-
PCB 167	6.0 ± 6.0	<LOD - 22.5 (3)	<LOD	-	<LOD	-
PCB 169	<LOD	-	<LOD	-	<LOD	-
PCB 170	20.4 ± 2.4	13.3 - 35.6	<LOD	-	<LOD	-

a, number of samples above LOD indicated in parentheses if < 11

Table 6.2. (Cont'd.)

	Egg		Maternal Blood		Hatchling Blood	
Compound	**Mean (±SE)**	**Range[a]**	**Mean (±SE)**	**Range[a]**	**Mean (±SE)**	**Range[a]**
PCB 172	5.3 ± 1.6	<LOD - 9.6 (7)	<LOD	-	<LOD	-
PCB 174	<LOD	-	<LOD	-	<LOD	-
PCB 175	<LOD	-	<LOD	-	<LOD	-
PCB 176	<LOD	-	<LOD	-	<LOD	-
PCB 177	10.6 ± 0.4	8.8 - 13.8	<LOD	-	<LOD	-
PCB 178	<LOD	-	<LOD	-	<LOD	-
PCB 180+193	53.5 ± 7.5	33.9 - 100.4	114.0 ± 13.5	72.0 - 202.0	91.1 ± 11.9	55.0 - 165.2
PCB 183	14.8 ± 1.8	9.7 - 25.9	52.0 ± 12.1	19.8 - 134.2	63.8 ± 12.0	28.0 - 142.4
PCB 185	<LOD	-	<LOD	-	<LOD	-
PCB 187	16.1 ± 1.1	10.8 - 21.3	<LOD	-	<LOD	-
PCB 188	<LOD	-	<LOD	-	<LOD	-
PCB 189	<LOD	-	<LOD	-	<LOD	-
PCB 191	<LOD	-	<LOD	-	<LOD	-
PCB 194	<LOD	-	<LOD	-	<LOD	-
PCB 195	<LOD	-	<LOD	-	<LOD	-
PCB 196+203	30.2 ± 0.9	24.7 - 35.1	<LOD	-	<LOD	-
PCB 197	<LOD	-	<LOD	-	<LOD	-
PCB 199	11.4 ± 2.9	<LOD - 22.2 (8)	<LOD	-	<LOD	-
PCB 200	<LOD	-	<LOD	-	<LOD	-
PCB 201	<LOD	-	<LOD	-	<LOD	-
PCB 202	7.5 ± 2.9	<LOD - 15.3 (6)	<LOD	-	<LOD	-
PCB 205	<LOD	-	<LOD	-	<LOD	-
PCB 206	<LOD	-	<LOD	-	<LOD	-
PCB 207	<LOD	-	<LOD	-	<LOD	-
PCB 208	<LOD	-	<LOD	-	<LOD	-
ΣPCBs	553.6 ± 54.6	392.8 - 839.4	578.9 ± 85.6	316.4 - 1206.5	850.8 ± 105.2	559.4 - 1456.6

a, number of samples above LOD indicated in parentheses if < 11

Table 6.2. (Cont'd.)

	Egg		Maternal Blood		Hatchling Blood	
Compound	**Mean (±SE)**	**Range[a]**	**Mean (±SE)**	**Range[a]**	**Mean (±SE)**	**Range[a]**
4,4'-DDE	<LOD	-	<LOD	-	<LOD	-
4,4'-DDT	<LOD	-	<LOD	-	<LOD	-
2,4-DDT + 4,4-DDD	<LOD	-	<LOD	-	<LOD	-
2,4'-DDD	<LOD	-	<LOD	-	<LOD	-
2,4'-DDE	<LOD	-	<LOD	-	<LOD	-
oxychlordane	7.6 ± 2.0	<LOD - 21.0 (7)	<LOD	-	<LOD	-
trans-chlordane	18.3 ± 0.9	13.8 - 22.3	21.3 ± 2.7	11.5 - 33.4	42.5 ± 4.8	17.1 - 68.4
cis-chlordane	<LOD	-	<LOD	-	<LOD	-
trans-nonachlor	15.0 ± 6.1	<LOD - 71.3 (10)	<LOD	-	<LOD	-
cis-nonachlor	10.6 ± 3.3	<LOD - 21.7 (7)	<LOD	-	<LOD	-
dieldrin	63.1 ± 6.8	38.3 - 102.7	<LOD	-	<LOD	-
endrin	<LOD	-	<LOD	-	<LOD	-
aldrin	<LOD	-	<LOD	-	<LOD	-
heptachlor	<LOD	-	<LOD	-	<LOD	-
heptachlor epoxide	16.0 ± 3.4	5.9 - 40.8	<LOD	-	<LOD	-
endosulfan I	<LOD	-	<LOD	-	<LOD	-
HCB	22.6 ± 2.0	8.8 - 31.4	<LOD	-	<LOD	-
mirex	9.4 ± 1.1	<LOD - 12.8 (10)	161 ± 43.0	<LOD - 476.3 (10)	132.4 ± 34.0	<LOD - 340.6 (10)
octachlorosytrene	<LOD	-	<LOD	-	<LOD	-
pentachlorobenzene	<LOD	-	<LOD	-	<LOD	-
alpha-HCH	<LOD	-	<LOD	-	<LOD	-
beta-HCH	41.2 ± 5.8	20.8 - 78.9	<LOD	-	<LOD	-
gamma-HCH	172.3 ± 7.4	137.8 - 207.8	501.1 ± 59.7	231.3 - 899.8	939.0 ± 63.4	634.3 - 1256.2

a, number of samples above LOD indicated in parentheses if < 11

Table 6.2. (Cont'd.)

	Egg		Maternal Blood		Hatchling Blood	
Compound	**Mean (±SE)**	**Range[a]**	**Mean (±SE)**	**Range[a]**	**Mean (±SE)**	**Range[a]**
PBDE 17+25	<LOD	-	<LOD	-	<LOD	-
PBDE 30	<LOD	-	<LOD	-	<LOD	-
PBDE 33+28	12.9 ± 0.5	9.7 - 15.7	<LOD	-	<LOD	-
PBDE 47	21.5 ± 1.7	11.1 - 28.1	13.3 ± 1.2	7.2 - 20.1	27.7 ± 2.7	14.9 - 41.4
PBDE 49	<LOD	-	<LOD	-	<LOD	-
PBDE 66	<LOD	-	<LOD	-	<LOD	-
PBDE 71	<LOD	-	<LOD	-	<LOD	-
PBDE 75	<LOD	-	<LOD	-	<LOD	-
PBDE 85	<LOD	-	<LOD	-	<LOD	-
PBDE 99	32.0 ± 3.6	12.0 - 54.8	21.3 ± 3.7	5.0 - 52.8	55.3 ± 12.1	8.9 - 131.8
PBDE 100	19.3 ± 2.2	<LOD - 26.5 (10)	<LOD	-	<LOD	-
PBDE 116	<LOD	-	<LOD	-	<LOD	-
PBDE 119	<LOD	-	<LOD	-	<LOD	-
PBDE 138	<LOD	-	<LOD	-	<LOD	-
PBDE 153	27.4 ± 1.2	20.8 - 35.2	86.2 ± 10.3	37.5 - 151.4	<LOD	-
PBDE 154	11.3 ± .05	8.2 - 14.0	<LOD	-	<LOD	-
PBDE 155	4.9 ± 0.9	<LOD - 8.7 (9)	<LOD	-	<LOD	-
ΣPBDEs	129.3 ± 8.1	61.7 - 163.8	120.8 ± 14.1	57.5 - 224.3	83.0 ± 14.4	23.8 - 173.2
ΣPOPs	1286.8 ± 66.6	992.3 - 1568.7	1383.1 ± 190.0	727.1 - 2834.9	2047.6 ± 175.8	1428.9 - 3320.6
% lipids	8.9 ± 0.2	6.8 - 10.9	1.5 ± 0.1	1.1 - 2.1	1.6 ± 0.1	1.4 - 2.0

a, number of samples above LOD indicated in parentheses if < 11

Correlations between eggs, maternal blood and hatchling blood

Concentrations of POPs in *C. mydas* eggs were significantly correlated with both maternal and hatchling blood for the compounds detected in all three sample types. The egg concentrations of ΣPCBs were significantly correlated with ΣPCB concentrations in both the maternal and hatchling blood (Figure 6.1; $R^2 > 0.93$, $P < 0.005$). Similarly, the egg concentrations of ΣPBDEs were significantly correlated with ΣPBDE concentrations in both the maternal and hatchling blood (Figure 6.2; $R^2 > 0.71$, $P < 0.005$). The egg concentrations of γ-HCH, trans-chlordane and mirex were significantly correlated to respective concentrations in both the maternal and hatchling blood (Figure 6.3; $R^2 > 0.83$, $P < 0.005$).

The custom ANCOVAs that tested for homogeneity of the egg-blood regression slopes produced variable results for the different POP compounds. For ΣPCBs and trans-chlordane, the slopes were found to be significantly different ($P < 0.05$). For ΣPBDEs, γ-HCH and mirex, the slopes of the maternal blood-egg and hatchling blood-egg regressions were parallel ($P > 0.08$). Furthermore, full-factorial ANCOVAs indicated that the elevation of the maternal blood-egg regression was higher than the hatchling blood-egg regression for ΣPBDEs and mirex ($P < 0.05$). However, elevation of the hatchling blood-egg regression was higher than the maternal blood-egg regression for γ-HCH ($P < 0.005$).

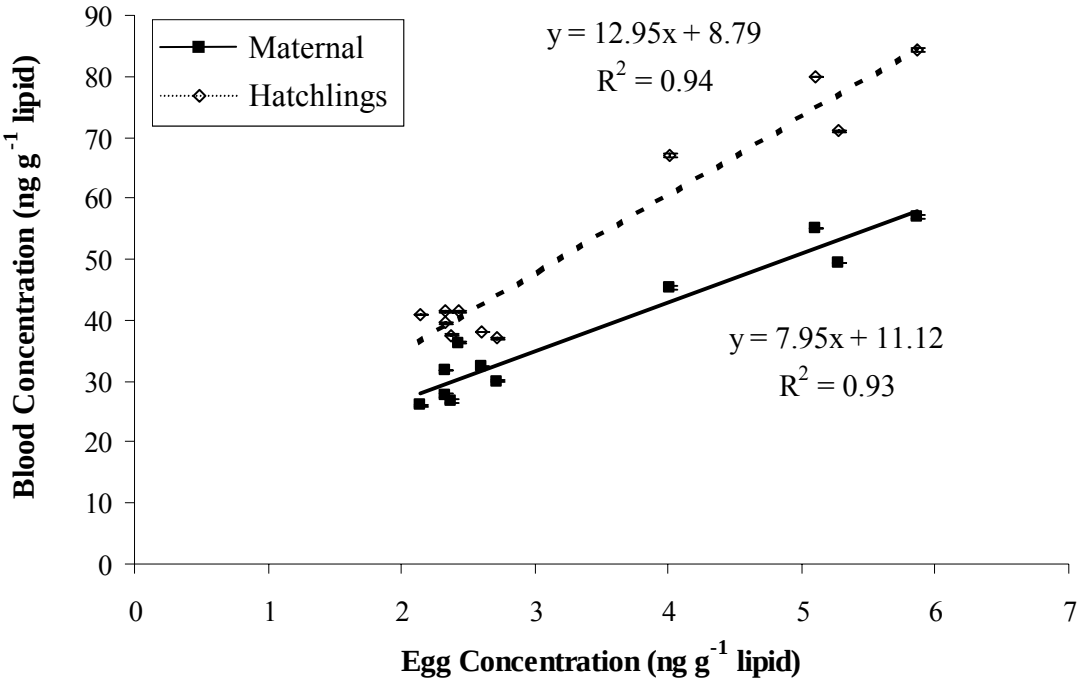

Figure 6.1. Relationship between mean egg concentration (± SE) and blood concentrations of maternal and hatchling blood for the ΣPCBs in *Chelonia mydas* nesting at Ma'Daerah, Terengganu, Malaysia.

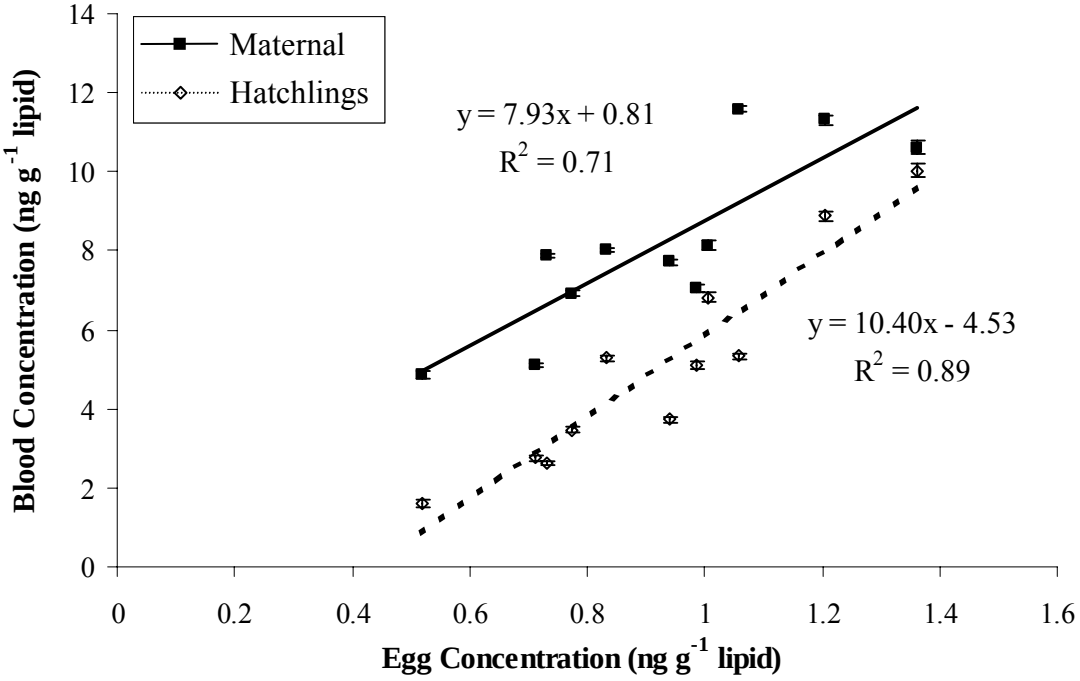

Figure 6.2. Relationship between mean egg concentration (± SE) and blood concentrations of maternal and hatchling blood for the ΣPBDEs in *Chelonia mydas* nesting at Ma'Daerah, Terengganu, Malaysia.

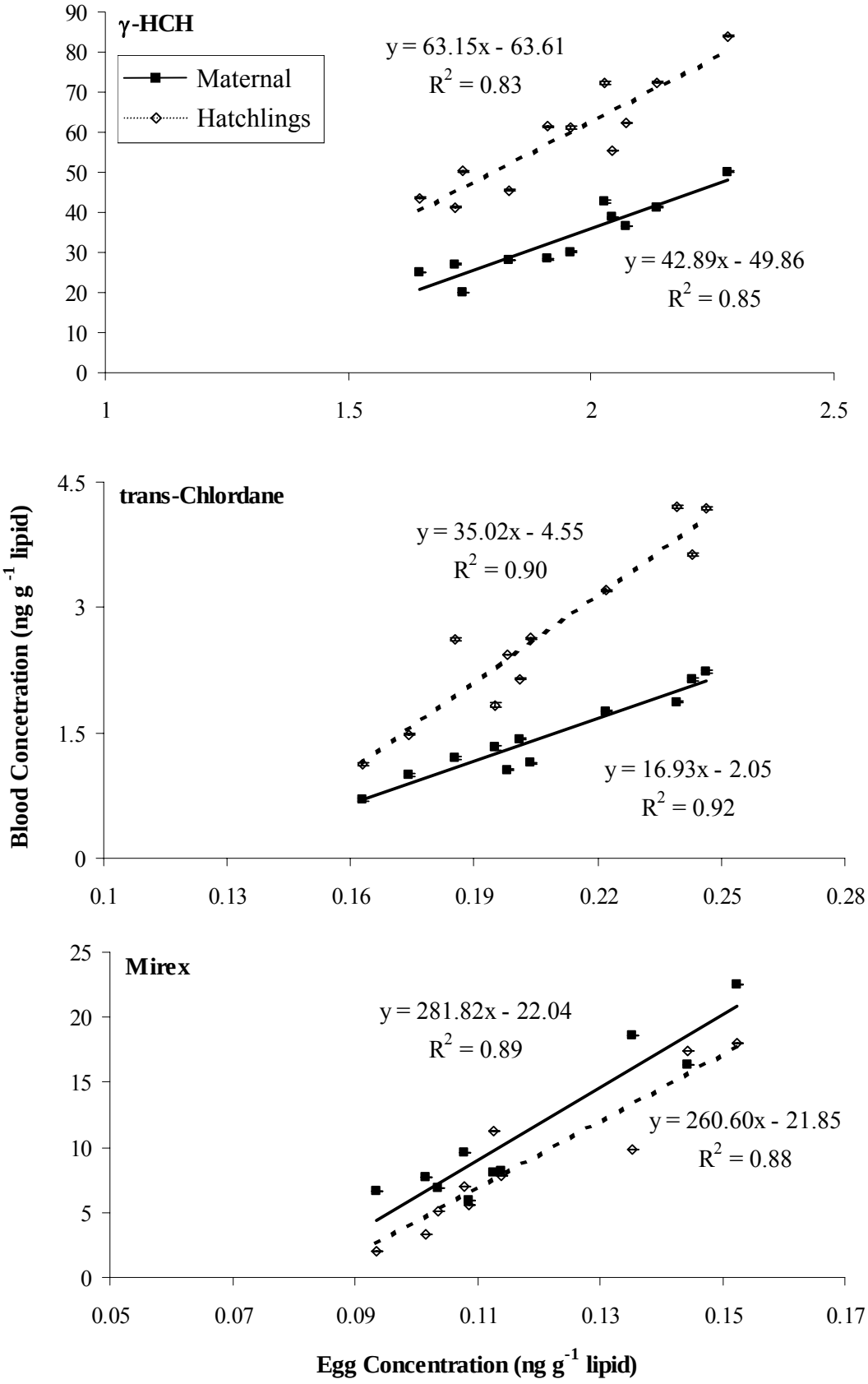

Figure 6.3. Relationship between mean egg concentration (± SE) and blood concentrations of maternal and hatchling blood for γ-HCH, trans-chlordane and mirex in *Chelonia mydas* nesting at Ma'Daerah, Terengganu, Malaysia.

Comparison of egg contamination among clutches

For the 11 *C. mydas* clutches examined from Ma'Daerah, the mean ΣPOP concentration in the eggs was < 2 ng g^{-1} (wet mass) and ranged between 0.99 ± 0.10 and 1.57 ± 0.06 ng g^{-1} (Figure 6.4). Furthermore, there were only two groups of clutches with different mean ΣPOP concentrations. Eggs from clutches 221, 232, 234, 260 and 264 had significantly higher mean ΣPOP concentrations than clutches 228, 229, 233, 259, 263 and 274 (Figure 6.4; LSD: $P < 0.05$). There was no correlation between maternal curved carapace length (CCL) and mean ΣPOP concentration of the eggs (linear regression: $R^2 = 0.26$, $P = 0.11$). Similarly, there was no significant correlation between mean ΣPOP concentration of each clutch and the clutch sequence number (linear regression: $R^2 = 0.34$, $P = 0.06$).

Analysis of similarity indicated that the presence and concentrations of POP compounds in eggs from different clutches were different but eggs within the same clutch were similar (ANOSIM: $R = 0.993$, $P = 0.001$). This was confirmed by the *n*MDS plot (stress = 0.09) that showed eggs in the same clutches to be well grouped and separated from other clutches (Figure 6.5). The *n*MDS plot further illustrated that eggs with the same symbol shape, whether filled or open, were well grouped and separated from other groups (see Figure 6.5).

When the POP concentration data of the clutches from known feeding grounds (see Chapter 5) were combined with data from the present study and ANOSIM was repeated, the presence and concentration of POPs of all 14 clutches remained well separated (Figure 6.6; ANOSIM: $R = 0.991$, $P = 0.001$).

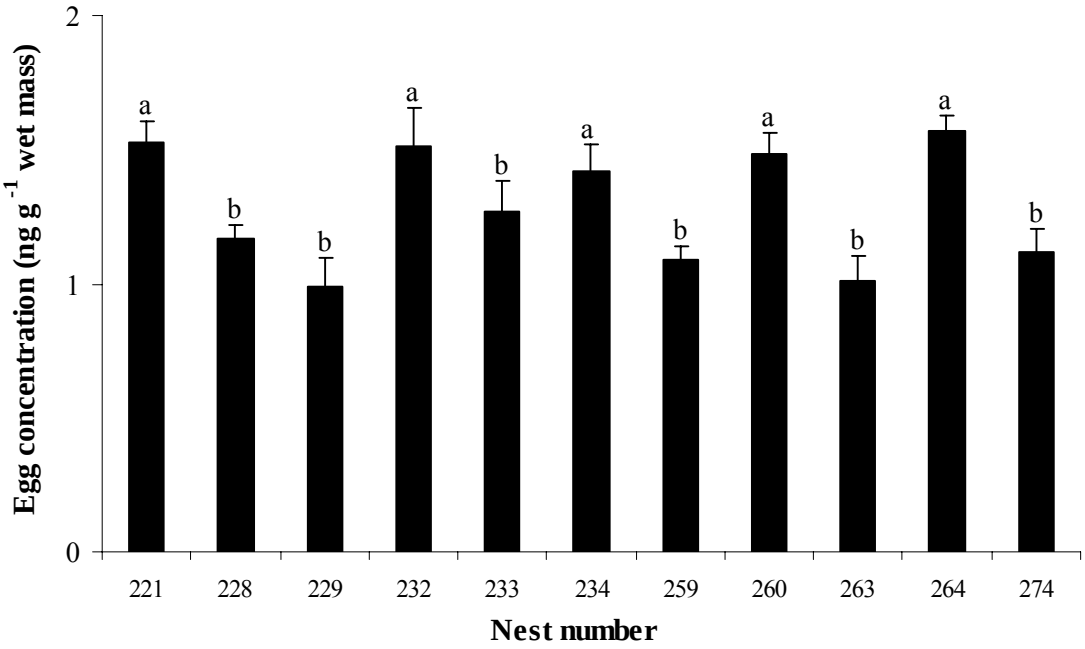

Figure 6.4. The mean ΣPOP concentration for *Chelonia mydas* eggs from 11 clutches incubated in the Ma'Daerah hatchery, Terengganu. Peninsular Malaysia. Letters refer to significantly different values ($P < 0.05$).

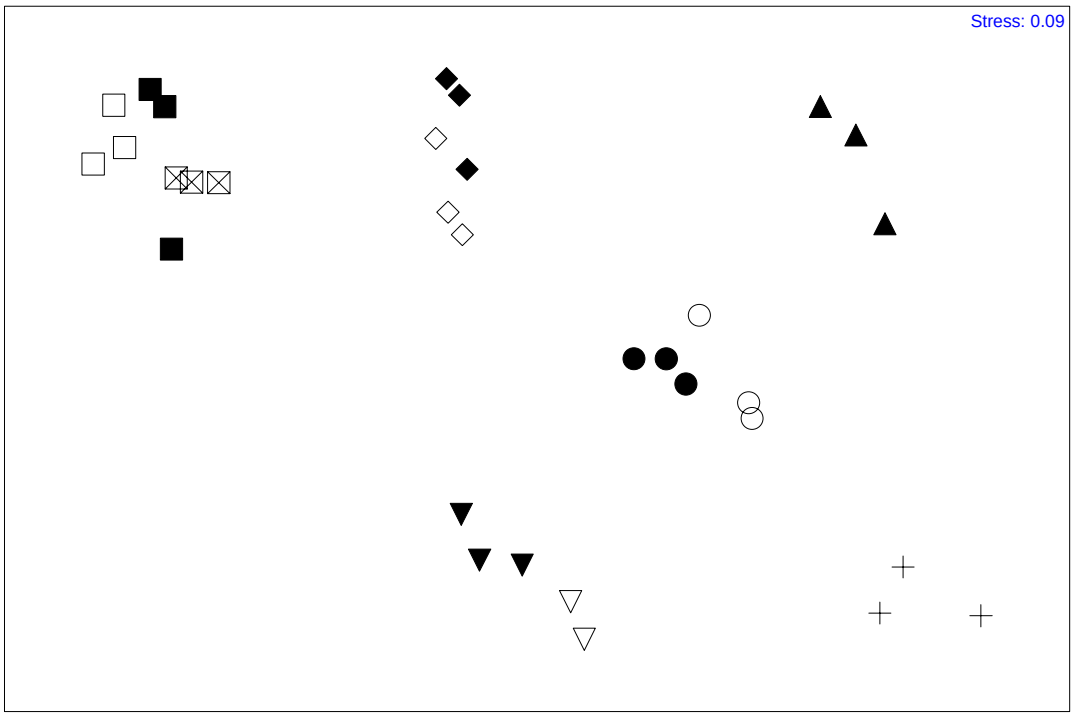

Figure 6.5. The *n*MDS plot of egg POP concentrations for 11 *Chelonia mydas* nesting at Ma'Daerah, Terengganu, Malaysia, 2004. Eggs from the same clutch are indicated by the same symbol. Six separate groups indicated by symbols with the same shape (open or filled).

Figure 6.6. The *n*MDS plot of egg POP concentrations of the 11 *Chelonia mydas* clutches in this study combined with the three clutches from Chapter 5. All clutches were from *C. mydas* nesting at Ma'Daerah, Terengganu, Malaysia. Eggs from the same clutch are indicated by the same symbol. Square symbols indicate the three clutches of *C. mydas* from known feeding grounds (see Chapter 5).

POP concentrations and hatchling development

Incubation of the 11 *C. mydas* clutches in the hatchery ranged from 52 to 57 days (Table 6.3). Only mass:straight carapace length (SCL) ratio of hatchlings was significantly correlated with the mean egg ΣPOP concentration (Figure 6.7; $R^2 = 0.65$, $P = 0.02$). There were no relationships between mean egg ΣPOP concentrations and hatching success, emergence success, the percentage of abnormal hatchlings, hatchling mass, hatchling SCL, or hatchling abnormality index. Furthermore, there was no significant correlation between hatchling mass:SCL and initial egg mass (linear regression: $R^2 = 0.22$, $P = 0.15$).

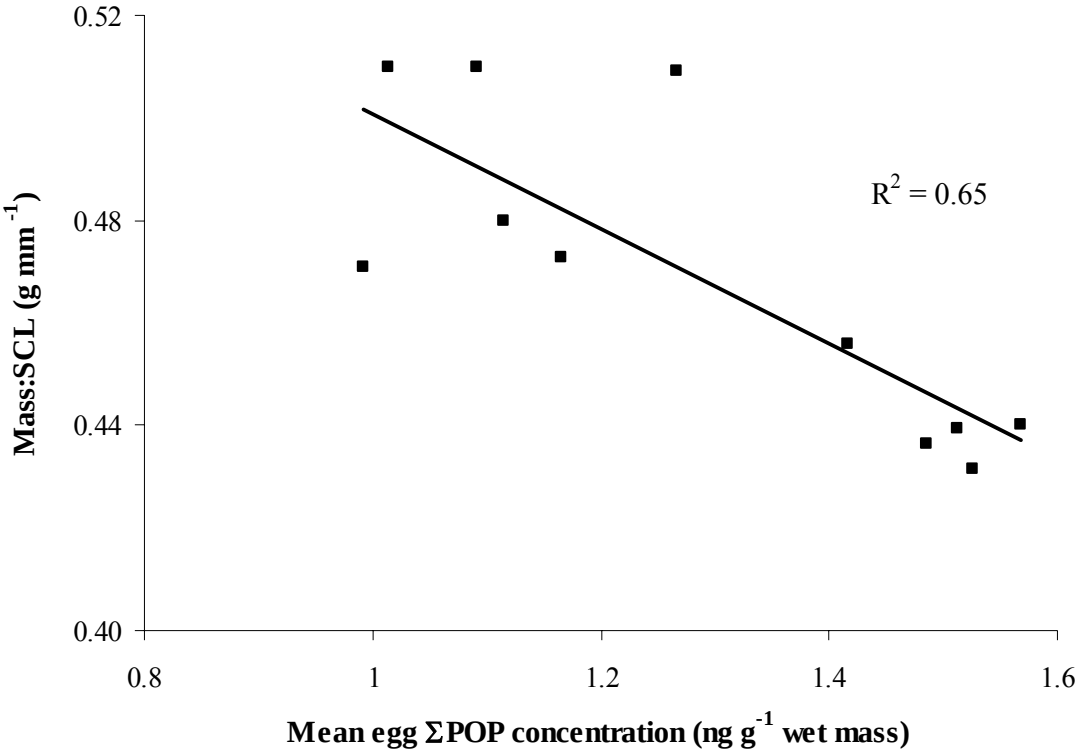

Figure 6.7. Relationship between the mean egg ΣPOP concentration (SE < 0.14) and the mean mass:straight carapace length (SCL) ratio (SE < 0.005) of *Chelonia mydas* hatchlings at Ma'Daerah, Terengganu, Peninsular Malaysia.

Table 6.3. Summary of incubation and hatchling morphometrics (mean ± SE, range) of clutches used in examination of maternal transfer of POPs in *Chelonia mydas* nesting at Ma'Daerah, Terengganu, Peninsular Malaysia.

	Incubation Details							Hatchling Details				
Nest	# Eggs	Initial egg mass (g)	Date Laid	Date Emerged	Incubation (days)	Hatched (%)	Emerged (%)	Mass (g)	SCL (mm)	Mass:SCL ($g\ mm^{-1}$)	Abnormal Index[a]	Nest Abnormality[b]
221	127	34.6 ± 0.8 (33.1 - 35.7)	21/6/04	16/8/04	56	89.8	77.2	19.5 ± 0.2 (18.0 - 21.0)	45.3 ± 0.2 (44.0 - 46.8)	0.43 ± 0.003 (0.41 - 0.46)	1.1 ± 0.3 (0 - 4)	60%
228	128	37.5 ± 0.8 (36.1 - 38.9)	22/6/04	17/8/04	54	82.0	71.1	22.1 ± 0.2 (20.7 - 23.7)	46.8 ± 0.2 (45.1 - 48.0)	0.47 ± 0.003 (0.45 - 0.49)	0.9 ± 0.2 (0 - 3)	60%
229	123	36.5 ± 0.8 (35.4 - 38.1)	22/6/04	15/8/04	54	96.7	94.3	21.6 ± 0.2 (19.2 - 22.9)	45.9 ± 0.2 (44.0 - 47.3)	0.47 ± 0.004 (0.42 - 0.50)	0.8 ± 0.2 (0 - 2)	55%
232	36	33.5 ± 0.3 (33.0 - 34.1)	23/6/04	19/8/04	57	91.7	91.7	19.8 ± 0.1 (17.8 - 20.7)	45.0 ± 0.2 (43.6 - 46.7)	0.44 ± 0.003 (0.40 - 0.47)	0.45 ± 0.1 (0 - 2)	40%
233	131	37.6 ± 0.3 (37.3 - 38.2)	23/6/04	14/8/04	52	93.9	89.3	23.8 ± 0.2 (21.3 - 25.2)	46.7 ± 0.2 (45.3 - 48.4)	0.51 ± 0.004 (0.47 - 0.53)	0.68 ± 0.2 (0 - 3)	45%
234	103	34.4 ± 0.8 (33.1 - 35.8)	23/6/04	16/8/04	54	94.2	91.3	21.7 ± 0.2 (20.1 - 23.4)	47.6 ± 0.2 (45.8 - 49.4)	0.46 ± 0.003 (0.43 - 0.49)	0.45 ± 0.2 (0 - 4)	25%
259	64	39.7 ± 0.4 (39.0 - 40.4)	30/6/04	22/8/04	53	95.3	87.5	23.9 ± 0.1 (22.9 - 24.9)	46.9 ± 0.2 (45.3 - 48.0)	0.51 ± 0.002 (0.50 - 0.54)	0.8 ± 0.2 (0 - 2)	55%
260	127	36.4 ± 0.9 (34.6 - 37.5)	30/6/04	21/8/04	52	96.1	93.7	20.1 ± 0.2 (18.5 - 21.4)	46.1 ± 0.2 (44.5 - 48.7)	0.44 ± 0.003 (0.40 - 0.46)	1.3 ± 0.2 (0 - 3)	75%
263	98	34.4 ± 1.3 (31.8 - 36.4)	1/7/04	23/8/04	53	98.0	92.9	23.1 ± 0.2 (18.0 - 21.1)	45.9 ± 0.2 (43.9 - 47.5)	0.51 ± 0.003 (0.46 - 0.52)	0.7 ± 0.4 (0 - 4)	40%
264	140	34.7 ± 0.8 (33.2 - 36.1)	1/7/04	27/8/04	57	55.7	52.9	20.4 ± 0.2 (18.9 - 21.8)	47.3 ± 0.2 (45.9 - 49.3)	0.44 ± 0.003 (0.41 - 0.47)	1.25 ± 0.4 (0 - 6)	45%
274	77	35.7 ± 0.6 (35.0 - 36.8)	3/7/04	27/8/04	55	98.7	96.1	21.8 ± 0.2 (18.6 - 22.9)	46.2 ± 0.2 (43.7 - 47.6)	0.48 ± 0.002 (0.41 - 0.51)	0.52 ± 0.2 (0 - 3)	50%

a, mean number of deviations from a normal scute count (see Chapter 2); b, percent of the hatchlings sampled from each nests that had at least one scute abnormality; SCL, straight carapace length

6.4 Discussion

POP concentrations in nesting females, eggs and hatchlings

Prior to the beginning of this thesis, blood concentrations of POPs had not previously been studied in *C. mydas*. However, blood concentrations of POPs in the nesting females and hatchlings of the present study were similar to the blood concentrations observed in the rehabilitating *C. mydas* sampled from Sea World, Australia (see Chapter 4). This is true for the overall concentrations of the major POP groups, although the specific compounds contributing to contamination varied considerably between these two studies. In relation to other sea turtle species, blood concentrations of POPs in the present study were > 1000 times lower than POP concentrations in the blood of juvenile *Caretta caretta* and *Lepidochelys kempi* (Keller et al. 2004a). This could be expected due to the higher trophic level occupied by *C. caretta* and *L. kempii* and the vast geographical separation of the populations sampled.

Egg concentrations of PCBs in the present study were similar to *C. mydas* eggs sampled from Peninsular Malaysia in other chapters of this thesis (Table 6.4). However, PCB concentrations were much lower than *C. mydas* and *C. caretta* eggs sampled in Florida (USA), Cyprus and Ascension Island (Thompson et al. 1974; McKenzie et al. 1999; Alam and Brim 2000; Alava et al. 2006). Interestingly, the range in the PCB concentrations observed in the present study was smaller than the Peninsular Malaysian market eggs (Chapter7). The narrower range in concentrations in the present study could be expected as the markets eggs were collected from a much larger geographical area and would most likely come from *C. mydas* foraging in a larger number of areas. The pesticide *p,p'*-DDE was not detected in the present study. This is consistent with all other *C. mydas* eggs analysed in this thesis with the exception of the eggs from one individual *C. mydas* nesting at Ma'Daerah and feeding off the coast of Vietnam (see Chapter 5). However, *p,p'*-DDE has previously been detected in *C. mydas* and *C. caretta* eggs from Heron Island, Florida (USA), Cyprus and Ascension Island (Thompson et al. 1974; Clark and Krynitsky 1980, 1985; Podreka et al. 1998; McKenzie et al. 1999; Alam and Brim 2000; Alava et al. 2006).

Table 6.4. Summary of the concentration (mean ± SE or range, in ng g^{-1} wet mass) of *p,p'*-DDE and ΣPCBs in sea turtle eggs from this thesis and previous studies.

Species	n	Location	*p,p'*-DDE	Σ PCBs	Reference
C. mydas	1	Cyprus	2.3	6.1	McKenzie et al. (1999)
	2	FL, USA	2.0 ± 2.0	-	Clark and Krynitsky (1980)
	10	Ascension Island	<LOD - 9	20 - 220	Thompson et al. (1974)
	15	Heron Island	1.7 ± 0.3	-	Podreka et al. (1998)
	55	Peninsular Malaysia	<LOD	0.15 - 3.69	Chapter 7
	3	Ma'Daerah	<LOD - 3.3	0.15 - 0.79	Chapter 5
	11	Ma'Daerah	<LOD	0.39 - 0.84	This study
	14	Peninsular Malaysia	<LOD	<LOD	Morrisey (2003)
	7	Peninsular Malaysia	<LOD	<LOD	Coufal (2002)
C. caretta	1	Cyprus	154	89	McKenzie et al. (1999)
	9	FL, USA	66 ± 63	-	Clark and Krynitsky (1980)
	55	FL, USA	56 - 150	-	Clark and Krynitsky (1985)
	22	FL, USA	0.5 - 1330	7 - 3930	Alava et al. (2006)
	20	FL, USA	-	1155 - 16730	Alam and Brim (2000)

Maternal transfer of POPs

This is the first study of its kind to investigate the transfer of POPs from nesting females to both eggs and hatchlings in sea turtles. Significant correlations between maternal blood and egg were observed for POP compounds that were detected in all sample types. This provides strong evidence that POP compounds accumulated in the nesting females during foraging are transferred to the eggs during vitellogenesis and oviposition. These eggs were collected directly from the cloaca during oviposition to avoid contamination from the surrounding environment. Any contamination observed in the eggs could therefore only have come from the nesting female.

The strong correlations between maternal blood and egg POP concentrations indicated that egg samples could be used to predict POP contamination of adult female *C. mydas*. Furthermore, due to the low variability in POP concentrations within clutches, a small sample (e.g. three eggs, as in this study) would be sufficient for this analysis. The collection of eggs for contamination screening is a much simpler sampling method than collecting blood from nesting females. Nesting under bushes and shrubs, as well as the short window of opportunity between oviposition and when the front flippers are engaged in nest filling, limits the access for blood sampling of nesting female sea turtles. Furthermore, eggs (30-50 g) represent a relatively higher sample mass compared to blood samples (10-15 g), and have significantly higher lipid content. This allows lower limits of detection to be reached under current methods and therefore increases the likelihood of identifying more compounds in each sample. Although egg sampling is destructive as it prevents the incubation of the eggs sampled, three eggs from a clutch represents < 0.5% of the reproductive output for each adult female *C. mydas* over a nesting season. Egg sampling could therefore be considered a useful and relatively non-disruptive method for determining POPs in *C. mydas.* Alternatively, the use of undeveloped eggs at the end of incubation could be explored to further reduce the destructive nature of egg sampling. However, further studies would need to be performed to determine if the POP concentrations in the eggs change over the duration of incubation and whether there were any external nest influences on egg POP concentrations.

There were also significant positive correlations in POP concentrations between eggs and hatchling blood. This indicated that the POPs in the eggs were being transferred to the hatchlings during embryonic development. Furthermore, the relationships between hatchling blood and egg concentrations were generally similar to the relationships between maternal blood and eggs, as reflected by the homogeneity in slopes for many compound groups. Many compound groups, however, demonstrated significant differences in elevation between the regression lines for maternal blood-egg and hatchling blood-egg relationships. This difference was expected, as POP concentrations are likely to be decreasing along the maternal blood – egg – hatching blood pathway.

POP concentrations in *C. mydas* were consistently higher in maternal and hatchling blood compared to eggs. Hatchling blood was expected to have higher concentrations of POPs than the eggs from which they developed. Firstly, the mass of a *C. mydas* hatchling is ~ 50-60% of the initial mass of the egg (Miller 1985). Therefore, if all compounds were transferred from the egg to the hatchling, POP concentration in the hatchling would be approximately double that of the egg. Furthermore, hatchlings do not feed in the first few days post-hatching and survive entirely on energy from the yolk (Dial 1987; Wyneken and Salmon 1992; Lohmann et al. 1997). Storage of lipids is therefore minimal in these first few days, resulting in high concentrations of lipids in the blood. Concentrations of the lipophilic POP compounds in the blood would therefore also be high during these early stages following hatchling emergence.

The higher concentrations of POPs observed in the maternal blood samples compared to eggs were consistent with the only previous study that analysed both maternal blood and eggs of sea turtles (Stewart et al. 2008). As previously mentioned, lipophilic POP compounds are mobilised with stored lipid during the breeding season. During vitellogenesis POP compounds associated with lipid molecules would be incorporated into to the egg yolks (Guillette and Crain 1996). However, in the interest of protecting offspring, there may be mechanisms that minimise the incorporation of toxic chemicals into the eggs during this process. This could in part explain why maternal blood POP concentrations were consistently higher than those in eggs. An alternative explanation could be related to the mobilisation of lipids to support egg production and provide energy to the nesting females during the breeding season (Kwan 1994; Hamann et al. 2002). The plasma triglycerides of *C. mydas* can increase from 2.92 ± 1.46 mmol L^{-1} in non-vitellogenic females to 13.11 ± 1.40 mmol L^{-1} during vitellogenesis (Hamann et al. 2002). During this process, lipids and POPs would enter the bloodstream and only the lipids would be consumed. The POPs may therefore remain in the bloodstream and concentrate above levels found in the eggs.

The transfer of POPs from nesting females to eggs and hatchlings would result in the reduction in chemical body burdens in adult females. However, the mobilisation of these chemicals during vitellogenesis may also have health consequences for nesting female sea turtles. While sequestered in stored lipids, the effects of POPs are likely to be minimal. However, when lipids are mobilised for vitellogenesis and migration, the

concurrent mobilisation of the lipophilic POP compounds may expose other parts of the body to the effects of these chemicals. Nesting female sea turtles may therefore be more susceptible to the effects of POPs during these stages of their lifecycle. This is also likely to periodically affect adult males as they also mobilise lipids to meet the energetic demands of breeding migrations. However, sub-adults and juveniles in foraging grounds may be able to sequester the majority of the POPs they accumulate during feeding and reduce the effects of these chemicals on their development and functioning.

Both of the models for higher maternal blood POP concentrations compared to eggs in *C. mydas* would be supported by increases in maternal blood POP concentrations over a nesting season. This could be tested by sampling individuals of a nesting population as they laid their first clutch of the season and re-sampling as they returned to lay subsequent clutches. Eggs could also be sampled on these occasions to investigate if there is regular transfer of POPs over different clutches. Marine mammals often offload the majority of toxins to their first offspring (Aguilar and Borrell 1994; Ylitalo et al. 2001). In sea turtles, it is possible that the POPs mobilised during vitellogenesis may concentrate more in the first clutch of each nesting season, as opposed to equal transfer to clutches over the entire season. In the present study, there was no correlation between the egg POP concentrations and the clutch sequence number. However, the relationship between clutch sequence and POP contamination would be better investigated in subsequent clutches of the same individuals.

Although POP concentrations were higher in maternal and hatchling blood, the number of POP compounds identified was higher in the eggs. This can be attributed to the differences in the limit of detection (LOD) that is largely affected by the mass of sample analysed. Egg mass at the time of collection was ~ 45 g allowing for analysis of 7-10 g aliquots. However, due to the relative difficulty in obtaining blood from nesting females, maternal blood samples ranged from 2 - 5 g. For hatchling blood, five samples from each nest were combined to produce a single sample of 1.5 - 2 g for that clutch. The LOD for egg samples was ~ 5 pg g^{-1} (wet mass), depending on actual sample mass and specific compound. The LOD for blood samples was up to seven times higher (~ 35 pg g^{-1}) than egg samples (see Chapter 3). Therefore, based on the observations that maternal and hatchling blood concentrations were generally higher

than egg samples, it could be expected that POP compounds at concentrations less than ~ 20 pg g^{-1} in the eggs would not be detected in the blood samples. This was true for most compounds, except for β-HCH and dieldrin. However, both of these compounds had unusually high interference in blood samples that were not observed in the egg samples. This did not allow reliable identification of these peaks in the chromatograms of blood samples.

POP variation in eggs due to foraging

When the mean egg ΣPOP concentrations were compared among clutches, there were only two significantly different groups of clutches. This may initially suggest that the *C. mydas* in this study have come from two different foraging areas of differing contamination levels. However, the ΣPOP concentrations do not represent the contributions of individual compounds to the contamination of *C. mydas* eggs. As illustrated in Chapter 5, analysis of similarity (ANOSIM) on matrices including the presence and concentration of all 125 POP compounds can provide more detailed information on contamination differences between individuals. Furthermore, it has been suggested that the differences in contamination profiles indicate individual *C. mydas* from different foraging areas. Analysis of similarity in the present study indicated that the POP contamination profiles of all clutches were statistically separated from each other. Also, further interpretation of the *n*MDS plot indicated there were six major groupings of eggs from the 11 clutches sharing similar POP contamination profiles. This suggests that the nesting *C. mydas* of this study may have actually come from six different foraging areas.

The use of multivariate analysis of similarity on matrices containing 125 POP compounds is a novel approach to identifying differences in foraging areas of a nesting population. In Chapter 5, the three *C. mydas* nesting at Ma'Daerah from different foraging areas had significantly separated contamination profiles. The separation of the 11 clutches in this study into six distinct groups further supports the use of this type of multivariate approach to investigate foraging ground differences. Furthermore, six distinct foraging areas from 11 individuals suggests that this nesting population of *C. mydas* may come from many smaller low density foraging areas as opposed to a small number of large highly populated foraging areas. However, the

differences between individuals from the same foraging areas are unknown. It is possible that although significantly separated in the ANOSIM analysis and *n*MDS plot, these groups of clutches may still represent *C. mydas* from the same foraging areas. Further analysis into the variation in chemical profiles of individual *C. mydas* from the same foraging area is therefore required before this concept can be validated.

When analysis of similarity was performed on data of the nesting *C. mydas* from known foraging areas (Chapter 5) combined with the 11 clutches of this study, there was no overlap in POP contamination profiles. This indicated that the *C. mydas* of this study were unlikely to have been foraging in the areas identified in Chapter 5. This was not unexpected as the likelihood of matching POP profiles with the three *C. mydas* of known foraging locations would be relatively low. *Chelonia mydas* nest, on average, every three years and lay, on average, three clutches per nesting season (Hirth 1980; Miller 1985; Van Buskirk and Crowder 1994; Miller 1997). Therefore, the 300-500 nests laid at Ma'Daerah each year (Ibrahim, unpublished data) would represent ~ 125 individuals nesting per year, indicating a total nesting stock > 400 *C. mydas* at the Ma'Daerah rookery. Identification of foraging areas for three individuals therefore represents < 1% of the total population. Furthermore, the geography of Southeast Asia includes many coastal areas within a typical *C. mydas* migration distance from Ma'Daerah. There are therefore a large number of possible foraging areas in the region that can be utilised by *C. mydas* nesting at Ma'Daerah.

A more comprehensive satellite tracking program would provide more information on the foraging areas used by the *C. mydas* population nesting at Ma'Daerah. With ~ 125 individuals nesting at Ma'Daerah each year and a mean inter-nesting period of three years, tracking 25 individuals per year over three consecutive years would identify the foraging grounds of ~ 20% of the population. However, with the wide range of potential foraging grounds throughout Southeast Asia, it is possible that this study would still not identify all foraging grounds of this *C. mydas* nesting population. Furthermore, depending on the success of transmitter deployment and battery life, a project of this nature, at current pricing levels, could cost in excess of AUD $300,000. The development of alternative and less expensive methods to determine the foraging ground variability of a sea turtle nesting population is therefore warranted.

In addition to differences in the foraging grounds utilised, there are also a number of factors that may influence the contamination of *C. mydas* foraging in the same area. The accumulation of POPs in *C. mydas* is likely to increase with time spent in a contaminated area. Older *C. mydas* would therefore have higher POP concentrations than younger individuals foraging in the same area. However, in the present study, there was no significant correlation between egg POP concentrations and the curved carapace lengths of the nesting females. The absence of a relationship between age and contamination indicated that these nesting females are unlikely to be from the same foraging area.

There may also be subtle differences in the contamination of sub-habitats within a greater foraging area. The localised feeding areas used by most *C. mydas* may therefore subject individuals to different contamination levels within the same foraging area (see Table 5.5 – Chapter 5). It is therefore possible that the *C. mydas* in the present study are foraging in the areas identified in Chapter 5 but are foraging in different sub-habitats within these areas. However, the POP profiles of the *C. mydas* in the present study are well separated from the turtles from Chapter 5, particularly from the individual foraging in Vietnam. It therefore remains unlikely that the *C. mydas* of this study are foraging in the areas identified in Chapter 5.

Effects of POP concentrations on hatchling development

The egg ΣPOP concentration was significantly correlated with the mass:length ratio of the emerged hatchlings. Mass:length ratios are commonly used in fish biology as an index of body condition, with higher ratios indicating better condition (Bolger and Connolly 1989). The condition index has also been used to compare the health of foraging *C. mydas* populations in Queensland, Australia (Arthur 2000). In sea turtle hatchlings, a reduced mass:length ratio may compromise the duration of offshore dispersal. Sea turtle hatchlings emerge from nests in an energetic frenzy and do not feed in the first 3-5 days as they swim continuously to the safer open ocean waters (Dial 1987; Wyneken and Salmon 1992). The energy demands of offshore dispersal must therefore be met by the store of residual yolk that they have at the time of emergence (Miller 1985). A decrease in mass:length ratio of sea turtle hatchlings could indicate reduced residual yolk and hence reduce the duration of offshore dispersal. Furthermore, there is evidence that larger turtle hatchlings survive better

than their smaller conspecifics due to reduced susceptibility to bird and fish predation, superior locomotion and increased mobility and agility (Haskell et al. 1996; Janzen et al. 2000b, a). The decrease in mass:length ratio with increasing egg POP concentrations observed in the present study may therefore indicate a negative influence of POP contamination on hatchling development that may affect their dispersal and survival. However, there are a number of maternal and environmental factors that can also influence the growth of oviparous reptile embryos, and should be considered in the interpretation of these results.

In freshwater and marine turtles, environmental factors such as nest temperature and moisture content have been found to influence hatchling growth and energy expenditure (Packard et al. 1993; Booth 1998; Booth and Astill 2001; Broderick et al. 2001; Packard and Packard 2001). Scute abnormalities of *C. mydas* hatchlings have also been associated with high nest temperatures (Schauble et al. 2003). Furthermore, nest depth can influence *C. mydas* hatchling energy expenditure by increasing the time taken to climb the egg chamber. A difference in nest depth of 25 cm can increase emergence lag by nearly two days (van de Merwe et al. 2005). This would lead to increased consumption of the residual yolk and hence reduce the mass of emerging hatchlings. However, despite this increased duration of expenditure of the residual yolk, mass:length ratios of *C. mydas* hatchlings have not been found to significantly change with nest depth (van de Merwe et al. 2005).

Regardless of the potential influences of nest factors on hatchling growth, most environmental factors were relatively constant among nests in the present study. For example, the incubation period ranged between 52 and 57 days. Therefore, based on the strong correlations between incubation period and mean nest temperature for *C. mydas*, mean nest temperatures were estimated to be between 29 and 30 $^{\circ}$C (Miller 1985). The high incidence of hatchling abnormalities observed in the present study are therefore unlikely to be due to high nest temperatures, as previously reported (Schauble et al. 2003). In addition, factors such as nest depth, moisture, particle size, organic content and salinity would have been relatively constant among nests due to the consistent construction of hatchery nests and the close proximity of the nests in the hatchery. Environmental factors are therefore unlikely to have had an influence on the differences in the mass:length ratios observed in this study.

Factors other than physical and environmental nest conditions may also account for the differences in the mass:length ratios observed between nests in the present study. The maternal influence on offspring size in animals is well recognised, and the amount of resource packaged into eggs can affect both maternal and hatchling fitness (Bernardo 1996). In freshwater turtles, egg size is generally related to maternal body size and age. It has been hypothesised that this relationship is determined by maternal resources, and morphological constraints (Congdon and Gibbons 1983; Iverson and Smith 1993; Bernardo 1996; Kuchling 1999; Clark et al. 2001; Wilkinson and Gibbons 2005). Furthermore, the initial egg mass can have an influence on embryonic development in oviparous reptiles. In the freshwater turtle, *Emydura signata*, the initial egg mass has been found to be directly related to hatchling size, mass and energy expenditure (Booth 1998). There are therefore strong maternal influences on both egg and hatchling size in freshwater turtles.

The maternal influences on egg mass and consequential effects on hatchling size may be less important in sea turtles. Morphological constraints on egg size are more specific to smaller turtles as the size of the oviduct, ilia and the posterior opening between the carapace and the plastron will tend to be more limiting in these species. Reproductive organ morphology is therefore less likely to be a major influence on egg size in sea turtles due to their relatively large body size. This is supported by the absence of a correlation between maternal size and egg mass in the present study. Furthermore, unlike the freshwater turtle, *E. signata*, the initial egg mass has minimal effect on hatchling growth and energy expenditure in *C. mydas* (Booth and Astill 2001). This is supported by the absence of a correlation between initial egg mass and mass:length ratios in the present study. Furthermore, the effect of initial egg mass on hatchling growth may not be detected in *C. mydas* due to smaller relative variation in *C. mydas* egg mass compared to *E. signata* (Booth 1998; Booth and Astill 2001). This is supported by the small variation in initial egg mass (33.0 - 40.4 g) observed in the present study.

The minimal influence of maternal investment and nest factors on mass:length ratios of *C. mydas* hatchlings supports the likelihood of egg POP concentrations influencing hatchling mass:length ratios. Persistent organic pollutants have been associated with abnormal skeletal development in marine mammals (Zakharov and Yablokov 1990;

Marsili et al. 1997). The relationship of POP contamination with mass:length ratios observed in the present study may therefore be due to disruption to skeletal development during incubation affecting straight carapace length (SCL). Furthermore, the mass:length ratio may be the hatchling development parameter that is most sensitive to influences from POP contamination. This may account for the fact that the remaining incubation and hatchling parameters measured were not significantly correlated with egg POP concentrations. Additionally, the narrow POP concentration range observed in this study may not be sufficient to elicit a response from the other hatchling development parameters. The ranges in values for the incubation and hatchling variables measured were also narrow. It is therefore unlikely that significant correlations would be observed within such narrow POP concentration and hatchling parameter ranges.

6.5 Conclusions

The correlations between maternal blood POP levels and the concentrations in the eggs indicated that there was significant maternal transfer of these chemicals during vitellogenesis. Furthermore, egg POP levels were also significantly correlated to hatchling blood concentrations, indicating that the chemicals were further transferred to the hatchlings during embryonic development. These findings therefore support the use of egg samples to estimate the contamination of *C. mydas* nesting populations.

The variation in the ΣPOP concentrations between clutches was minimal and only revealed two groups with significantly different POP concentrations. However, analysis of similarity revealed that there were six distinct groups of clutches in relation to the presence and concentration of 125 POP compounds. This indicated that these 11 nesting females may have come from six distinct foraging areas. However, the contamination profiles of the eggs in this study were not similar to the *C. mydas* eggs from known foraging grounds (from Chapter 5). It is therefore unlikely that the *C. mydas* in this study have come from these previously identified foraging grounds. However, changes in POP contamination in individuals over time and variations in contamination within foraging areas need to be further investigated before this can be validated.

The mass:length ratio of hatchlings declined significantly with increasing egg POP concentrations. With consistent environmental factors between clutches during development, this indicated that POPs may cause a disruption to hatchling development. However, this disruption seems to be subtle as other factors that would indicate disruption to development, such as hatching success and hatchling abnormalities, were not correlated with POP concentration in the eggs.

Chapter 7 - Chemical contamination of *Chelonia mydas* eggs in Peninsular Malaysia: Implications for conservation and public health

7.1 Introduction

Peninsular Malaysia has historically supported large nesting populations of *Chelonia mydas*, predominantly on the east coast in the state of Terengganu (Hendrickson and Alfred 1961; Hendrickson and Balasingham 1966). However, *C. mydas* nesting populations in Peninsular Malaysia have declined by > 80% since the 1950s, primarily due to the collection of eggs for human consumption (Hendrickson and Alfred 1961; Siow and Moll 1981; Limpus 1993a; Ibrahim 1994; Ibrahim et al. 2003). It has been estimated that prior to the late 1950s, nearly 100% of all sea turtle eggs laid on the beaches of Peninsular Malaysia were collected for human consumption at a rate of up to two million eggs per year (Hendrickson and Alfred 1961; Siow and Moll 1981).

Management of sea turtles in Peninsular Malaysia began in 1961 with the implementation of hatcheries to protect the eggs from collection (Siow and Moll 1981). Since then, conservation efforts have increased to include nesting beach protection through government gazetted areas, public education campaigns and fishing and egg collection legislation and enforcement. Under current legislation, the majority of eggs are protected with the remaining eggs allocated to collectors under a permit system (Ibrahim et al. 2003). In 2006, it was estimated that 90% of all *C. mydas* eggs laid in Peninsular Malaysia were relocated to hatcheries or protected *in situ* by the Department of Fisheries, Malaysia (Ibrahim, unpubl. data). However, this figure is dependent on Department of Fisheries budgetary allocations and can fluctuate from year to year. Furthermore, considering the high economic value of sea turtle eggs (~ USD $0.60 - 1.20 each), there is an incentive for permit holders to under-report egg collection. Eggs may even be harvested without permits and sold in black markets. The collection of eggs for human consumption therefore continues to pose a threat to *C. mydas* populations, although the magnitude of this impact has been greatly reduced in recent years.

The consumption of sea turtle products (tissues, eggs and blood) poses a number of public health concerns due to high lipid content and the presence of bacteria, parasites and environmental contaminants (Aguirre et al. 2006). Persistent organic pollutants (POPs) and heavy metals have been reported in the eggs of a number of *C. mydas* populations (see Figures 1.3 and 1.5 (Chapter 1): Thompson et al. 1974; Clark and Krynitsky 1980; Podreka et al. 1998; Godley et al. 1999; McKenzie et al. 1999; Coufal 2002; Lam et al. 2006). Many of these chemicals are carcinogenic and have been associated with neurological, cardiovascular, respiratory, endocrinological, developmental, reproductive and immunological dysfunction (Colborn et al. 1993; Chang 1996; Baird and Cann 2005; McKinlay et al. 2008). As a guide for consumption of foods containing these chemicals, the World Health Organisation (WHO) and other regional organisations have determined acceptable daily intake (ADI) of these compounds (FAO/WHO 2007). However, ADIs are determined on an individual compound basis. The effects of consuming food with multiple contaminants are not well understood.

The chemical contamination of *C. mydas* eggs may also have conservation implications. Persistent organic pollutants, in particular, are known endocrine disrupting chemicals (EDCs), and have been found to cause decreased fertility and hatching success, sex reversal and abnormal immune function in fish, birds, reptiles and mammals (Colborn et al. 1993; Guillette and Gunderson 2001; McKinlay et al. 2008). The most well documented effects of EDCs in oviparous reptiles are on sex reversal and hatchling development. Eggs of the American alligator (*Alligator mississippiensis*) with high levels of *p,p'*-DDE, *p,p'*-DDD, dieldrin and *cis*-chlordane experienced high egg mortality and abnormal development of the reproductive system of juveniles (Guillette and Crain 1996). Similarly, PCBs in the eggs of the freshwater snapping turtle (*Chelydra serpentina*) were associated with embryonic mortalities and deformities (Helwig and Hora 1983; Olafsson et al. 1983; Bryan et al. 1987). Furthermore, PCBs, chlordanes and *p,p'*-DDE have been found to cause sex reversal in developing embryos of the red-eared slider turtle, *Trachemys scripta* (Bergeron et al. 1994; Willingham and Crews 1999).

The chemical contamination of *C. mydas* eggs in Peninsular Malaysia potentially has both conservation and human health consequences. The availability of *C. mydas* eggs

in the markets of Peninsular Malaysia provides an opportunity to collect a sample of the population's eggs and assess the level of chemical contamination. This information can be used to predict the effects of these chemicals on the *C. mydas* populations and the potential harm they may cause to the humans consuming them.

7.2 Methods

Market egg collection

In August 2006, a survey was conducted by road to identify markets in Peninsular Malaysia selling *C. mydas* eggs for human consumption. Effort was concentrated on the east coast and southern part of the west coast as this is where the majority of sea turtle nesting occurs in Peninsular Malaysia (Hendrickson and Alfred 1961; Hendrickson and Balasingham 1966). Over six days, a 1,115 km route covering ~ 730 km of coastline was taken from Kota Bharu through Kuantan, Kuala Lumpur, Johor Bahru and back up the east coast to Kuantan (Figure 7.1). Each of the 33 markets encountered on this route was surveyed for *C. mydas* eggs. A random sample of 3-4 eggs was purchased from each market where eggs were being sold. In larger markets, where there were multiple vendors, eggs were taken from a random sample of the vendors. At the time of collection, vendors were asked about the nesting location and the approximate date the eggs had been collected.

The frozen eggs were transported in an insulated box surrounded by ice packs as carry-on luggage on a commercial flight from Kuala Lumpur to Brisbane. Upon arrival in Australia, the eggs were still frozen solid and were immediately taken to the Organics Section, Queensland Health Scientific Services, in Coopers Plains where they were kept frozen (-20 $^{\circ}$C) until analysis.

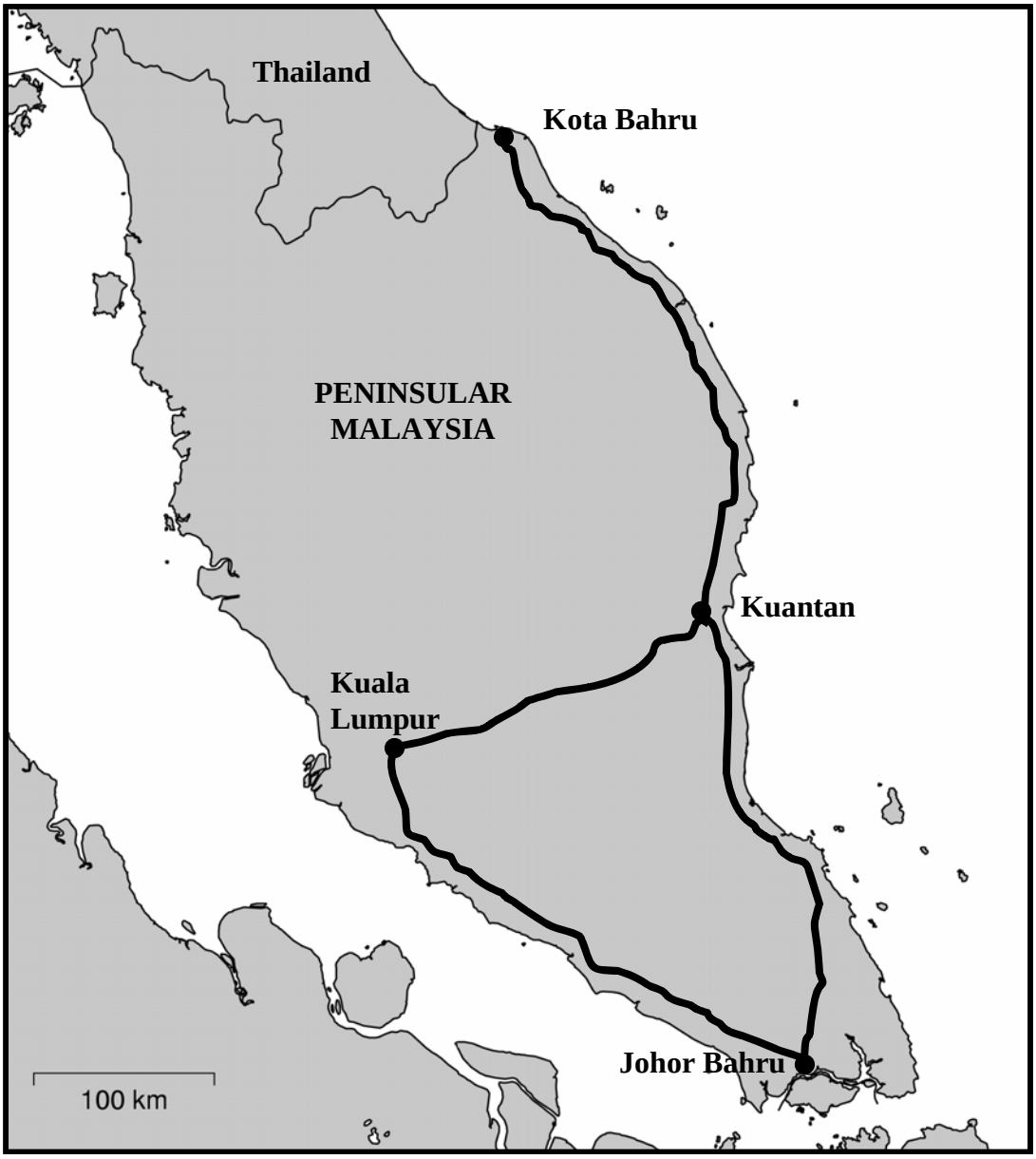

Figure 7.1. Map of Peninsular Malaysia indicating the route taken and coastline covered in the survey for markets selling *Chelonia mydas* eggs for human consumption. Map created using Maptool (SEATURTLE.ORG 2002).

Chemical analysis

All egg samples were prepared and analysed for POPs and metals using methods outlined in Chapters 2 and 3. Briefly, POPs were analysed using GC-MS/MS following accelerated solvent extraction in dichloromethane, gel permeation chromatography and Florisil™ column clean-up. Percent lipid of each egg was determined gravimetrically following extractions. Following acid digestions, heavy metals copper, zinc, selenium, arsenic, cadmium and lead were analysed using ICP-

MS and mercury was analysed with CV-AAS. Standard reference materials (SRMs), pooled and blank samples were run alongside samples for quality control.

Screening risk assessments

Screening risk assessments (SRAs) were performed for a number of the major POPs and metals. More comprehensive ecological risk assessments (ERAs) were not possible due to the lack of toxicological information for *C. mydas* and other sea turtle species. However, in the absence of such species-specific toxicological information, SRAs provide an adequate estimation of the potential effects of environmental contaminants.

For each compound, hazard quotients (HQs) were calculated for each compound by dividing the measured concentration (MEC) by the predicted no effect concentration (PNEC). The PNECs were generally estimated from toxicological studies on the effects of these compounds on sea turtles and other oviparous reptiles. However, in cases where this information was not available, PNECs were estimated from bird data. When using species other than sea turtles a number of assumptions and uncertainty factors needed to be considered in the calculations of PNECs (USACHPPM 2000). The HQs were calculated for both a best and worst case scenario (Equations 7.1 and 7.2). In cases where there was limited information and a maximum and minimum PNEC could not be determined, the same value was used for both HQs.

$$HQ_{best} = \frac{MEC(\min)}{PNEC(\max)} \quad \text{….. Equation 7.1}$$

$$HQ_{worst} = \frac{MEC(\max)}{PNEC(\min)} \quad \text{….. Equation 7.2}$$

There was only one previous study on the toxicological effects of POPs on sea turtles. However, there were a number of studies on freshwater turtles that have investigated the effects of POPs on sex reversal. Based on these studies, the PNECs could be determined for *p,p'*-DDE, dieldrin and PCBs. The PNEC for *p,p'*-DDE was estimated as 543 ng g^{-1}, based on study on *C. mydas* that found no sex reversal of developing embryos up to a concentration of 543 ng g^{-1} (Podreka et al. 1998). A study on red-eared slider turtles (*Trachemys scripta*) found no significant sex reversal when

embryos were dosed with 5 μL of 2.6 μM dieldrin (Willingham and Crews 1999). Based on an estimated penetration of 30% and an average egg mass of ~ 10 g, this dose represented egg concentration of ~ 40 ng g^{-1} dieldrin. Therefore, after applying an uncertainty factor of 10 for inter-species differences, the PNECs for dieldrin were estimated at 4 ng g^{-1}. The effects of PCBs on sex reversal have also been studied on *T. scripta* (Bergeron et al. 1994). In this study, the topical administration of 10 μg of single PCB compounds to *T. scripta* eggs produced no sex reversal. Based on an estimated penetration of 30% and an average egg mass of 11.4 g, these doses represented egg concentrations of ~ 260 ng g^{-1}. Therefore, after applying an uncertainty factor of 10 for inter-species differences, the PNEC for a single PCB was 26 ng g^{-1}.

Studies on the toxicological effects of heavy metals on sea turtles do not exist and are scarce for oviparous reptiles and birds. Furthermore, the toxicological effects of essential metals have generally been limited to invertebrates and fish. Due to the profound difference in ovipary of these species, the estimation of PNECs for *C. mydas* from invertebrate and fish studies was not considered valid. The PNECs in the present study were therefore limited to the toxic metals and selenium that were estimated from reptile and bird toxicological studies. The PNEC for lead was calculated from the no observed adverse effect level (NOAEL) determined for *T. scripta* by Burger et al. (1998). An uncertainty factor of 100 was applied to the NOAEL value of 100 μg g^{-1} (x 10 for inter-species differences, x 10 for the use of a sub-chronic NOAEL). A PNEC of 1 μg g^{-1} was therefore estimated for lead. There was no toxicological information on oviparous reptiles for selenium, cadmium or arsenic. The PNECs for selenium were therefore estimated as 0.34 and 6 μg g^{-1}, based on assumptions and calculations from bird egg studies detailed in Lam et al. (2006). The PNEC for cadmium was estimated from the NOAELs of two dosing studies on broiler chickens. In studies by Voleda et al. (1997) and Leach et al. (1979), egg cadmium concentrations of 1.3 μg g^{-1} and 0.14 μg g^{-1}, respectively, had no influence on embryo mortality or eggshell thinning. An uncertainty factor of 100 was applied to each of these NOAELs to account for inter-species differences and the use of sub-chronic exposure. This resulted in lower and upper estimates for the PNEC for cadmium to be 0.013 and 0.0014 μg g^{-1}, respectively. The PNEC for arsenic was

estimated from a feeding study on mallard hens (Stanley et al. 1994). There were no observable adverse effects on duckling production, mortality or hatch success in eggs with arsenic concentrations of 1.8 μg g^{-1}. Therefore, after the uncertainty factor of 100 was applied to this NOAEL, the PNEC for arsenic was estimated at 0.018 μg g^{-1}.

Human health risk assessments

The potential effects of consuming *C. mydas* eggs containing POPs and metals on human health was investigated using risk assessment parameters outlined by the National Research Council (NRC) and the National Academy of Sciences (NAS) of the United States (NRC/NAS 1983). The establishment of a risk assessment for the effects of chemicals in food on public health involves identification of the hazard, determination of the relationship between dose and response, assessment of exposure and characterisation of the risk.

Most POPs and heavy metals have been identified to have adverse effects on humans and wildlife (see Chapter 1). As a result, the joint FAO/WHO expert committee on food additives (JECFA) has calculated acceptable daily intakes (ADIs) for these chemicals (FAO/WHO 2007; WHO 2008). The ADIs are based on human and animal experiments that investigate the no observable adverse effect levels (NOAEL) of these chemicals and are generally presented as μg kg^{-1} body weight day^{-1}. Maximum residue levels (MRLs) permitted in food are further calculated from the ADIs and indicate the maximum concentration of chemicals allowed in food products at the point of sale. The MRLs also take into account the contamination and relative consumption of different food types, and vary slightly between countries with different diet preferences (NRC/NAS 1983; FAO/WHO 2000). They are not a true indication of risk to human health and will therefore not be further considered in this risk assessment.

The human health risk assessment generally includes the percentage of people within a population with a chemical consumption level above the ADIs for each compound. This is calculated from known consumption rates and contamination concentrations of different food products (Van Oostdam et al. 2005). However, the rate of *C. mydas* egg consumption in Peninsular Malaysia was not investigated in the present study, nor is it

well understood. Instead, the maximum concentration of each of the major POP and metal compounds reported was used to evaluate risk by expressing the contaminant concentration as percent of ADI in a single *C. mydas* egg (Equation 7.3). This calculation was based on a 65 kg person consuming a single *C. mydas* egg of mass 35 g.

$$\text{Max. percent of ADI in one egg} = \left(\frac{C \times EM}{ADI \times PM}\right) \text{x } 100 \qquad \text{….. Equation 7.3}$$

C = maximum concentration of contaminant reported in *C. mydas* eggs ($\mu g\ g^{-1}$)
EM = mass of *C. mydas* egg (= 35 g)
ADI = acceptable daily intake ($\mu g\ kg^{-1}\ day^{-1}$)
PM = person mass (= 65 kg)

Statistical analysis

Persistent organic pollutants (POPs) were initially arranged into three major groups: 1) PCBs - sum of all 83 polychlorinated biphenyl congeners; 2) OCPs - sum of all 23 organochlorine pesticides; and 3) PBDEs - sum of all 19 polybrominated diphenyl ether congeners. The OCPs were then further arranged into the major individual compounds (mirex, endosulfan I, pentachlorobenzene, dieldrin and heptachlor epoxide) and the three major OCP groups: 1) HCHs - α, β and γ - hexachlorocyclohexane; 2) chlordanes - oxychlordane, *trans/cis*-chlordane, *trans/cis*-nonachlor; and 3) DDTs - 2,4'-DDE, 4,4'-DDE, 2,4'-DDD, 2,4'-DDT, 4,4'-DDD and 4,4'-DDT. Mean POP and metal concentrations ($\pm$ SE) were calculated with all values below the limit of detection assigned a value half the limit of detection. This produced the least amount of bias while not requiring the use of complex iteration software (Helsel 1990). The number of individual POP compounds in each egg was also calculated.

Differences in egg contamination among the vendor-reported nesting areas were investigated. Egg collection sites were grouped into five major nesting areas of Peninsular and Borneo, Malaysia: 1) Sabah (Borneo Malaysia); 2) Peninsular west coast (Perak); 3) Peninsular northeast coast (Redang/Perhentian Islands and Punti Bari); 4) Peninsular east-central coast (Paka and Cherating); and 5) Peninsular

southeast coast (Tioman Island). One-factor analysis of variance (ANOVA) was performed to identify significant differences in four variables among the major nesting areas: 1) the mean ΣPOP concentration, 2) the number of POP compounds, 3) the mean Σ essential metal concentration, and 4) the mean Σ toxic metal concentration. Least significant difference (LSD) post hoc analyses were performed in cases of significant ANOVA results ($P < 0.05$).

Multivariate statistics were used to further investigate differences in the POP composition of eggs from different nesting areas. Metals were not further analysed in this way due to the less distinct separation in metal profiles of the *C. mydas* from different foraging areas (see Chapter 5). A Bray-Curtis similarity matrix with no data transformation was constructed for POPs using Primer v5 (PRIMER-E, UK). Each POP compound was entered as a separate variable and each egg was analysed as an individual sample. Eggs were then grouped into the aforementioned five major nesting areas. To investigate similarity in the chemical composition between these nesting areas, analysis of similarity (ANOSIM) was performed on the Bray-Curtis matrix and non-metric multi dimensional scaling (*n*MDS) plot was constructed (PRIMER-E, UK).

Differences in egg contamination among markets were investigated by pooling the eggs from each market. A one-factor ANOVA was performed to identify significant differences in four variables among the markets: 1) the mean ΣPOP concentration, 2) the number of POP compounds, 3) the mean Σ essential metal concentration, and 4) the mean Σ toxic metal concentration. Least significant difference (LSD) post hoc analyses were performed in cases of significant ANOVA results ($P < 0.05$).

For all ANOVAs, the assumptions of normality and homogeneity of variance were checked by interpretation of residuals plotted against the dependent variables and Levene's test of equality of error variances, respectively.

7.3. Results

Chelonia mydas eggs were found in nine of the 33 markets visited (Figure 7.2). All of these markets were on the east coast of Peninsular Malaysia and ranged from multiple vendors selling hundreds of eggs each (in the larger town markets) to a single vendor

selling 50-100 eggs at a roadside fish market. According to the vendors, the eggs had been collected from seven different nesting sites (Figure 7.3), at times ranging from the previous night to ten days prior to purchase for this study. The nesting areas from where the eggs had been collected ranged from nearby beaches to as far away as Sabah in Borneo Malaysia (Table 7.1).

Table 7.1. The nesting locations and time between collection and purchase for this study of *Chelonia mydas* eggs collected from markets in Peninsular Malaysia.

Market	**Location eggs collected**	**Distance from market (km)**	**Days since collection**
Kota Bharu	Turtle Island (Borneo)	1800	10
	Redang/Perhentian Islands	80	7
Pasir Putih	Tioman Island	400	8
Penarik	Punti Bari	2	1
Kuala Terengganu	Redang Island	65	1
	Turtle Island (Borneo)	1700	3
	Perak	300	2
Paka	Paka beach	1	1
Dungun	Paka beach	1	3-7
Mersing	Tioman Island	55	1-7
Cukai	Cherating	10	7-10
Kuantan	Turtle Island (Borneo)	1700	3-6

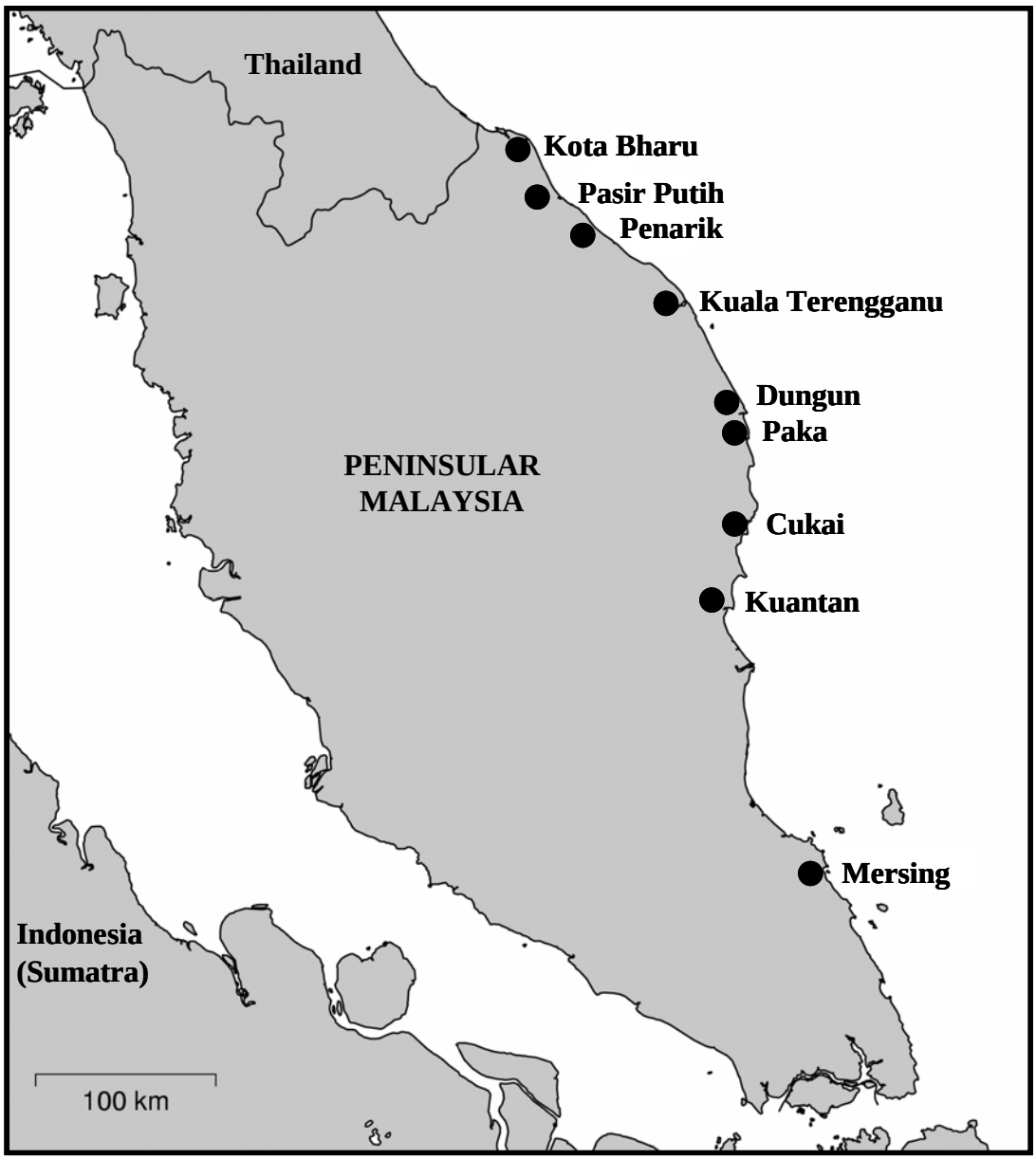

Figure 7.2. Locations of markets in Peninsular Malaysia selling *Chelonia mydas* eggs for human consumption. Map generated using Maptool (SEATURTLE.ORG 2002).

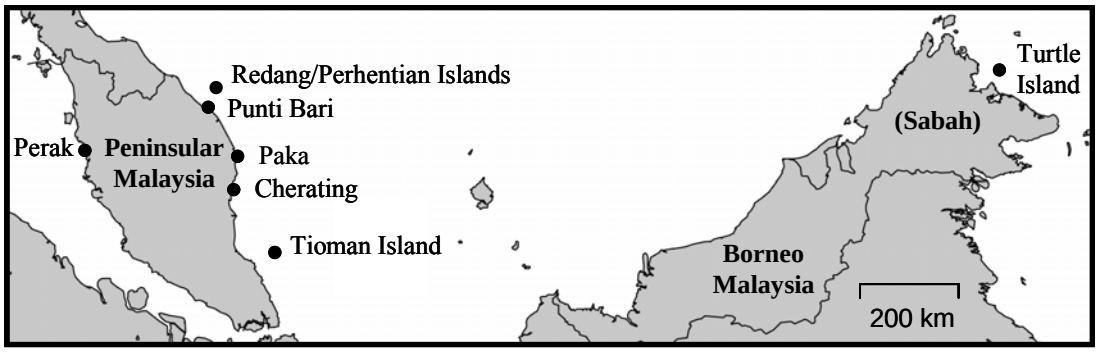

Figure 7.3. Nesting areas from where the *Chelonia mydas* eggs had been collected for sale in the markets of Peninsular Malaysia. Map generated using Maptool (SEATURTLE.ORG 2002).

Organochlorine pesticides (OCPs), PCBs, chlordanes and HCHs were identified in all 55 of the eggs analysed (Table 7.3). The OCPs and PCBs were the most highly concentrated POP compounds, while chlordanes and HCHs were generally found at trace levels. The PBDEs and DDTs were identified at trace levels in 84% of the eggs analysed (Table 7.3). There was a consistent recurrence of the particular compounds that contributed to the concentration of the POP groups (Table 7.2). The range of all POP concentrations was large and there was considerable variation within and between markets in both the overall concentration and the relative contributions from the individual compounds (Figures 7.4 to 7.6). Percent lipids ranged from 7.3% to 13.5% (mean ± SE = 9.3 ± 0.1%).

Zinc, copper and selenium were identified in all of the 55 eggs analysed. However, cobalt was not detected in any of the eggs. Zinc was the most highly concentrated element in each egg. For the toxic metals, arsenic was the most common element with the highest concentrations, detected in 65% of the eggs sampled. Cadmium and lead were less common, being detected in 49% and 11% of the eggs, respectively. The range of all metal concentrations was large (Table 7.3) and there was considerable variation within and between markets in both the overall concentration and the relative contributions from the individual elements (Figures 7.7 and 7.8).

Table 7.2. The compounds contributing to the concentration of the different POP groups in *Chelonia mydas* eggs from markets in Peninsular Malaysia.

POP group	Major compounds
PCBs	18, 28+31, 52, 56, 74, 99, 118, 132+153, 138+158, 156, 170, 178, 180+193, 183, 187, 194, 196+203, 202
OCPs	dieldrin, endosulfan I, heptachlor epoxide, hexachlorobenzene, mirex
Chlordanes	oxychlordane, *trans*-chlordane, *trans*-nonachlor
HCHs	β-HCH, γ-HCH
DDTs	4,4'-DDE, 4,4'-DDT
PBDEs	47, 99, 100, 153, 154

Table 7.3. Mean (± SE) and range of the concentrations of the POPs (pg g^{-1} wet mass) and metals (μg g^{-1} wet mass) in *Chelonia mydas* eggs from the markets of Peninsular Malaysia.

Compound	Mean ± SE	Range (n[a])
POPs		
ΣPCBs	470.5 ± 83.3	146.6 - 3691.5
ΣOCPs	394.9 ± 43.1	169.5 - 2286.7
ΣChlordanes	57.5 ± 9.4	24.7 - 514.2
ΣHCHs	68.8 ± 8.7	13.2 - 230.1
ΣDDTs	83.5 ± 18.3	LOD - 701.9 (46)
ΣPBDEs	21.4 ± 6.6	LOD - 352.7 (46)
ΣPOPs	1096.6 ± 432.5	432.5 - 6266.9
No. POPs	27.0 ± 0.8	17 - 45
Essential Metals		
Copper	0.526 ± 0.023	0.056 - 1.073
Zinc	15.34 ± 0.93	1.33 - 39.48
Selenium	0.464 ± 0.026	0.049 - 0.836
Σ Essential	16.33 ± 0.96	1.44 - 40.92
Toxic Metals		
Arsenic	0.097 ± 0.011	LOD - 0.351 (36)
Cadmium	0.009 ± 0.001	LOD - 0.029 (27)
Lead	0.031 ± 0.003	LOD - 0.124 (6)
Σ Toxic	0.138 ± 0.012	LOD - 0.380 (46)
Lipids (%)	9.33 ± 0.14	7.31 - 13.54

a, number of samples above the limit of detection in parentheses if < 55

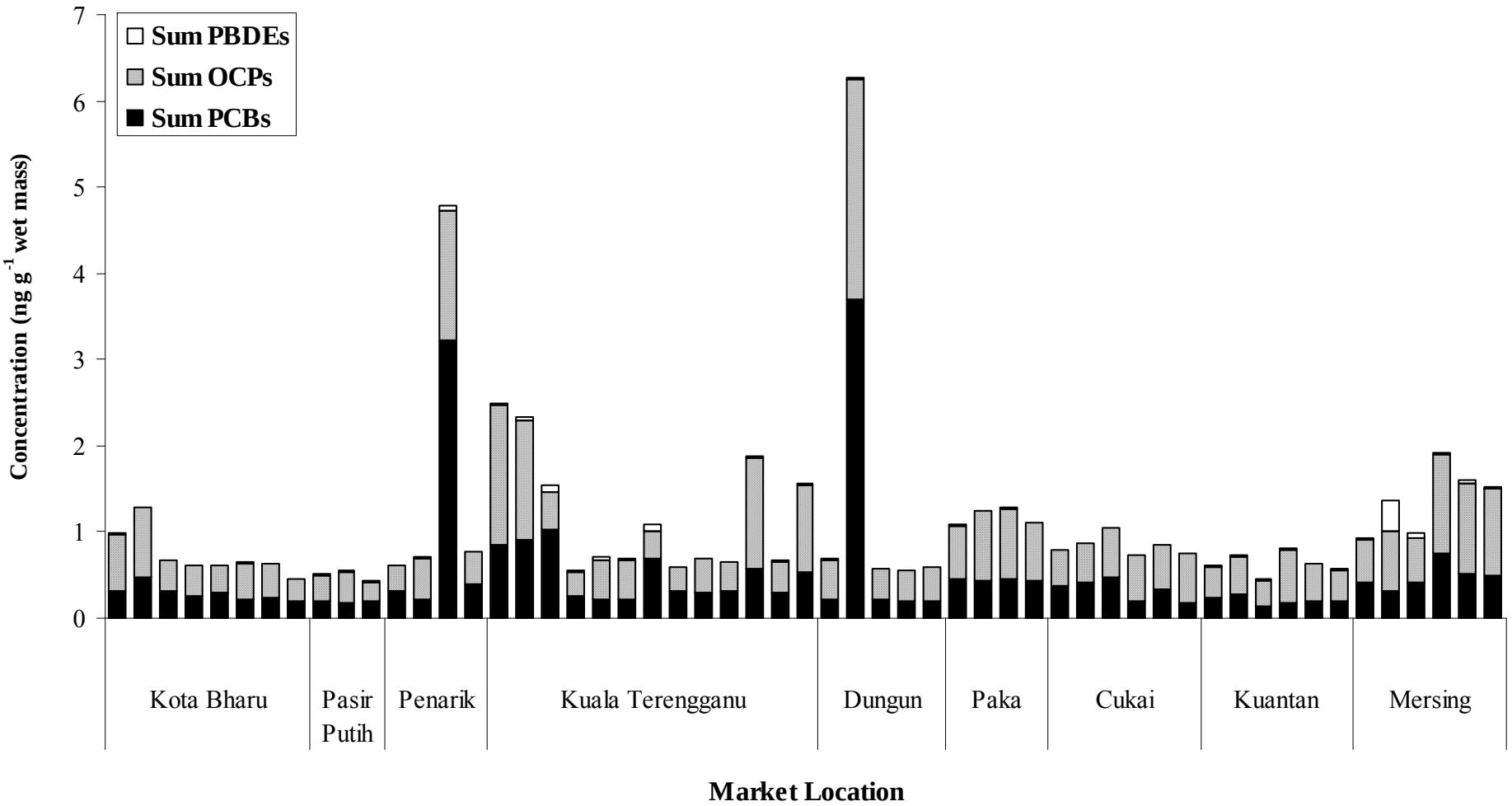

Figure 7.4. Concentrations (ng g^{-1} wet mass) of polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and organochlorine pesticides (OCPs) in the *Chelonia mydas* eggs collected from markets in Peninsular Malaysia.

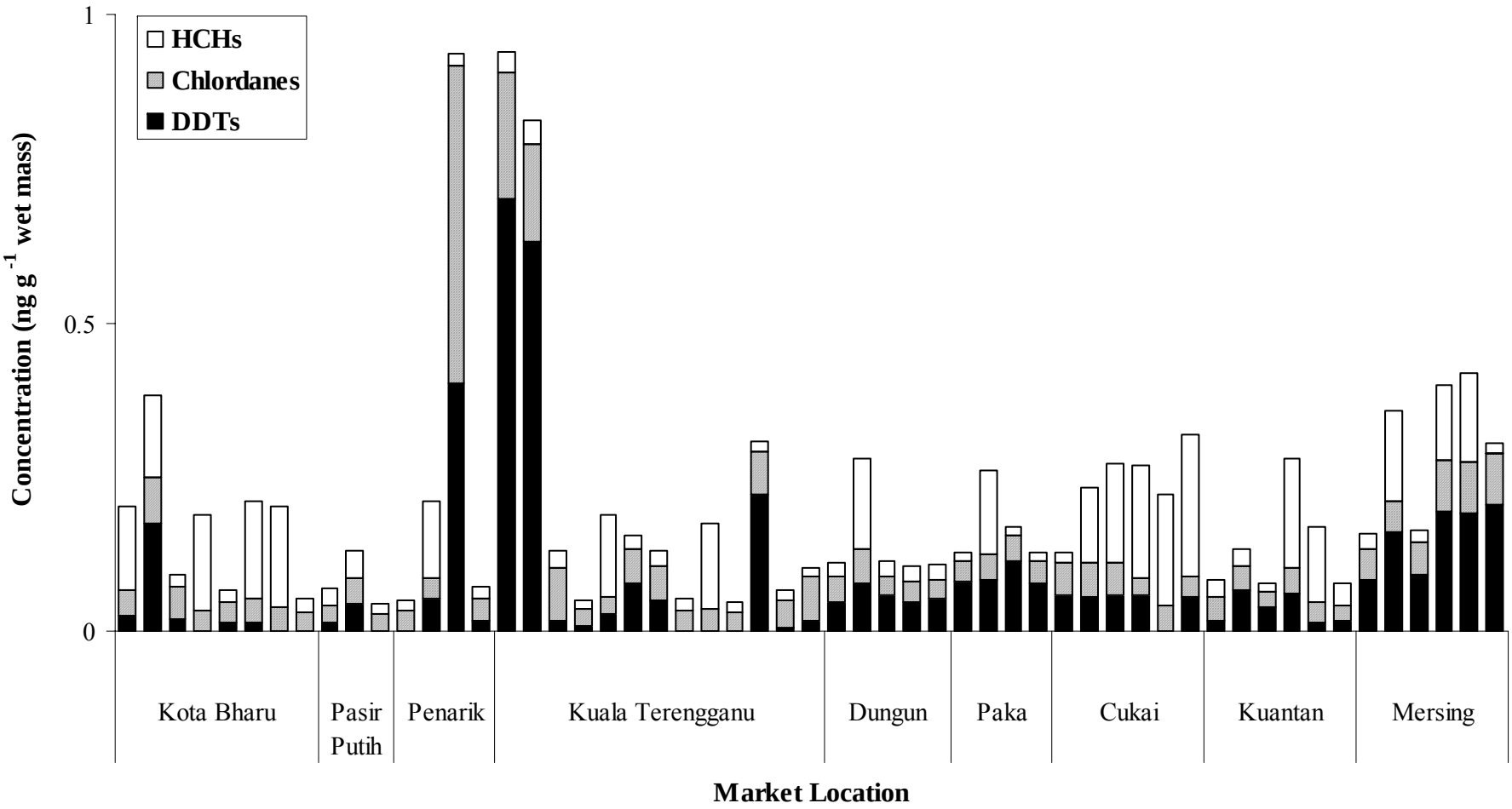

Figure 7.5. Concentrations (ng g^{-1} wet mass) of the major OCP groups in the *Chelonia mydas* eggs collected from markets in Peninsular Malaysia. HCHs - α, β and γ - HCH; chlordanes - oxychlordane, *trans/cis*-chlordane, *trans/cis*-nonachlor; DDTs - 2,4'-DDE 4,4'-DDE 2,4'-DDD 2,4'-DDT 4,4'-DDD 4,4'-DDT.

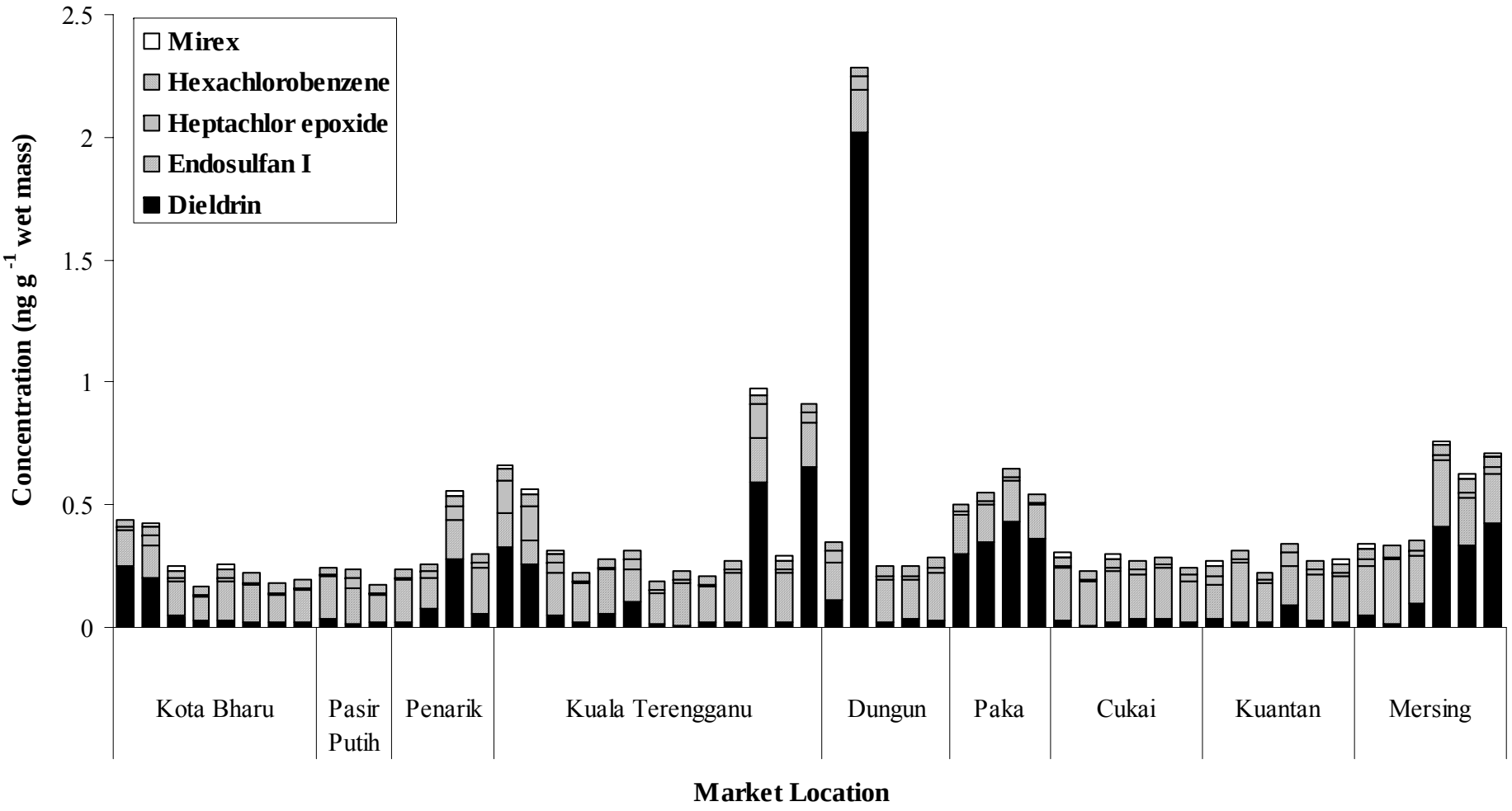

Figure 7.6. Concentrations (ng g^{-1} wet mass) of the major individual organochlorine pesticide compounds in the *Chelonia mydas* eggs collected from markets in Peninsular Malaysia.

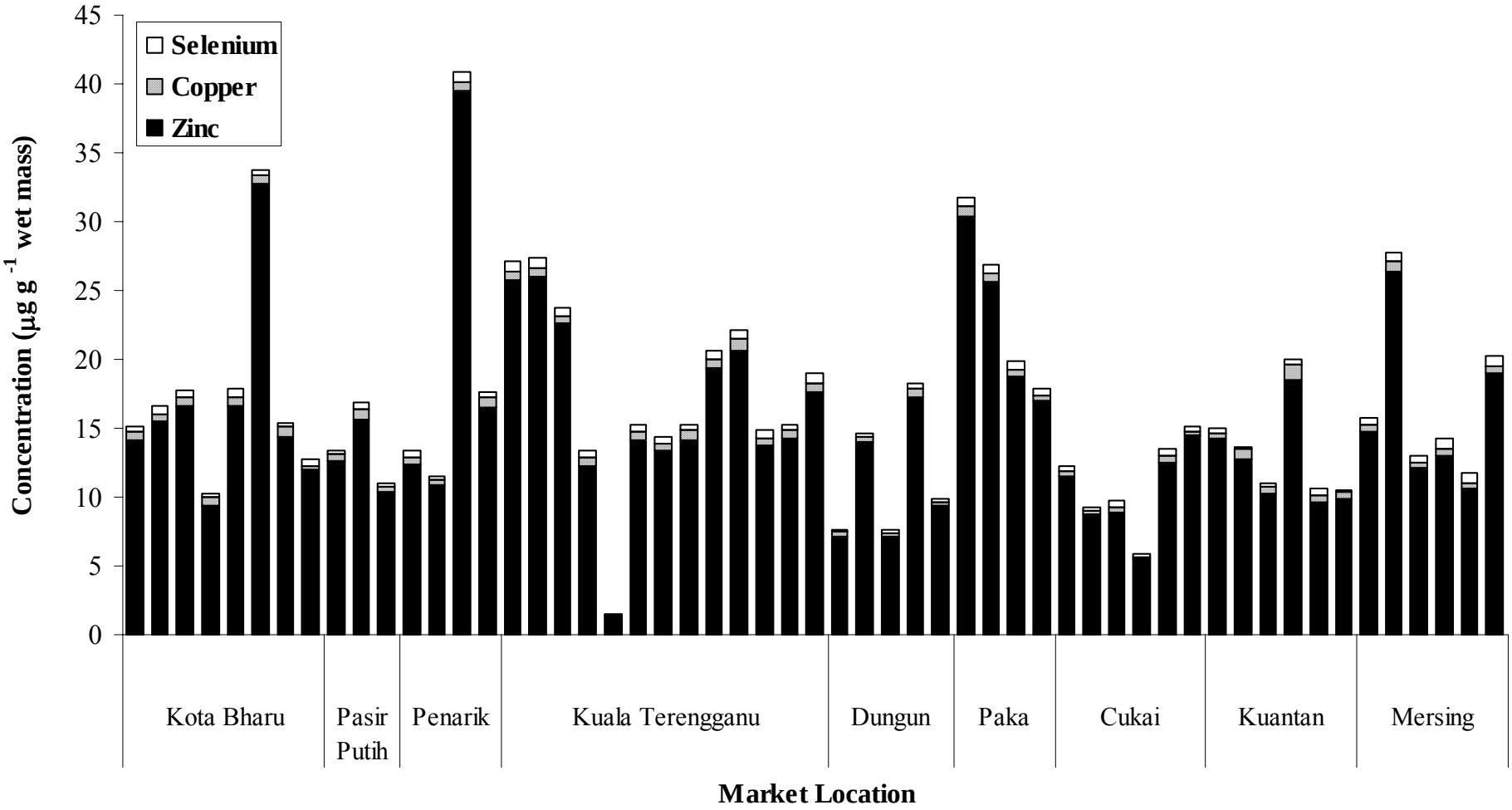

Figure 7.7. Concentrations of essential metals in the *Chelonia mydas* eggs collected from markets in Peninsular Malaysia.

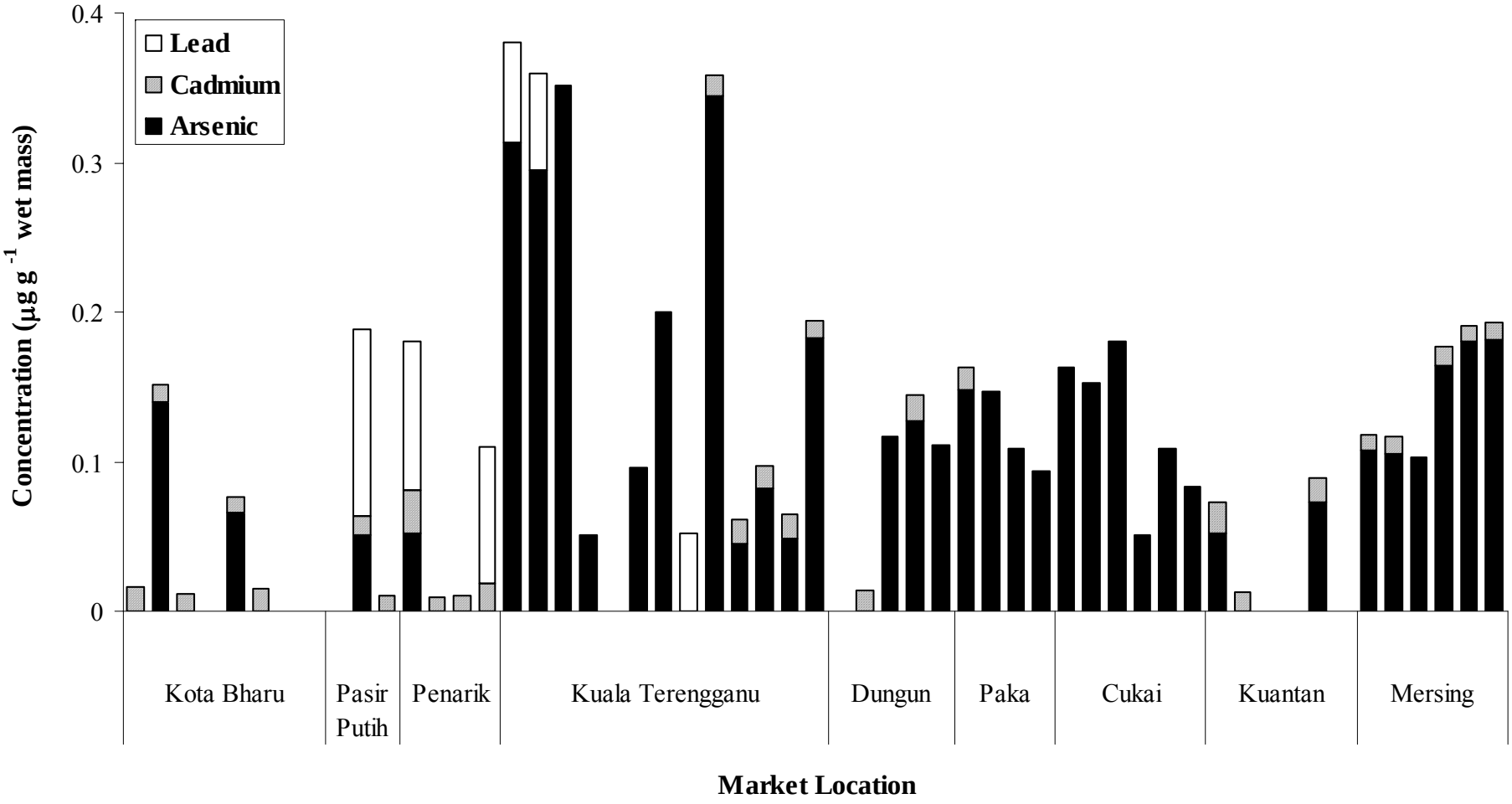

Figure 7.8. Concentrations of toxic metals in the *Chelonia mydas* eggs collected from markets in Peninsular Malaysia.

There was no significant difference in the mean ΣPOPs concentrations (ANOVA: F = 1.01; df = 8, 46; P = 0.441) or Σ essential metals (ANOVA: F = 2.05; df = 8, 46; P = 0.061), or the total number of POPs (ANOVA: F = 1.99; df = 8, 46; P = 0.069) between markets (Figures 7.9 and 7.10). However, there was a significant difference in the Σ toxic metals concentration between markets (Figure 7.10; ANOVA: F = 2.50; df = 8, 46; P = 0.024). Eggs from Kuala Terengganu had significantly higher Σ toxic metal concentration than eggs from Kota Bharu (LSD: P = 0.001), Penarik (LSD: P = 0.048), Dungun (LSD: P = 0.047) and Kuantan (LSD: P = 0.002). Eggs from Mersing had significantly higher Σ toxic metal concentration than eggs from Kuantan (LSD: P = 0.029) and Kota Bahru (LSD: P = 0.029).

There was no significant difference in the ΣPOP concentrations (ANOVA: F = 0.99; df = 4, 50; P = 0.424) or the total number of POP compounds (ANOVA: F = 2.05; df = 4, 50; P = 0.102) between the major nesting areas (Figure 7.11). Similarly, there was no significant difference in the Σ essential metal concentration (ANOVA: F = 1.67; df = 4, 50; P = 0.172) or Σ toxic metals (ANOVA: F = 0.98; df = 4, 50; P = 0.429) between nesting areas (Figure 7.12).

There was a significant difference in the POP concentration profiles between clutches (ANOSIM: R = 0.21, P = 0.001). However, an R value < 0.25 indicated that the groups were not very well separated (Clarke and Gorley 2001). This was confirmed by the *n*MDS plot (stress = 0.12) that showed considerable overlap of the groups (Figure 7.13). However, more detail was obtained by investigating the differences between each of the pair-wise combinations of groups (Table 7.4). Eggs from Sabah were best separated from the eggs from the west coast of Peninsular Malaysia, although statistically different to eggs from all other nesting sites, except the east-central coast of Peninsular Malaysia. Eggs collected from the east coast of Peninsular Malaysia were all similar to each other and also similar to eggs collected from the west coast.

Table 7.4. Results of pairwise comparisons of analysis of similarity (ANOSIM) tests comparing the difference in POP contamination profiles of eggs *C. mydas* eggs collected from five different nesting areas.

Nesting site comparison	**R**	**P**
Sabah – Northeast Peninsular (Redang, Perhentian, Bari)	0.244	**0.009**
Sabah – East-central Peninsular (Paka, Cherating)	0.056	0.095
Sabah – Southeast Peninsular (Tioman)	0.359	**0.002**
Sabah – West Peninsular (Perak)	0.518	**0.019**
Northeast Peninsular - Southeast Peninsular	0.031	0.252
Northeast Peninsular - East-central Peninsular	0.215	1
Northeast Peninsular – West Peninsular	-0.089	0.636
East-central Peninsular - Southeast Peninsular	0.199	1
East-central Peninsular - West Peninsular	0.297	0.086
Southeast Peninsular - West Peninsular	-0.001	0.459

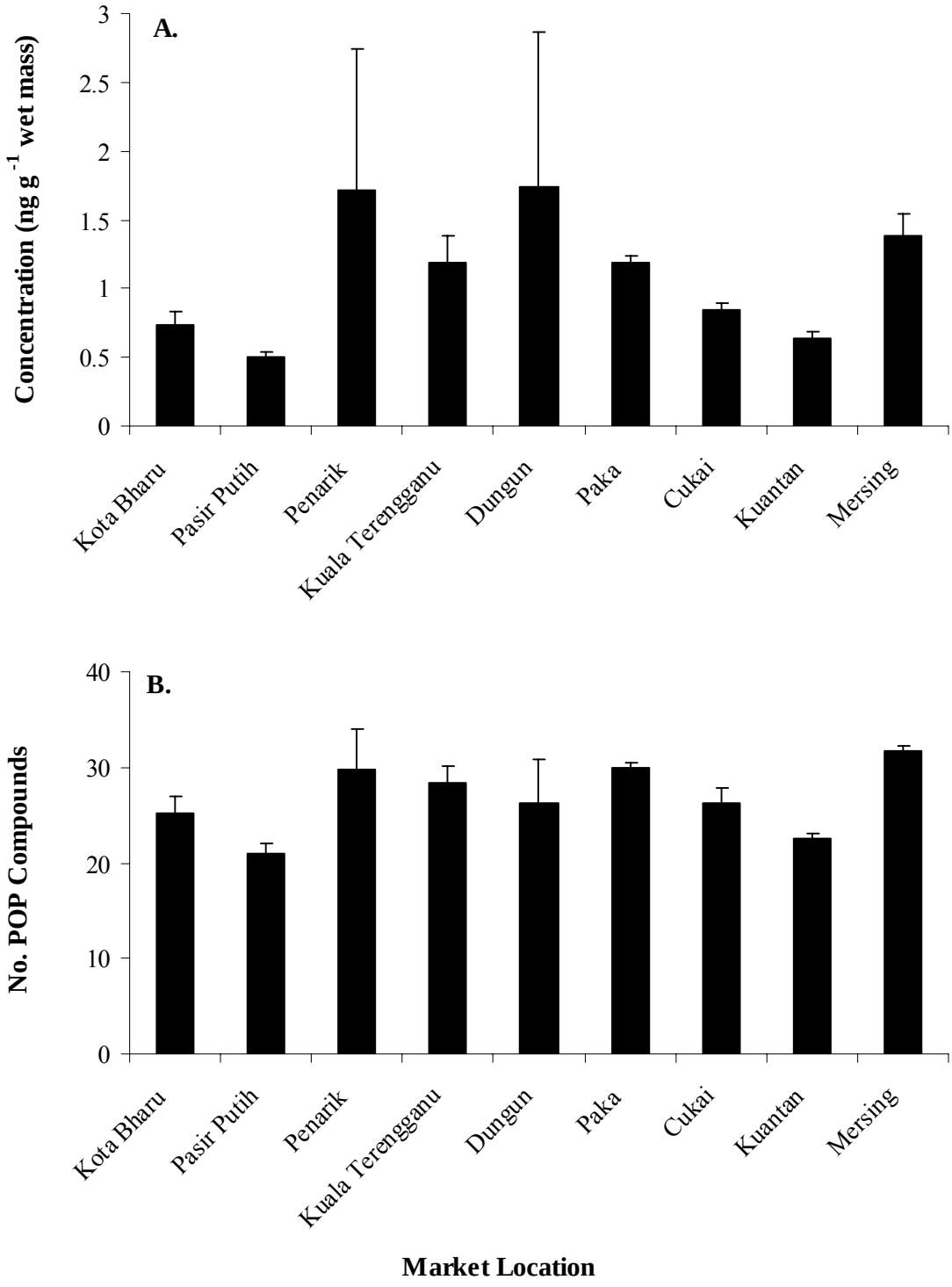

Figure 7.9. Mean (+ SE) ΣPOP concentration (A) and number of POPs (B) in *Chelonia mydas* eggs collected from markets of Peninsular Malaysia.

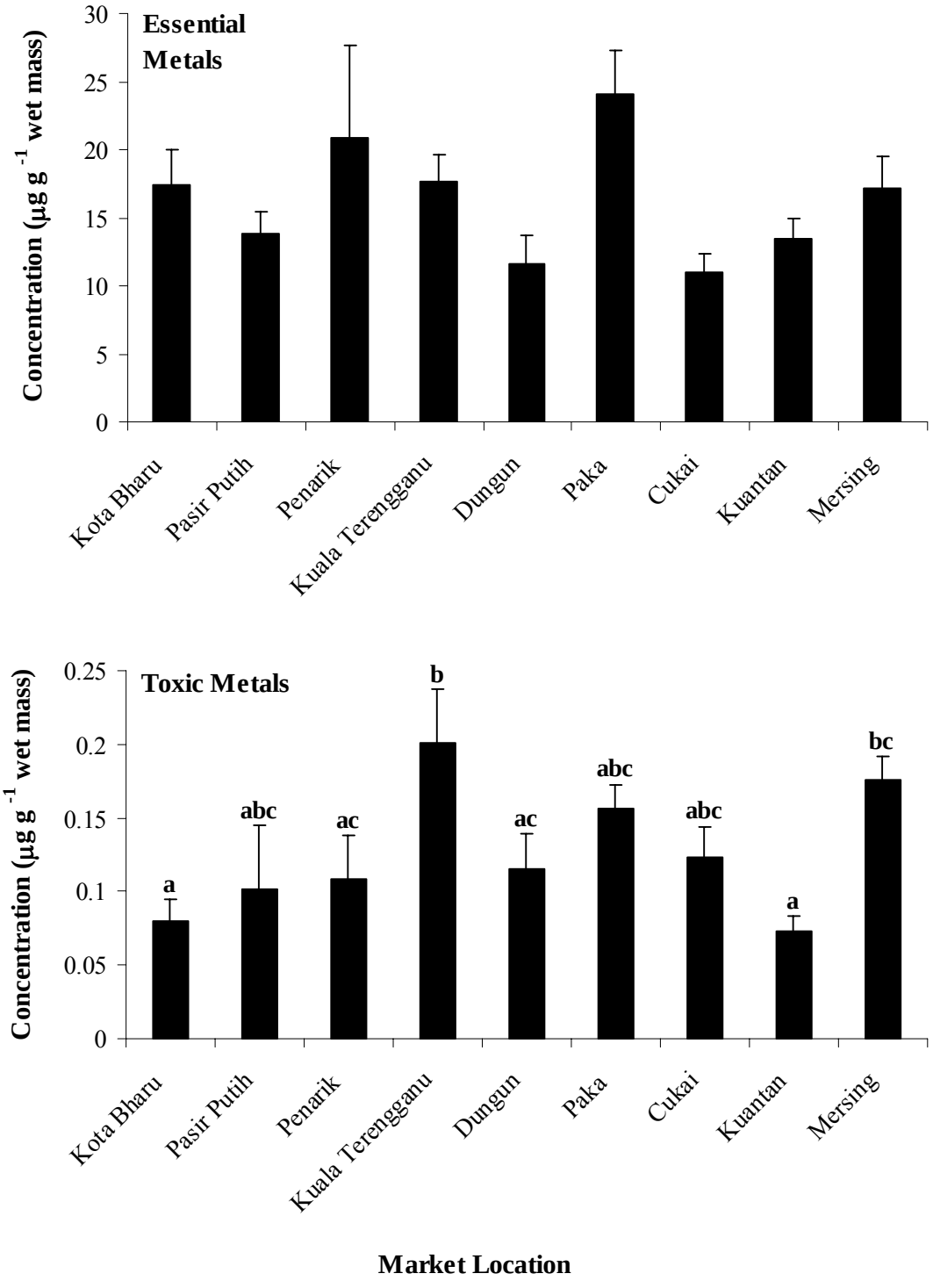

Figure 7.10. Mean (+ SE) concentrations of Σ essential and Σ toxic metals in the *Chelonia mydas* eggs collected from markets in Peninsular Malaysia. Letters refer to significantly different values ($P < 0.05$).

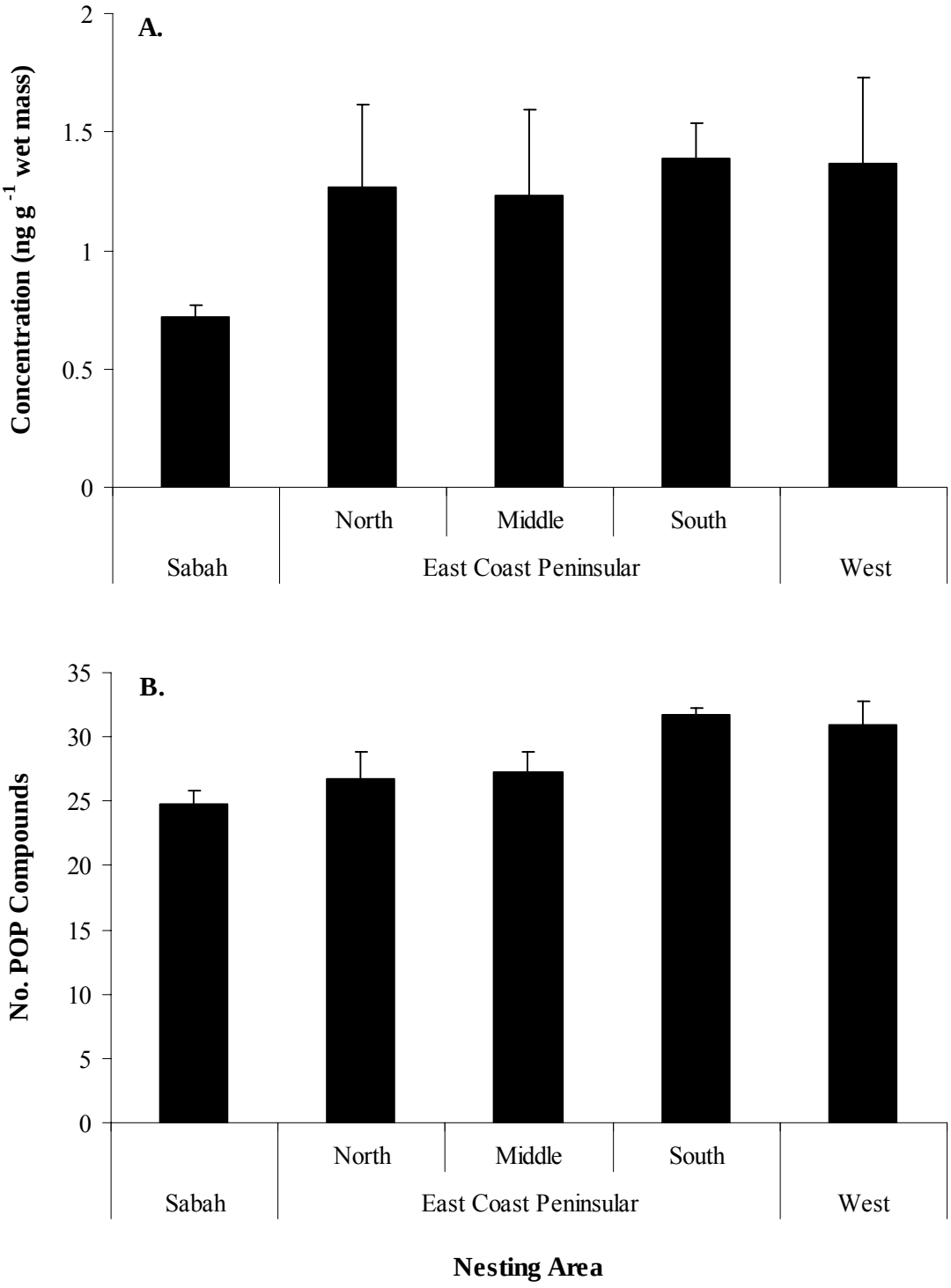

Figure 7.11. Mean (+ SE) ΣPOP concentration (A) and number of POPs (B) in *Chelonia mydas* eggs collected from major nesting areas and sold in markets of Peninsular Malaysia.

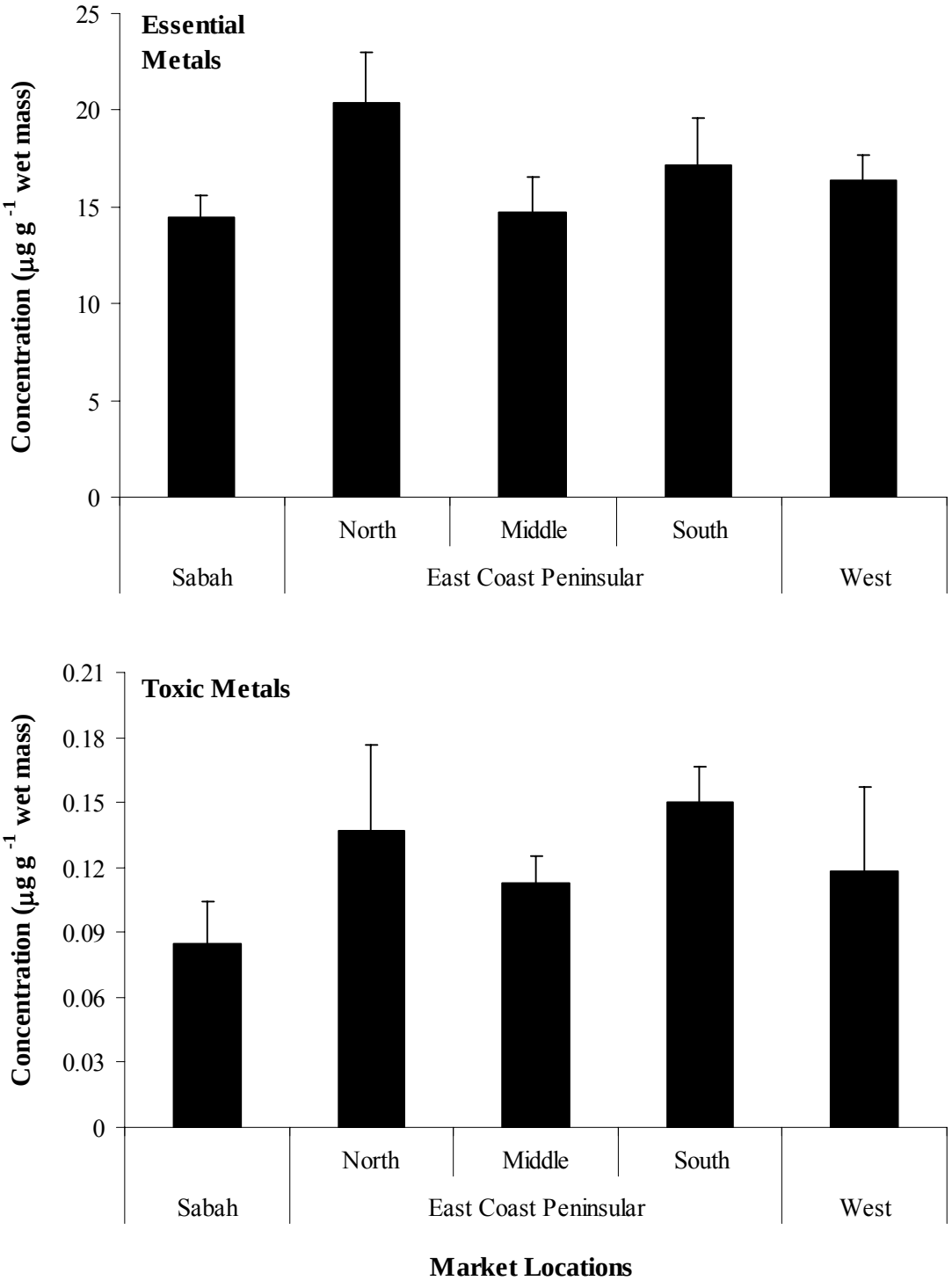

Figure 7.12. Concentration (mean + SE) of Σ essential and Σ toxic metals in *Chelonia mydas eggs* collected from major nesting areas and sold in markets in Peninsular Malaysia for human consumption.

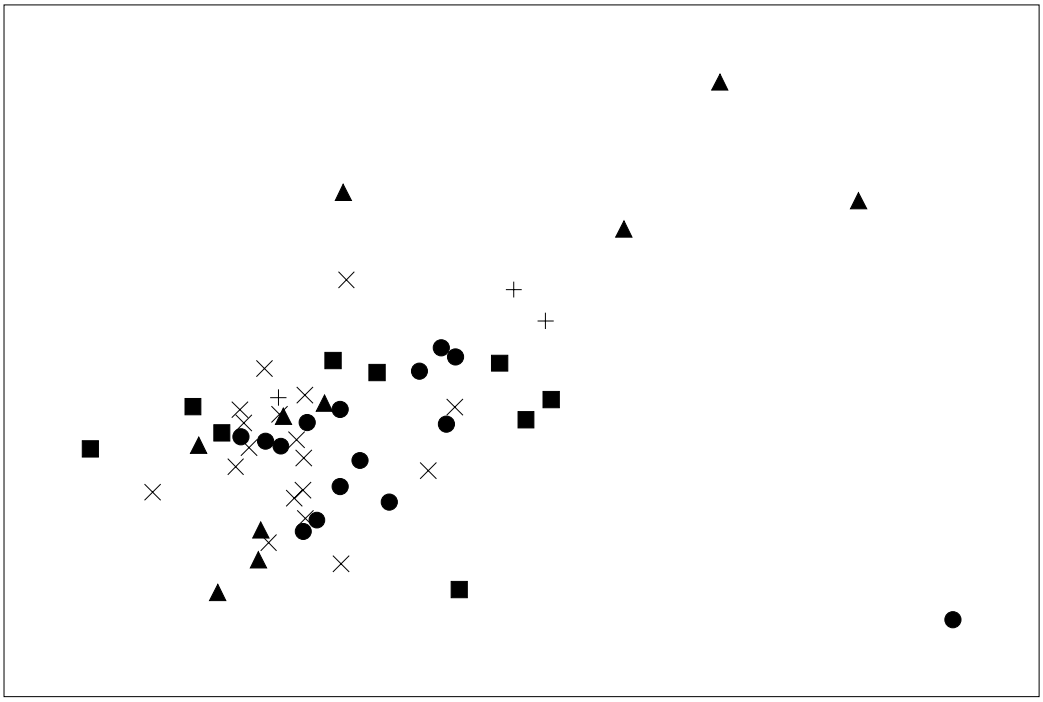

Figure 7.13. *n*MDS plot of egg POP concentrations for *Chelonia mydas* egg collected from markets in Peninsular Malaysia. Eggs collected from: Sabah (3) and the west (2), northeast (%), east-central (#) and southeast (!) coasts of Peninsular Malaysia.

Screening risk assessments for green turtle eggs

The worst and best-case hazard quotients were calculated for a number of the toxic metals and POP compounds reported in the eggs of this study (Table 7.5). The worst-case hazard quotients ranged from < 0.01 to 0.51 for POPs and from 0.1 to 19.5 for heavy metals (Table 7.5).

Table 7.5. The best and worst case hazard quotients (HQs) for metals and POPs identified in *Chelonia mydas* eggs from markets in Peninsular Malaysia.

Compound	MEC_{max}	MEC_{min}	$PNEC_{min}$	$PNEC_{max}$	HQ_{worst}	HQ_{best}
POPs (ng g^{-1})						
DDTs[a]	0.702	0	543	543	< 0.01	0
Dieldrin	2.02	0.008	4	4	0.51	< 0.01
Tri-CB[b]	0.512	0	26	26	< 0.01	0
Tetra-CB[c]	2.00	0.060	26	26	< 0.01	< 0.01
ΣPCBs[d]	3.69	0.147	26	26	0.14	< 0.01
Metals (μg g^{-1})						
Lead	0.124	0	1	1	0.1	0
Selenium	0.836	0.049	0.34	6	2.5	< 0.01
Cadmium	0.029	0	0.0014	0.013	0.2	0
Arsenic	0.351	0	0.018	0.018	19.5	0

a, sum of DDTs used for MECs but value for PNEC derived from effects of *p,p'*-DDE
b, the sum of tri-chlorinated biphenyls was used for the MECs but the value for PNEC derived from effects of a single tri-chlorinated biphenyl
c, the sum of tetra-chlorinated biphenyls was used for the MECs but the value for PNEC derived from effects of a single tetra-chlorinated biphenyl
d, the sum of all polychlorinated biphenyls (PCBs) was used for the MECs but the value for PNEC derived from effects of a single tetra-chlorinated biphenyl

Human health risk assessments

The maximum percent of ADI in one *C. mydas* egg ranged from < 0.1 to 30,247 % for POP compounds and from 0.37 to 12.2 % for metals (Table 7.6).

Table 7.6. The maximum percent of acceptable daily intake (ADI) in one *Chelonia mydas* egg for the major POP and metal compounds reported in eggs from Peninsular Malaysia.

Compound	ADI[a] ($\mu g\ kg^{-1}\ day^{-1}$)	Egg concentration ($ng\ g^{-1}$ wet mass)	Maximum percent of ADI in one egg[b]
POPs			
PBDEs	100	<LOD - 0.353	< 0.1
DDT	20	<LOD - 0.702	< 0.1
Endosulfan	6	0.104 - 0.271	< 0.1
PCBs	1	0.147 - 3.69	0.2
HCH	0.3	0.013 - 0.231	< 0.1
Dieldrin	0.1	0.008 - 2.02	1.1
Mirex	0.07	<LOD - 0.027	< 0.1
Chlordane	0.05	0.025 - 0.514	0.6
c-PCBs[d]	2×10^{-6}	0.014 - 1.29	30,247
Metals			
Zinc	300-1000	1,330 - 39,480	9.1[c]
Copper	50-500	56 - 1,070	1.5[c]
Selenium	12.5	49 - 836	4.6
Lead	3.6	<LOD - 124	2.4
Arsenic	2	<LOD - 351	12.2
Cadmium	1	<LOD - 29	2.0

a, acceptable daily intake (ADI) determined by the joint FAO/WHO expert committee on food additives (JECFA) and the International Programme on Chemical Safety (FAO/WHO 2007; WHO 2008)
b, maximum percent of ADI in one egg was calculated on a 65 kg person consuming a single 35 g *C. mydas* egg
c, calculated from most conservative ADI and maximum concentration of zinc reported
d, coplanar PCBs

7.4 Discussion

Egg availability in markets

The limited availability of *Chelonia mydas* eggs for human consumption is indicative of current sea turtle conservation efforts in Peninsular Malaysia. Egg sale was confined entirely to towns on the east coast and occurred in only nine of the 33 markets surveyed. The localised availability of eggs on the east coast seemed to be more related to culture than limitation in supply. *Chelonia mydas* nesting is almost entirely restricted to the coasts and islands of the east coast of Peninsular Malaysia. People in these areas would therefore have access to the freshest product. However, the apparent long distance transportation of eggs from Sabah and Perak to east coast

markets indicated that supply could meet the demand in other areas of the country if the demand existed. When surveying the west coast between Kuala Lumpur and Johor Bahru, the request for *C. mydas* eggs often invoked reactions indicating sea turtle egg consumption was not common in these areas. This was supported by conversations with west coast residents and Department of Fisheries staff who indicated that consumption of sea turtle eggs was predominantly a cultural practice of east coast residents.

The long distance transportation of eggs to east coast markets also indicated that the demand for sea turtle eggs might not currently be met locally in this area due to the current level of egg protection. Between 1996 and 2006, estimations of *C. mydas* egg protection in Peninsular Malaysia increased from 13% to 94%. This effectively decreased the number of eggs available for collection and consumption per year from > 170,000 to < 15,000 (Department of Fisheries Malaysia, unpubl. data). This is a drastic decline in supply that may need to be supplemented to meet sustained demand over this period. However, the importation of *C. mydas* eggs from areas like Sabah and Perak to compensate for the decline in local supply has ethical and legal implications that sea turtle managers need to consider. Conservation of *C. mydas* in the Southeast Asian region would be compromised if the protection of eggs in one area increases the collection of eggs from other areas. This would particularly be a concern if collecting pressure increased in relatively less disturbed, and more difficult to patrol, nesting habitats. Furthermore, the transport of *C. mydas* eggs from Sabah to Peninsular Malaysia is illegal under the Convention on the Conservation of Migratory Species of Wild Animals (Bonn, Germany, June 23 1979) and Sabah state law (*The Wildlife Conservation Enactment (No. 6),* 1997). Immediate conservation efforts must therefore increase patrolling and monitoring of egg transport between Borneo and Peninsular Malaysia. However, long-term solutions lie in reducing the demand for the consumption of sea turtle eggs in the region, perhaps by introducing a moratorium on egg sale, at least until stocks recover.

Egg contamination and conservation

Studies on the concentrations of POPs and metals in sea turtle eggs are scarce. There are a limited number of studies with low sample sizes on PCBs and organochlorine pesticides (chlordanes, dieldrin and DDTs) in *C. mydas* and *C. caretta* eggs (Clark

and Krynitsky 1980; Podreka et al. 1998; Godley et al. 1999; McKenzie et al. 1999; Alam and Brim 2000; Coufal 2002; Morrisey 2003; Alava et al. 2006; Lam et al. 2006). Polybrominated diphenyl ethers (PBDEs) have not been reported in any sea turtle eggs to date. Similarly, arsenic has only once previously been reported in sea turtle eggs and other metals have been reported in a limited number of studies on *C. mydas* and *C. caretta* eggs, and *D. coriacea* eggshells (Hillstead et al. 1974; Stoneburner et al. 1980; Sakai et al. 1995; Vazquez et al. 1997; Godley et al. 1999; Lam et al. 2006). Furthermore, the analysis in previous studies has been limited to a small number of compounds of interest. The analysis of eight metals and 125 POP compounds in 55 eggs from markets all over Peninsular Malaysia therefore provides important information for *C. mydas* egg contamination in the Southeast Asian region. This information can also provide a useful reference for other sea turtle populations worldwide.

The concentrations of POPs and metals observed in *C. mydas* eggs of this study varied considerably from other studies on sea turtle eggs. The PCB concentrations in the present study were approximately 10 times higher than eggs from a 1974 study on the Ascension Island *C. mydas* population (Thompson et al. 1974), and similar to more recent studies on *C. caretta* eggs from Florida, USA (Alam and Brim 2000; Alava et al. 2006). In contrast, concentrations of DDTs in this study were more than 10 times lower than *C. mydas* eggs from Florida, Ascension Island, Heron Island and Cyprus (Thompson et al. 1974; Clark and Krynitsky 1980; Podreka et al. 1998; McKenzie et al. 1999). All metal concentrations were considerably lower than those observed in *C. caretta* eggs. This would be expected due to the carnivorous diet and higher trophic level of *C. caretta*. In comparison to previous studies on *C. mydas* eggs in Peninsular Malaysia, essential metal concentrations were similar (Morrisey 2003). However, toxic metal and POP concentrations were significantly higher in the present study (Coufal 2002; Morrisey 2003).

There was no significant difference in the ΣPOP or Σ metal concentrations, or the total number of POP compounds between eggs from the five major nesting areas. Furthermore, there was a large amount of variation in egg concentrations within each nesting area. Such variation was expected as a nesting population generally consists of turtles from different foraging areas (Balazs 1994; Liew et al. 1995; Cheng 2000;

Godley et al. 2002; Seminoff et al. 2008). As chemical contamination occurs nearly exclusively through feeding (Langston and Spence 1995), individuals from different foraging areas would have chemically distinct egg contamination profiles (see Chapters 5 and 6). However, it must also be considered in the present study that eggs were allocated to different nesting areas based on information from the market vendors selling the eggs. Vendors may have incorrectly reported nesting areas to promote fresher product and/or conceal illegal importation from areas outside Peninsular Malaysia. This may have further contributed to the variation in chemical contamination in eggs allocated to different nesting areas.

Closer examination of the POP data using ANOSIM revealed that eggs from different nesting areas had significantly different contamination profiles. However, the grouping of the eggs from the same nesting areas on the *n*MDS plot was not as well defined as in previous chapters (Chapters 5 and 6). This was expected as the Ma'Daerah nesting area studied in Chapter 6 illustrated widespread variation in contamination profiles among the clutches of different nesting females. Variation in chemical profiles in the present study may also be further emphasised by incorrect reporting of nesting areas by vendors and/or the combination of different nesting areas into a more regional statistical unit.

Despite the expected variations among clutches within the assigned nesting areas in the present study, the chemical profiles of the different nesting areas showed some separation in chemical profiles. The eggs from Sabah were best separated from the eggs from the west coast of Peninsular Malaysia. These were the two most geographically separated nesting areas and it is unlikely that nesting turtles from these areas would share common foraging areas. The eggs from Sabah were also significantly separated from the eggs from the east coast Peninsular Malaysia, except for the east-central area. However, there was considerable overlap in the contamination profiles between the east coast sites ($R < 0.36$). This may indicate that there are some common foraging areas used by *C. mydas* nesting in Sabah and Peninsular Malaysia. Eggs from nesting areas on the east and west coasts of Peninsular Malaysia had similar contamination profiles. This was expected due to the relatively close proximity of these nesting areas and the high possibility that nesting females from these sites would share common foraging areas. However, as mentioned

in previous chapters, care must be taken in associating different chemical profiles with different foraging areas. There are many other factors that may lead to variation in chemical contamination profiles that may account for these differences. Similarly, turtles foraging in completely different areas may have very similar contamination profiles.

The effects of egg contamination on sea turtles are not well understood due to the ethical and logistical constraints of toxicological studies on these endangered vertebrates. However, parallels can be drawn from the effects of POPs and metals in other species. Although not well documented in reptiles, the major effects of metals occur at a cellular level. Toxic metals can generate free radicals and disrupt cell functioning by binding to the functional sites of proteins. Many of the toxic metals alter the functioning of DNA polymerase, the enzyme responsible for the repair of damaged DNA (Sanders et al. 1996). The damage to cellular proteins activates the rapid synthesis of stress proteins that repair and protect the targeted proteins (Sanders et al. 1996). There is also a metal specific stress response. This involves the production of metallothioneins (MTs), lysosomes, mineralised and organic-based concretions that bind to the metals and limit their ability to alter the function of proteins. However, despite the cellular response to metal toxicity, protection from toxic metals is not complete. Many metals have been associated with cancer as well as neurological, cardiovascular, respiratory, gastrointestinal, endocrinological, developmental, renal, hepatic, cognitive, reproductive and immunological diseases (Chang 1996; Baird and Cann 2005).

Persistent organic pollutants (POPs) can act as endocrine disrupting chemicals, altering the development and function of the endocrine system (Colborn et al. 1993; Guillette et al. 1996; Guillette and Gunderson 2001; McKinlay et al. 2008). Following a significant chemical spill in Lake Apopka, Florida, U.S.A., high levels of *p,p'*-DDE, *p,p'*-DDD, dieldrin and *cis*-chlordane were found in American alligator (*Alligator mississippiensis*) eggs. Male juveniles of this population had poorly organised testes. Ovaries from 6-month-old females had polyovular follicles, multinucleated oocytes (normal ovarian follicle contains a single oocyte that has a single nucleus) and abnormal plasma sex steroid hormone concentrations (Guillette and Crain 1996). Similarly, PCBs in the eggs of the freshwater snapping turtle (*Chelydra serpentina*)

have been linked to embryonic mortality and reproductive deformities (Helwig and Hora 1983; Olafsson et al. 1983; Bryan et al. 1987).

Studies using the topical administration of POPs have also found a number of compounds that cause sex reversal in the developing embryos of oviparous reptiles that exhibit temperature dependent sex determination. The application of polychlorinated biphenyl (PCB) congeners, 2',4',6'-trichloro-4-biphenyl or 2',3',4',5'-tetrachloro-4-biphenyl at a concentration of ~ 9 μg g^{-1} produced significant sex reversal in *Trachemys scripta* (Bergeron et al. 1994). Based on calculations using a mean egg mass of 11.4 g and estimation of 30% penetration, this represented sex reversal at egg concentrations of ~ 2.6 μg g^{-1}. Furthermore, there is evidence of synergistic effects of PCBs as these two PCB congeners applied together produced significant sex reversal at an egg concentration of ~ 0.26 μg g^{-1} (Bergeron et al. 1994). The topical administration of a range of pesticides has also been found to produce sex reversal in *T. scripta* at concentrations between 0.16 and 18 μM (Willingham and Crews 1999). Based on a mean egg mass of 10 g and 30% penetration into the eggs, sex reversal was observed in *T. scripta* at egg concentrations of 19, 12, 23, 950 and 15 ng g^{-1} of *trans*-nonachlor, *cis*-nonachlor, arochlor 1242 (PCB mixture), *p,p'*-DDE and chlordane, respectively. In the only study of this kind on sea turtles, developing *C. mydas* embryos exposed to *p,p'*-DDE at concentrations of up to 2.66 μg μL^{-1} resulted in internal egg concentrations of 543 ng g^{-1}. However, no sex reversal of *C. mydas* hatchlings was observed at these concentrations (Podreka et al. 1998).

The screening risk assessments indicated that the POP concentrations observed in the *C. mydas* eggs collected from markets in Peninsular Malaysia were unlikely to cause sex reversal in the developing embryos. Dieldrin posed the highest risk to sex reversal in these *C. mydas* eggs, although a worst-case HQ of 0.51 indicated that this risk was minimal. However, the metal concentrations observed in the *C. mydas* eggs collected from the markets in Peninsular Malaysia indicated a higher risk of disruptions to embryonic development. In particular, the arsenic concentrations (worst-case HQ = 19.5) indicated a relatively high risk of embryonic mortality and reduced hatch success in these *C. mydas* eggs.

There are many uncertainties in the calculation of PNECs for the screening risk assessment due to the low number of toxicological studies specific to *C. mydas* eggs. Furthermore, the present study calculated PNECs from dosing studies on reptiles and birds. In doing so, the amount of chemical that penetrated the eggs was estimated from the amount admninistered to the outside of the eggshells, as it was generally not calculated in these studies. A linear relationship between dose and penetration resulting in approximately 30% of the chemical applied penetrating the eggshell was assumed. This was based on the results of a study on the topical administration of DDE to *C. mydas* eggs (Podreka et al. 1998). However, a dosing pilot experiment in the present study indicated that the penetration of DDE in *C. mydas* eggs was exponentially related to the dose applied (see Appendix C). Further investigation into the dose response of eggs to topical administration of toxic chemicals is therefore required to improve the validity of using dosing studies to calculate the HQs used in screening risk assessments.

The HQs were also calculated on the toxicological effects of single compounds acting alone. The present study is the first of its kind to analyse and identify such a large number of POP and metal compounds in *C. mydas* eggs. The large number of POP compounds observed in the eggs of this study (mean: 27.0 ± 0.8, range: 17-45) could therefore be important in terms of combined effects. The combined effect of PCBs has been reported in *T. scripta* (Bergeron et al. 1994). The combination of a tri-chlorinated biphenyl with a tetra-chlorinated biphenyl produced sex reversal at the same concentration that produced no effect when the PCB compounds were administered individually. The effect of multiple PCB compounds on sex reversal may therefore be more important than the HQs of the present study have indicated.

Despite the low risk of hatchling sex reversal at current egg POP concentrations in Peninsular Malaysia, egg contamination should be monitored over time to ensure that this risk does not increase. If the sex ratio becomes too female biased, reproduction in the population may be compromised. Furthermore, future study into the effects of POPs on the development of the reproductive system of *C. mydas* is warranted. There may be more subtle effects of POPs that do not result in complete sex reversal, but development of dysfunctional reproductive organs and other abnormalities. This should also be the focus of future chemical contamination research in sea turtles.

The effects of POPs on embryonic development are considered organisational, where the morphology of tissues are permanently modified (Guillette et al. 1995). Organisational effects are dependent on exposure during critical periods of sensitivity, bioaccumulation versus degradation, and secretion and the biological availability of the chemicals (Guillette et al. 1995). The POP concentrations of *C. mydas* eggs also reflect the contamination of nesting females (see Chapter 6). As *C. mydas* are seasonal breeding animals, they may also be affected by organisational effects of POPs (Guillette et al. 1995). For example, the development of the follicles, ovaries and oviducts in nesting female *C. mydas* may be affected during the breeding season. Furthermore, the effects of POPs on adult *C. mydas* can also be activational, where the function of a normally organised tissue is altered (Guillette et al. 1995). The POP concentrations observed in *C. mydas* eggs in the present study may therefore also have health implications for the nesting females of these populations. Furthermore, the large number of compounds observed in the eggs could have combined harmful effects on the adult females of these *C. mydas* populations.

The relatively high risk of disruption to hatchling development associated with the reported *C. mydas* egg arsenic concentrations was also interesting in context of the high occurrence of scute abnormalities observed in *C. mydas* hatchlings of Peninsular Malaysia (van de Merwe 2002; Morrisey 2003; Schauble et al. 2003; Ibrahim et al. 2004; Schauble et al. 2004). Abnormalities in the morphology of hatchlings can indicate disruption to development, although some variation in development due to genetic plasticity is normal (Miller 1985). The frequency of abnormal scute counts is generally < 1% in marine turtle hatchlings (0.6% of 5666 eggs, McGehee 1979; < 1% of 2811 eggs, Blanck and Sawyer 1981; 0.17% of 90,000 eggs, Miller 1982). However, in the hatcheries of Peninsular Malaysia, up to 89% of *C. mydas* hatchlings in a single nest had abnormal scute counts (van de Merwe 2002). Furthermore, in a study on hatcheries and i*n situ* sites in Peninsular Malaysia, > 90% of *C. mydas* nests had at least one hatchling with scute abnormalities (Morrisey 2003).

Morphological abnormalities observed in hatchlings of oviparous reptiles can occur due to a number of factors, including maternal and/or paternal genetics, chance mutations of genes or as a result of environmentally induced developmental problems (Janzen 1993; Packard and Packard 2001). Although there were no correlations

between egg POP concentration and hatchling scute abnormalities observed in Chapter 6, the range of egg POP concentrations was quite small. It would therefore be of interest to further investigate the role of chemical contaminants in the development of abnormalities in *C. mydas* hatchlings, particularly at higher POP concentrations. The combined effects of multiple compounds on hatchling development would also be particularly relevant in relation to the large numbers of compounds reported in the *C. mydas* eggs in the present study. The effects of chemical compounds on sea turtle development could be tested with chemical dosing experiments. Studies using different compounds and combinations of compounds could investigate the effects of individual compounds, and any synergistic effects of multiple compounds on hatchling development and sexual differentiation. Alternatively, retrospective analysis of abnormal and intersex hatchlings collected and stored for previous studies could be used to investigate correlations with POP contamination and developmental abnormalities in sea turtles.

Egg contamination and human health

The POP and metal concentrations observed in *C. mydas* eggs sold in markets also present public health concerns for the people of Malaysia and the surrounding region. Sea turtle eggs are a traditional food source in many coastal areas of Malaysia and other countries in Southeast Asia. They provide a good source of protein and are often perceived to have medicinal properties. Furthermore, sea turtle eggs provide an important source of income to many communities (Ibrahim 1994). However, the chemicals found in the *C. mydas* eggs of this study are known to have a number of harmful effects to humans (see Chapter 1). In particular, PCBs and *p,p'*-DDE in human males have been associated with increased sperm abnormalities and negative effects on sperm DNA, motility, ability to penetrate oocytes and counts (Guo et al. 2000; Hauser et al. 2003; Hsu et al. 2003; Rignell-Hydbom et al. 2004). There are therefore potential human health hazards associated with consuming *C. mydas* eggs from markets in Peninsular Malaysia.

The most alarming statistic from the assessment of the human health risk associated with consuming *C. mydas* eggs in Peninsular Malaysia was related to coplanar PCBs. According to this risk assessment, the consumption of a single *C. mydas* egg can result in the intake of > 300 times the accepted daily intake (ADI) of coplanar PCBs,

as determined by the joint FAO/WHO expert committee on food additives (FAO/WHO 2007). Even though this represents the maximum risk calculated, coplanar PCBs were detected in all 55 *C. mydas* eggs analysed. Even the consumption of an egg with the lowest concentrations of coplanar PCBs reported would result in the intake of more than three times the ADI. Coplanar PCBs are considered particularly toxic due to their structure and are often considered in the same context as dioxins and furans, some of the most toxic chemicals known (McKinlay et al. 2008). The reported concentrations of coplanar PCBs therefore create serious health risks to the human population in Peninsular Malaysia consuming *C. mydas* eggs. Furthermore, the high risks to human health associated with consumption of coplanar PCBs indicate that the low HQs calculated for *C. mydas* development may have been underestimated. The effects of coplanar PCBs on the development of *C. mydas* embryos should therefore be investigated in future studies.

The other POP and metal compounds observed in the *C. mydas* eggs in the present study pose considerably less health risk to humans. Arsenic was the next highest ranked chemical in terms of risks to human health. The consumption of a single egg *C. mydas* egg in Peninsular Malaysia would result in the intake of 9.5% of the ADI of arsenic. Therefore, > 10 *C. mydas* eggs would need to be consumed daily for the ADI of arsenic to be exceeded. The sea turtle egg consumption patterns of people in Malaysia were not included in this study, but it is unlikely that humans would consume > 10 *C. mydas* eggs on a daily basis. The financial cost of this alone would be equivalent to 10 restaurant meals. Furthermore, the availability of eggs in the markets would unlikely be able to support this level of consumption in the coastal communities over the sea turtle nesting season.

Care must be taken in interpreting the human health risk assessments in the present study. Firstly, the risk assessments were calculated on the consumption of *C. mydas* eggs only. It is possible that other foods consumed by the people of Peninsular Malaysia are also contaminated with POPs and heavy metals. If these chemicals were also being consumed through other dietary intake, the contribution from *C. mydas* eggs would become more significant. This is particularly likely in coastal communities of Southeast Asia due to high levels of contaminants reported in seafood (Agusa et al. 2007). A more comprehensive total diet study (TDS) would therefore

need to be conducted to include all foods consumed by the communities in Peninsular Malaysia that eat *C. mydas* eggs. Secondly, the ADIs are generally calculated on an individual compound basis (FAO/WHO 2007). The effects of consuming foods with multiple chemical compounds are not well understood. Despite the fact that the coplanar PCB concentrations were already a high risk, the large number of chemicals found in the *C. mydas* eggs may further increase the risks of consuming these eggs. Furthermore, many of the compounds detected in *C. mydas* eggs are persistent and will bioaccumulate in humans, indicating that the threats to human health may increase with prolonged egg consumption.

The mean concentrations of POPs and metals and the mean number of POPs in each egg were highly variable within each market. This was expected as eggs in each market had often been collected from a number of different nesting areas that had already been shown to have variable egg contamination. The large amount of variability within markets may also have contributed to the similarity observed in essential metal and POP concentrations and the number of POP compounds per egg. Only the total concentration of toxic metals differed significantly between markets. Eggs from Kuala Terengganu and Mersing had significantly higher concentrations of toxic metals than markets in Kota Bharu, Penarik, Dungun and Kuantan. This information could be interpreted as eggs from Kota Bharu, Penarik, Dungun and Kuantan markets are safer to eat. However, care must be taken in making this assumption. Although the toxic metal concentrations were significantly lower in some markets, the variation was still high. Many eggs sold in these markets would therefore still have relatively high toxic metal concentrations.

The amount of variation in chemical contamination observed within markets and nesting areas indicates that there are no places in Peninsular Malaysia to obtain *C. mydas* eggs that are relatively safe to eat. The chemical contamination of *C. mydas* eggs in markets of Peninsular Malaysia is therefore a lottery in terms of the concentrations and number of compounds. Considering the potential consequences of eating food with such a large number of toxic compounds, consumption of *C. mydas* eggs from markets in Peninsular should be minimised or avoided completely.

Incidentally, the chemical contamination reported in the *C. mydas* eggs in Peninsular Malaysia may assist conservation efforts in the region. The health risk of consuming eggs with high numbers of chemical compounds could potentially reduce the impact of egg collection on sea turtle populations in Southeast Asia. One of the biggest challenges to sea turtle conservation agencies in this region is to reduce the demand for consumption of sea turtle eggs. This could be achieved by awareness campaigns highlighting the health consequences of consuming sea turtle eggs with such a large number of toxic compounds. If effective, this would presumably reduce the collection of sea turtle eggs for human consumption and contribute significantly to sea turtle conservation efforts in the region.

7.5 Conclusions

The collection of *Chelonia mydas* eggs for human consumption has been the major factor in the severe decline of this species in Peninsular Malaysia. However, the chemical contamination of the eggs observed in this study may also have conservation consequences. The hazard quotients for POPs and heavy metals indicated that arsenic concentrations presented a relatively high risk of reducing hatching success. The remaining POP and metal compounds presented minimal risk to the developing embryos. However, the egg concentrations also represent contamination of the nesting female *C. mydas* (see Chapter 6). The chemicals observed in the eggs in the present study may therefore have effects on the functioning of nesting female *C. mydas*. Furthermore, there were a large number of chemical compounds detected in each egg. The potential combined effects of this large number of compounds are unknown but potentially harmful to the development of *C. mydas* embryos and nesting females.

The chemical contamination of *C. mydas* eggs in Peninsular Malaysia also has potential effects on human health. The concentrations of coplanar PCBs observed in the *C. mydas* eggs present a serious human health concern. Consumption of a single egg represents an intake of 3-300 times the accepted daily intake of these compounds. Coplanar PCBs are particularly harmful due to their structure and are generally considered in the same context as dioxins and furans, some of the most dangerous chemicals known. Furthermore, the high risk to human health associated with the coplanar PCB concentrations reported indicates that the low risks to *C. mydas* embryonic development may have been underestimated. The concentrations of the

other POP and metal compounds pose a less serious risk. However, the potential effects of the large number of compounds reported in the eggs of this study are unknown and could potentially pose further health risks to humans. Furthermore, the variation in egg contamination indicated that there were no safe markets to purchase eggs from for human consumption. These data suggest that a total diet study on villagers who rely on *C. mydas* eggs as a seasonal food source should be conducted. If warranted, a public health advisory campaign may need to be conducted to reduce cumulative effects of human exposure to egg contamination. Incidentally, recommended reduction or outright ban of human turtle egg consumption would have a positive impact on efforts to conserve turtle populations in the region.

Chapter 8 - General discussion

Prior to the beginning of the present study, research on chemical contamination in sea turtles had generally been opportunistic in nature, limited to sampling stranded and deceased animals. There had been no systematic approach to investigating the important aspects of accumulation and transfer of toxic chemicals in sea turtles. Furthermore, methods for analysing pollutants in sea turtles were limited by the relatively high limits of detection and the small numbers of compounds detectable. As a result, the threat of chemical contamination in sea turtles was generally not well understood. Therefore, the objectives of the present study were to further develop methods and approaches for analysing and monitoring key chemical pollutants in sea turtles and to use these methods to investigate a number of important aspects of the accumulation and transfer of contaminants in *C. mydas* at different lifecycle stages. This chapter consolidates the information from all previous chapters and highlights the contributions of this study to sea turtle contaminant research. This chapter also discusses the results of previous chapters in the context of the overall threat of chemical contamination in sea turtles.

Methodological advances in sea turtle research

The present study has made a number of methodological advances that have improved the capacity of chemical contamination research in sea turtles. The development of the gas chromatography with coupled mass spectrometry (GC-MS/MS) method that could report 125 (23 OCPs, 83 PCBs and 19 PBDEs) persistent organic pollutants (POPs) at a limit of detection (LOD) of 5-35 pg g^{-1} was paramount to meeting the objectives of this thesis, and indeed future studies on chemical contamination in sea turtles. This method was an improvement on previous techniques using gas chromatography with electron capture detection (GC-ECD). The GC-ECD methods were limited by their relatively high LODs (~ 1 ng g^{-1}) and small number of compounds detectable. Lowering the LOD (to < 35 pg g^{-1}) was particularly important for research on *C. mydas* as this herbivorous species generally has lower contaminant concentrations than carnivorous sea turtle species and other marine vertebrates. The GC-MS/MS method developed in the present study was also an improvement on methods using a combination of GC-ECD and gas chromatography with mass

spectrometry (GC-MS). These methods could detect large numbers of POP compounds to a limit of detection of ~ 5 pg g^{-1}. However, they required multiple injections of a single sample into a number of gas chromatographs with various columns and detectors. It is therefore only possible to replicate these methods in well-equipped and highly-funded laboratories. The single injection of the GC-MS/MS method developed in the present study therefore improves the time and cost efficiency of POP analysis in sea turtles, and has made this research available to a wider range of laboratories and research groups.

The contribution of the GC-MS/MS method to sea turtle contaminant research was further supported by its accuracy and precision. Recoveries of the internal standards were generally 60-95%, with a gradual decrease in recovery with increasing chlorination of the PCBs. However, the use of closely related mass labelled internal standards in the calculations of the POP concentrations effectively eliminated the implications of reduced recovery. The accuracy of the method was validated against blood, egg and tissue standard reference materials (SRMs) from the National Institute of Standards and Technology (NIST), and reported to within 70% and 60% of the certified and reference values, respectively. Furthermore, there was minimal intra-batch variability, with coefficients of variation < 20% and generally < 5%.

The GC-MS/MS method developed in the present study also has implications for the analysis of POPs in other matrices. Due to the consistency in the accuracy and precision of the method in *C. mydas* blood, tissue and egg, adaptations to other environmental matrices such as sediment, water and vegetation are expected to be minimal. This method has therefore not only advanced the capabilities of POP analysis in sea turtles, but may also increase the scope for environmental contamination research in general.

The investigation into the correlations in contaminant concentrations between blood, carapace and internal tissue samples of *C. mydas* in the present study has also advanced the capabilities of contaminant monitoring in live sea turtle populations. Due to ethical constraints of taking tissue from live animals, the majority of previous studies on chemical contamination in sea turtles have been on tissues of dead and stranded animals. To better understand the contamination of live sea turtles, there was

a need to establish non-lethal methods that could give an accurate and reliable estimation of the chemicals in the internal tissues of *C. mydas*. The significant blood-tissue and carapace-tissue correlations observed in the present study provided the first known evidence on how non-lethal blood and carapace samples represent the internal metal and POP concentrations of sea turtles. However, it is important to note that the *C. mydas* sampled in this component of the present study were recovering from various ailments and injuries. Factors such as body condition, change in diet during rehabilitation and the stage of recovery may have influenced these results and therefore need to be considered when relating these findings to wild populations. However, regardless of these rehabilitation specific factors, the blood-tissue and carapace-tissue correlations indicated that blood and carapace samples are good non-lethal methods for predicting the internal tissue contamination of *C. mydas*. These methods thus provide a reliable but relatively non-invasive tool for assessing and managing the pollution challenges faced by sea turtles.

The final contribution of the present study to sea turtle contaminant research methodologies was the preliminary investigation into the response of *C. mydas* eggs to the topical administration of DDE in ethanol (see Appendix C). This technique has been widely used for investigating sex reversal in temperature dependent sex determined reptiles and is potentially a useful technique for investigating the effects of chemicals on the development of sea turtle embryos. However, prior to wholesale use of this technique in sea turtle studies, investigation into the response of sea turtle eggs to application of chemicals was warranted. Although only a pilot study, the exponential increase in egg concentration in relation to increased exposure to DDE contradicted the straight-line relationship assumed in previous studies. The results of this pilot study also indicated the potential problem of cross-contamination of DDE to adjacent eggs and an influence of DDE on the rate of development and morphological abnormalities in the embryos. These types of incubation experiments must therefore be carefully designed to ensure that these unintended factors do not influence the targeted responses (e.g. sex reversal).

Contamination of Chelonia mydas *in important lifecycle stages*

The methods developed during the early phases of the present study were used to investigate chemical contamination in the major lifecycle stages of *C. mydas*. The

present study was the first to follow the movement of nesting female *C. mydas* to foraging grounds and link this to the contamination of their eggs. The use of satellite telemetry to identify the habitats used for nesting, migration and foraging was important information for the management of the Ma'Daerah *C. mydas* population. The identification of inter-breeding, migration and foraging habitats indicated the need for extended coastal protection in Peninsular Malaysia, as well as highlighting the other countries in Southeast Asia where collaborative conservation efforts must be focussed. However, it was the incorporation of the chemical contamination of the eggs of these nesting females that made the present study unique.

Marine animals accumulate chemicals nearly exclusively through feeding and a nesting population of *C. mydas* is generally composed of individuals from a range of different foraging areas (Balazs 1994; Liew et al. 1995; Cheng 2000; Godley et al. 2002; Seminoff et al. 2008; present study). The location and variation of contamination of foraging areas is therefore important in understanding the threat of chemical contamination in a nesting *C. mydas* population. Multivariate analysis of similarity in the contaminant profiles indicated that the eggs from the three *C. mydas* that migrated to separate foraging areas had significantly different POP and metal concentration profiles. Furthermore, the significant correlations between eggs and blood reported in another component of this study indicated that the egg contamination profiles represented those of nesting females. It was therefore proposed that the chemical profiles of *C. mydas* eggs could be used to investigate the variation in foraging areas of a nesting population. The multivariate analysis of metals and POPs may have advantages over nutrient stable isotope analyses that have traditionally been used for investigating trophic relationships and movement of marine animals. While stable isotope analyses separate nutrient sources according to the predominant producer types, the signatures are not temporally constant (due to continual metabolism of nutrients) and provide limited distinction between animals feeding on the same food source in different areas (Peterson and Fry 1987). Metals and POPs are more conservative and may therefore provide a more stable and distinctive separation between foraging areas. However, there are a number of factors, such as age, sex and specific foraging range that could account for variation in chemical profiles between individuals of the same foraging area. These variations between individuals within a foraging area must therefore be further investigated

before the use of chemical profiles to indicate foraging ground variability of a nesting population can be validated.

The potential use of chemical contamination profiles to investigate variation in foraging grounds of a nesting population was further supported by more extensive analysis of the Ma'Daerah *C. mydas* nesting population. The eggs from 11 individual *C. mydas* nesting at Ma'Daerah had chemically distinct POP profiles. Furthermore, closer investigation of *n*MDS plots indicated that these clutches were further arranged in to six distinct groups. This may indicate that the 11 *C. mydas* sampled have come from six different foraging grounds. However, as aforementioned, the variations between individuals within a distinct foraging area need to be further investigated before these conclusions can be made. It is, however, reasonable to expect that variations among individuals from the same foraging area would be less than those among individuals foraging in areas with significantly different pollutant levels.

There is evidence of low variability in chemical contamination within locations in studies on mussels in the Southeast Asian region. The Asia-Pacific Mussel Watch Program has used mussels as bioindicators of tributyltin and organochlorine contamination in the coastal waters of the Asia-Pacific since 1994 (Tanabe et al. 2000). Mussels, being sedentary, give a reliable estimation of chemical contamination in an area and are hence widely used to monitor chemical pollution in coastal environments. A recent review by Monirith et al. (2003) that included data from 103 sites over four years (1994, 1997, 1998 and 2001), indicated that the variation in mussel contamination between sites in Southeast Asia was far greater than the variation within sites. *Chelonia mydas*, although not a sedentary species, tend to forage in specific areas over extended periods (See Table 5.7: Mendonca 1983; Brill et al. 1995; Renaud et al. 1995; Whiting and Miller 1998; Seminoff et al. 2002; Makowski et al. 2006; present study). They are thus able to record contaminant profiles that reflect the environmental levels in the foraging grounds. This supports the proposal that *C. mydas* foraging within different locations will acquire distinct chemical profiles that may be used to determine the foraging variability of a nesting population.

The maternal transfer of POPs to eggs and hatchlings during the nesting season was also investigated in the present study. The lipophilic nature of POPs infers that they may be transferred from nesting female sea turtles to eggs during vitellogenesis and further to hatchlings during development. However, this process had not previously been fully investigated in sea turtles. The present study identified significant correlations in POP concentrations between maternal blood and eggs, indicating that these lipophilic compounds are being transferred to eggs during vitellogenesis. Over time, this has the potential to reduce the chemical contamination of nesting female *C. mydas*, as similar 'off-loading' of lipophilic contaminants (during gestation and lactation) has been documented in marine mammals (Aguilar and Borrell 1994; Lee et al. 1996; Ylitalo et al. 2001). However, it may also compromise adult female health as the mobilisation of lipids and POPs during breeding seasons would remove the POPs from their sequestered state and increases their circulation around the body. As *C. mydas* are generally aphagic during the breeding season (Bjorndal 1985, 1997), stored lipids are required to meet the high energy demands of egg production and migration (Bjorndal 1982; Kwan 1994; Hamann et al. 2002). The *C. mydas* eggs in the present study comprised ~ 9% lipid, indicating that the eggs of ~ 36 g would require ~ 3.2 g of lipid per egg. Nesting female *C. mydas* laying an average of three clutches of ~ 100 eggs each would therefore require the mobilisation of ~ 1 kg of lipid for yolk production alone. Furthermore, elevated plasma triglyceride levels reported in nesting *C. mydas* throughout the nesting season indicate that large amounts of lipids are also mobilised to meet the energy demands of this process (Hamann et al. 2002). The simultaneous mobilisation of lipophilic POP compounds during this lifecycle phase may therefore increase the susceptibility of nesting female *C. mydas* to the effects of these toxic chemicals. Thus, elevated contaminant levels in nesting female *C. mydas* may compromise nesting activities and reduce the reproductive outputs of a population over a breeding season.

The transfer of POPs from nesting female *C. mydas* to eggs may also compromise hatchling development and survival. A significant correlation was observed in POP concentrations between the eggs and hatchling blood, indicating further transfer of these compounds to hatchlings during embryonic development. Furthermore, the significant correlation between increasing egg POP concentrations and decreasing hatchling mass:length ratios provided evidence of the adverse effects of egg

contamination on hatchling development at the reported concentrations. However, there was no evidence of an effect of POPs on other nest and hatchling parameters, such as hatching success, emergence success and hatchling abnormalities. This may be due to the low concentrations and ranges of POPs reported in the eggs investigated. The effects of POPs on hatchling development at these low concentrations may be subtle and only reflected in the changes in mass:length ratios. However, reduced mass:length ratios may indicate a decrease in residual yolk, which would compromise the duration of hatchling offshore dispersal (Miller 1985; van de Merwe et al. 2005). Additionally, it could represent reduced size refuge from near-shore predators (Janzen et al. 2000b, a). The relatively low concentrations of POPs reported in the present study may therefore have conservation implications for this population. Furthermore, the mass:length ratio of hatchlings may be an early indicator of POP contamination in sea turtles. This easily measured hatchling parameter could therefore be used by conservationists and managers to monitor the POP contamination in *C. mydas*, before more elaborate and potentially expensive assessments are made.

The effects of POPs on hatchling mass:length ratios observed at the low concentrations reported in the present study also highlights the importance of investigating the effects of multiple POP compounds. Previous studies on the effects of POPs on the development of oviparous reptiles have been limited to one or two compounds at a time. However, there may be synergistic effects of multiple compounds on hatchling development. Many different compounds at individually low concentrations may therefore have considerable effects on the development of oviparous reptiles. This further emphasises the contribution of the GC-MS/MS method developed in the present study that can report 125 compounds for each sample. Furthermore, with up to 62 individual POP compounds reported in the *C. mydas* eggs of this study, the effects of multiple compounds on sea turtle development should be the focus of future research.

The analysis of POPs in blood samples from two distinct *C. mydas* populations in Southeast Queensland, Australia and the east coast of Peninsular Malaysia also allowed for a direct comparison in *C. mydas* contamination between two geographically separated regions. Interestingly, the overall POP concentrations were similar for the *C. mydas* from these two regions. However, different PCB congeners,

PBDE congeners and organochlorine pesticides were identified in the *C. mydas* from the two sites. The geographical separation of these two regions extends beyond known migration distances of *C. mydas*. It is therefore very unlikely that there is any overlap in the foraging areas of these populations. The different chemical compounds observed in these two *C. mydas* populations therefore likely reflect different usage and accumulation patterns of these chemicals in these regions. The different compounds observed in the different populations also further supports the use of chemical profiles to identify sea turtles from different foraging grounds. Furthermore, if such geographically separated populations are equally affected in terms of overall contamination, it is likely that chemical pollution is a widespread problem for *C. mydas* populations globally. It is therefore important to investigate the contamination of other *C. mydas* populations, even if nesting is occurring in apparently pristine areas.

Implications of egg contamination on conservation and public health

In the final chapter of the present study, Peninsular Malaysia was used as a case study to investigate the conservation implications of chemical contamination of *C. mydas* eggs. The collection of eggs for human consumption in Peninsular Malaysia also provided an opportunity to assess the potential effects of *C. mydas* egg contamination on public health. Availability of *C. mydas* eggs in markets was restricted to the east coast of Peninsular Malaysia. However, the reported nesting areas from where these eggs were collected ranged from a few kilometres from the point of sale to thousands of kilometres away, in Sabah, Borneo Malaysia. This indicated that although protection of *C. mydas* eggs in Peninsular Malaysia is currently > 90%, demand for eggs for human consumption in this region may be met by further away sources. Therefore, in an area like Southeast Asia, where many countries occupy a small geographic range, conservation efforts for protecting *C. mydas* must be considered on a regional scale.

The chemical contamination of the eggs collected from markets in Peninsular Malaysia was also used to assess the risks to this *C. mydas* population. A screening risk assessment (SRA) of the POP concentrations observed in the *C. mydas* eggs collected from the markets in Peninsular Malaysia indicated a relatively low risk of hatchling feminisation of these populations. However, the risk of hatchling mortality

and reduced hatch success at the current arsenic concentrations could be considered relatively high. This is the first study of its kind to produce a contamination risk assessment for sea turtles including such a wide range of heavy metals and POPs. Therefore, due to the large numbers of chemicals reported in the *C. mydas* eggs of the present study, this detailed and comprehensive risk assessment could form a template for assessing the risks to other sea turtle populations. However, the SRAs in the present study were calculated on a single compound basis. The potential combined effects of the large numbers of POP and metal compounds observed in these eggs may further increase the risks to hatchling development in these *C. mydas* nesting populations. Future risk assessments should therefore consider the effects of multiple compounds in their calculations.

The human health risks associated with consuming *C. mydas* eggs from markets in Peninsular Malaysia were very high in terms of the concentrations of coplanar PCBs reported (14 - 1290 pg g^{-1} wet mass). The consumption of a single *C. mydas* egg could represent 3-300 times the acceptable daily intake (ADI) of these chemicals. Furthermore, a single *C. mydas* egg would contain 9.5 % of the ADI for arsenic, indicating that the consumption of > 10 eggs per day would exceed the ADI for that compound. However, due to the high economic cost and limited seasonal availability of *C. mydas* eggs, it is unlikely the people of Peninsular Malaysia could sustain the regular consumption of 10 eggs per day. Despite this, the risk assessments calculated in the present study could be used to warn the people of Peninsular Malaysia of the potential harm associated with *C. mydas* egg consumption. Toxic compounds, such as PCBs and toxic metals can have a wide range of effects on human health, including cancers and other physiological diseases (Colborn et al. 1993; Chang 1996; Baird and Cann 2005; McKinlay et al. 2008). It could therefore be recommended from the results of the present study that the consumption of *C. mydas* eggs in Peninsular Malaysia be minimised or completely stopped to reduce the risk of these harmful effects.

The human health risks associated with consumption of *C. mydas* eggs analysed in the present study also imply that the risks calculated for the Peninsular Malaysian *C. mydas* population may have been underestimated. The significantly lower ADI for coplanar PCBs compared to other PCB congeners, indicates that these particular

congeners are considerably more toxic to humans. In fact, they are often considered in the same context as dioxins and furans, which are some of the most toxic chemicals known. However, the coplanar PCBs were not considered separately in the SRAs for *C. mydas* in the present study. This was due to the absence of data on the effects of coplanar PCBs on the development and sex reversal in oviparous reptiles and birds. Further research into the effects of coplanar PCBs in oviparous reptiles and birds is likely to reveal more toxic effects of these chemicals. This would greatly increase the accuracy of future risk assessments of sea turtle populations containing these compounds.

The risks of consuming *C. mydas* eggs must also be considered in relation to the total diet of people of Peninsular Malaysia. The human health risk assessments in the present study were calculated on the consumption of *C. mydas* eggs only. They did not take into account the intake of these harmful chemicals from other food sources in the diets of people in Peninsular Malaysia. If POPs and heavy metals are being consumed from other food sources, the risks of eating contaminated *C. mydas* eggs would be elevated. It is likely that the communities that consume sea turtle eggs will also regularly consume other seafood. There are current concerns with the consumption of potentially contaminated fish, shellfish and crustaceans living and feeding in polluted coastal areas (Agusa et al. 2007). However, the potential risks of consuming these species may be more directly associated with localised pollution and could be reduced by collecting from more pristine areas or solving local pollution issues. The contamination of sea turtle eggs is more cryptic. Although sea turtles may lay their eggs in relatively unpolluted areas, the results of the present study indicate that contamination is occurring in foraging grounds that may be thousands of kilometres away from the point of collection and experiencing significantly different levels of contamination. The consumption of sea turtle eggs therefore poses a variable risk that is difficult to accurately predict or determine. Consumption of sea turtle eggs should therefore be avoided to minimise potentially harmful effects of chemical contamination.

It is also important to emphasise the potential increased risks associated with consuming *C. mydas* eggs with such a large number of chemical pollutants. Reporting POPs in turtle eggs has previously been limited by technical difficulties in detecting

these chemicals at trace concentrations. However, the low LOD of the method developed in the present study has increased the number of POP compounds that can be reported in sea turtle eggs. The health risk assessments of the present study were calculated on a single compound basis, as the effects of the consumption of multiple chemicals are not well known. However, the large number of POPs and metals reported in the eggs from Peninsular Malaysia may have combined effects on human health, further contributing to the risks of consuming this food source.

Incidental to the human health risk of consuming *C. mydas* eggs is the possibility that egg contamination may assist conservation efforts for this species. If the consumption of *C. mydas* eggs is reduced due to the health risks, the pressure on collection of the eggs for sale in markets would be reduced. This could potentially contribute to the recovery of these *C. mydas* populations in Peninsular Malaysia and the surrounding region. However, this is unlikely to be an advantage to this *C. mydas* population if the chemical contamination of *C. mydas* in this region is increasing and the effects of these chemicals become more prevalent. This is dependent on the continued use and accumulation of these chemicals in the Southeast Asian region. Although the production and use of many of these chemicals have been banned worldwide, some are still being used in this region. And with increasing development and industrialisation of Southeast Asian nations, the threat of chemical contamination to the marine environment is likely to continue.

Once again, analysis of mussels in this region can give an indication of temporal trends in coastal contamination, and hence assist in determining the prognosis for sea turtles in this region with respect to chemical pollution. Comparison of two Asia-Pacific Mussel Watch studies indicate that the concentrations of organochlorine compounds (DDTs, PCBs, chlordanes and HCHs) remained relatively constant from 1994 to 2005 (Monirith et al. 2003; Ramu et al. 2007). This would imply that the exposure of sea turtles to these compounds has not been increasing recently. The temporal trends of metal contamination are more difficult to interpret, as they are less regularly included in the Asia-Pacific Mussel Watch studies. However, investigation of a more localised study in Taiwan indicated a steady increase in copper and zinc contamination between 1991 and 1998 (Jeng et al. 2000). Similarly, increases in metal concentrations were observed in Hong Kong from 1998 to 2000 (Liu and Kueh 2005),

although following the pollutant abatement scheme in late 2001, there were decreases in the concentrations of copper, lead, zinc and arsenic in coastal mussels (Liu and Kueh 2005; Fang et al. 2008). However, cadmium concentrations in this area have remained relatively stable from 1983 to 2005 (Fang et al. 2008) and mercury concentrations continued to steadily increase from 1998 to 2003 (Liu and Kueh 2005).

It seems that chemical contamination in the marine environment of Southeast Asia is either remaining stable or increasing, although localised reductions can be made if abatement plans are implemented. However, chemicals such as POPs and heavy metals are resistant to breakdown and hence very persistent in the marine environment. They can therefore bioaccumulate and biomagnify in animals, such as sea turtles, and can continue to have effect beyond their time of release into the marine environment. The threat of contamination to sea turtles is therefore likely to continue into the future and warrants continual monitoring. Further research into the effects of chemical pollutants on sea turtles is also required so that the implications of current and future contamination levels can be better understood.

Conclusions

The findings of this thesis have further advanced the knowledge in sea turtle chemical contamination and enhanced the capacity for further research in this area. The development of new methods for detecting 125 POP compounds in sea turtle eggs, blood and tissue at trace levels has made investigations into chemical contamination in sea turtles more comprehensive and possible for a wider range of laboratories. Furthermore, the use of blood and carapace sampling has been validated as reliable and effective methods for determining the internal tissue contamination of sea turtles. And finally, the response of *C. mydas* eggs to the topical administration of POPs has indicated a number of important considerations in using this method to investigate the effects of chemicals on sea turtle incubation and development.

The analysis of POPs and metals in *C. mydas* at various lifecycle stages has also furthered the knowledge of contaminant accumulation and transfer patterns in sea turtles. The present study identified variations in *C. mydas* contamination due to different foraging areas and, therefore, proposed the use of chemical profiles to investigate foraging ground variability of a nesting population. This also indicated

that each *C. mydas* population must be treated individually when assessing chemical contamination and that foraging grounds need to be identified to assess chemical contamination risks to nesting populations. Although the overall effects of chemical pollutants on *C. mydas* populations are still largely unknown, the SRAs indicated a considerable risk of embryonic mortality and reduced hatch success associated with the concentrations of POPs and metals reported. In addition, the considerable human health risks associated with the consumption of contaminated *C. mydas* eggs calculated in this study indicate that the effects of contaminants on *C. mydas* may be greater than predicted. Although these risk assessments have been calculated on an individual compound basis, up to 62 different POP compounds were reported in a single egg. There is therefore potentially further risk associated with the combined effects of multiple contaminants. This is supported by the reduction in hatchling mass:length ratio with increasing POPs concentrations, despite individual compounds being at relatively low concentrations. Further investigation into the combined effects of POPs and metals on embryonic development are therefore required to more adequately assess the risk of these chemicals to *C. mydas* populations.

The similar level of contamination of the two geographically separated *C. mydas* populations investigated in the present study indicates that the threat of chemical pollution to sea turtle populations may be widespread. The assessment and monitoring of the contamination of other sea turtle populations around the world is therefore warranted. Furthermore, the POP and metal concentrations reported in the *C. mydas* populations of the present study were relatively low compared to other higher trophic level sea turtle species. This indicates that the risks of chemical contamination to other sea turtle populations may be higher than those estimated for *C. mydas* in the present study. Furthermore, with chemical pollution increasing in coastal areas and the persistent nature of these chemicals in the environment, it is important to continue chemical contamination research in sea turtles. This will ensure that the accumulation patterns and potential risks are monitored and will best equip managers with the information and early warning signs to deal with the problem of chemical contamination in sea turtles as it progresses.

Appendix A

Table A. Compounds and their initial concentrations (ng g^{-1}) in the calibrants prepared by the National Institute of Standards and Technology (NIST) on December 7, 2005 and January 12, 2007.

		Concentration (ng g^{-1})	
SRM	**Compound**	**Dec 7, 2005**	**Jan 12, 2007**
2261	HCB	106.97	264.82
	aldrin	107.83	266.94
	heptachlor	107.51	266.14
	2,4'-DDE	107.47	266.06
	4,4'-DDE	107.47	266.06
	2,4'-DDT	106.55	263.77
	mirex	108.26	268.00
	gamma-HCH	107.22	265.44
	heptachlor epoxide	107.51	266.14
	cis-chlordane	107.22	265.44
	trans-nonachlor	108.01	267.38
	diedrin	107.22	265.44
	2,4'-DDD	107.26	265.53
	4,4'-DDD	108.33	268.17
	4,4'-DDT	106.94	264.73
2262	PCB 1	118.05	245.17
	PCB 8	122.50	254.42
	PCB 18	117.50	244.03
	PCB 29	117.38	243.78
	PCB 28	118.17	245.42
	PCB 104	118.44	245.99
	PCB 44	117.26	243.54
	PCB 66	117.10	243.21
	PCB 101	116.20	241.33
	PCB 87	118.17	245.42
	PCB 77	119.74	248.69
	PCB 154	116.20	241.33
	PCB 118	117.85	244.77
	PCB 188	118.48	246.07
	PCB 153	116.47	241.90
	PCB 105	116.59	242.15
	PCB 138	115.76	240.43
	PCB 126	118.56	246.24
	PCB 187	116.87	242.72
	PCB 128	117.57	244.19
	PCB 201	118.20	245.50
	PCB 180	117.61	244.27
	PCB 170	116.75	242.48
	PCB 195	117.14	243.29
	PCB 206	114.23	237.24

Table A. (Cont'd.)

		Concentration (ng g^{-1})	
SRM	**Compound**	**Dec 7, 2005**	**Jan 12, 2007**
2262	PCB 209	117.73	244.52
	PCB 50	118.56	246.24
	PCB 194	12.60	26.18
	PCB 52	118.01	245.09
2275	oxychlordane	108.85	295.56
	alpha-HCH	114.18	310.03
	beta-HCH	113.42	307.96
	cis-nonachlor	111.82	303.62
	trans-chlordane	112.43	305.27
	endrin	110.68	300.52
	endosulfan I	109.61	297.62
	endosulfan II	112.01	304.13
	endosulfan sulfate	111.36	302.38
2274	PCB 31	117.26	263.94
	PCB 49	116.74	262.77
	PCB 95	117.10	263.58
	PCB 99	117.42	264.30
	PCB 110	116.54	262.32
	PCB 149	116.54	262.32
	PCB 151	116.26	261.68
	PCB 156	116.78	262.86
	PCB 169	116.18	261.50
	PCB 183	115.26	259.43
	PCB 194	115.66	260.33
PCB III	PCB 45	117.21	279.82
	PCB 56	110.28	263.28
	PCB 63	109.13	260.52
	PCB 70	114.33	272.93
	PCB 74	120.10	286.71
	PCB 82	120.68	288.09
	PCB 92	113.75	271.55
	PCB 107	116.06	277.06
	PCB 132	115.48	275.69
	PCB 146	119.52	285.33
	PCB 157	110.28	263.28
	PCB 158	107.97	257.77
	PCB 163	119.52	285.33
	PCB 174	116.06	277.06
	PCB 193	116.06	277.06

Table A. (Cont'd.)

		Concentration (ng g^{-1})	
SRM	**Compound**	**Dec 7, 2005**	**Jan 12, 2007**
PCB IV	PCB 79	105.18	268.53
	PCB 106	125.90	321.42
	PCB 112	142.15	362.92
	PCB 114	134.18	342.58
	PCB 119	48.76	124.50
	PCB 121	109.00	278.29
	PCB 127	71.39	182.27
	PCB 130	137.05	349.90
	PCB 137	106.14	270.97
	PCB 159	81.91	209.13
	PCB 165	76.18	194.48
	PCB 166	175.30	447.55
	PCB 167	175.94	449.18
	PCB 172	80.32	205.06
	PCB 175	171.15	436.97
	PCB 176	174.66	445.92
	PCB 177	102.31	261.21
	PCB 178	44.62	113.92
	PCB 185	103.59	264.46
	PCB 189	84.46	215.64
	PCB 191	72.99	186.34
	PCB 193	97.85	249.81
	PCB 196	104.22	266.09
	PCB 197	115.38	294.57
	PCB 200	107.09	273.41
	PCB 199	114.42	292.13
	PCB 202	111.87	285.62
	PCB 203	173.07	441.85
	PCB 205	111.23	283.99
	PCB 207	86.06	219.71
	PCB 208	136.41	348.27
	pentachlorobenzene	123.66	315.72
PBDE 26	PBDE 30	51.15	339.46
	PBDE 17	4.11	27.25
	PBDE 25	21.38	141.90
	PBDE 33	12.10	80.32
	PBDE 28	50.02	331.91
	PBDE 75	68.56	454.96
	PBDE 49	7.13	47.30
	PBDE 71	50.59	335.68

Table A. (Cont'd.)

		Concentration (ng g^{-1})	
SRM	**Compound**	**Dec 7, 2005**	**Jan 12, 2007**
PBDE 26	PBDE 47	40.59	269.32
	PBDE 66	8.69	57.66
	PBDE 100	75.20	499.00
	PBDE 119	59.67	395.94
	PBDE 99	39.89	264.73
	PBDE 116	41.20	273.41
	PBDE 85	68.85	456.90
	PBDE 155	38.12	252.94
	PBDE 154	49.79	330.38
	PBDE 153	51.24	340.05
	PBDE 138	57.35	380.58
	PBDE 156	52.99	351.63
	PBDE 183	49.58	329.00
	PBDE 191	49.14	326.07
	PBDE 181	47.26	313.64
	PBDE 190	46.91	311.31
	PBDE 203	35.43	235.10
	PBDE 205	51.91	344.49
	PBDE 206	49.35	327.45
PBDE 209	PBDE 209	27.17	290.48
HBCD Mixture	alpha-HBCD	6.92	278.54
	beta-HBCD	6.89	277.04
	gamma-HBCD	6.93	278.74
octachlorosytrene	octachlorosytrene	112.07	137.66

Appendix B

Table B. The mass labelled, deuterated and fluorinated compounds combined on June 1, 2006 by NIST to make the internal standard solution in ethanol.

Compound	Solvent	Source	Concentration in ethanol (ng g^{-1})
^{13}C-PCB 28	nonane	Wellington Laboratories	142.35
^{13}C-PCB 52	10% toluene in nonane	Wellington Laboratories	138.69
^{13}C-PCB 118	5% toluene in nonane	Wellington Laboratories	139.26
^{13}C-PCB 153	nonane	Wellington Laboratories	133.89
^{13}C-PCB 180	nonane	Wellington Laboratories	136.49
^{13}C-PCB 194	10% toluene in nonane	Wellington Laboratories	136.16
^{13}C-PCB 206	nonane	Cambridge Isotope Laboratories	115.86
4'-F-PBDE 208	isooctane	Chiron	113.06
^{13}C-PBDE 209	toluene	Wellington Laboratories	135.68
^{13}C-HCB	nonane	Cambridge Isotope Laboratories	143.39
^{13}C-oxychlordane	nonane	Cambridge Isotope Laboratories	136.68
^{13}C-trans-chlordane	nonane	Wellington Laboratories	71.40
^{13}C-trans-nonachlor	nonane	Cambridge Isotope Laboratories	134.27
^{13}C-dieldrin	nonane	Cambridge Isotope Laboratories	134.00
^{13}C-4,4'-DDE	nonane	Cambridge Isotope Laboratories	141.35
4,4'-DDD-*d* 8	isooctane	in-house solution	140.42
^{13}C-4,4'-DDT	nonane	Cambridge Isotope Laboratories	145.69
^{13}C-PCB 77	isooctane	in-house solution	2.36
^{13}C-PCB 126	isooctane	in-house solution	2.36
^{13}C-PCB 169	isooctane	in-house solution	2.36
^{13}C-αHBCD	10% toluene in nonane	Wellington Laboratories	32.36
^{13}C-γHBCD	10% toluene in nonane	Wellington Laboratories	26.40
F-PBDE 47	isooctane	Chiron	129.70
PBDE 104	isooctane	Accustandard	151.75
F-PBDE 160	isooctane	Chiron	68.44
^{13}C-methyl triclosan	nonane	Wellington Laboratories	120.81

Appendix C

The response of *Chelonia mydas* eggs to the topical administration of the pesticide dichlorodiphenyl-dichloroethane (DDE)

Introduction

Persistent organic pollutants (POPs) can act as endocrine disrupting chemicals, altering the development and function of the endocrine system (Colborn et al. 1993). In oviparous reptiles, many POPs are eco-estrogenic and mimic the actions of natural estrogen in the development and function of the reproductive system (Guillette et al. 1996). Following a significant chemical spill in Lake Apopka, Florida, USA, high levels of *p,p'*-DDE, *p,p'*-DDD, dieldrin and *cis*-chlordane were found in alligator (*Alligator mississippiensis*) eggs. Male juveniles of this population had poorly organised testes, ovaries from 6-month-old females had polyovular follicles and multinucleated oocytes (normal ovarian follicle contains a single oocyte that has a single nucleus) and abnormal plasma sex steroid hormone concentrations were observed (Guillette and Crain 1996). Similarly, PCBs in the eggs of the freshwater snapping turtle (*Chelydra serpentina*) have been linked to embryonic mortality and reproductive deformities (Helwig and Hora 1983; Olafsson et al. 1983; Bryan et al. 1987).

A number of eco-estrogenic chemicals have also been found in the eggs of the green sea turtle, *Chelonia mydas* (Thompson et al. 1974; Clark and Krynitsky 1980; Podreka et al. 1998; McKenzie et al. 1999). *Chelonia mydas* is listed as endangered by the IUCN (Seminoff 2002) and is susceptible to threats that may cause changes to natural sex ratios (Mrosovsky and Yntema 1980). The investigation into the effects of eco-estrogens on development and sex reversal in *C. mydas* is therefore an important area of sea turtle conservation research, although it has received limited attention in the literature to date. However, the absence of highly polluted sea turtle habitats has limited the opportunity to investigate the effects of these chemicals on sea turtle development. More experimental approaches to determining the effects of eco-estrogens on sea turtle development are therefore required.

Topical administration of eco-estrogens has been widely used to investigate the effects of eco-estrogens on sexual differentiation in developing embryos of oviparous reptiles that exhibit temperature dependent sex determination (TSD). This method involves dissolving eco-estrogenic chemicals in various solvents (usually ethanol) and applying the solutions to the outside of developing eggs during the period of gonadal differentiation. In sea turtles, the development of male or female gonads is determined by temperature, generally during the middle third of incubation. Male hatchlings are produced at lower temperatures and females at higher temperatures (Mrosovsky and Yntema 1980; Miller and Limpus 1981; Yntema and Mrosovsky 1982; Standora and Spotila 1985). Past studies using topical administration of eco-estrogens to oviparous reptile eggs have generally focussed on the feminising effects of these chemicals at male producing temperatures. The topical administration technique results in significantly higher survival than methods using direct injection of these chemicals into eggs (Crews et al. 1991), and has been successful in determining sex reversal thresholds in freshwater turtles for a number of natural and environmental estrogens.

Topical administration of the natural estrogens estradiol-17β and β-estradiol 3-benzoate produced female hatchlings at male producing temperatures in *Trachemys scripta* embryos (Crews et al. 1991; Bergeron et al. 1994; Willingham and Crews 1999). Similarly, two polychlorinated biphenyl (PCB) congeners, 2',4',6'-trichloro-4-biphenyl and 2',3',4',5'-tetrachloro-4-biphenyl, produced significant sex reversal in *T. scripta* at a concentration of 9 ppm (Bergeron et al. 1994). Furthermore, evidence of a synergistic effect of PCBs was found when the combination of these two PCB congeners produced significant sex reversal at a dose concentration of < 1 ppm (Bergeron et al. 1994). The pesticides, *trans*-nonachlor, *cis*-nonachlor, arochlor 1242 (PCB mixture), *p,p'*-DDE and chlordane also have been found to produce sex reversal in *T. scripta* at concentrations ranging from 0.16 to 18 μM (Willingham and Crews 1999). However, these studies did not measure the penetration of these chemicals into the eggs. It is therefore unknown what egg concentrations produce these effects in freshwater turtles. An understanding into the penetration of eco-estrogens following topical administration is therefore required to give an ecological relevance to these types of experiments.

In the only study of this kind on sea turtles, developing *Chelonia mydas* embryos exposed to *p,p'*-DDE in ethanol at concentrations of up to 2.66 μg μL^{-1} showed no significant sex reversal (Podreka et al. 1998). This study did measure the DDE in the eggs and found a linear relationship between DDE applied and DDE that penetrated the eggshell. Approximately 34% of the applied DDE penetrated the eggshell, with 8% incorporating into the embryo during the middle third of incubation. However, the range in concentrations investigated by Podreka et al. (1998) was minimal. The effects of DDE on the development of *C. mydas* eggs may occur at higher concentrations. Furthermore, the penetration of DDE into the egg at higher dosing concentrations may deviate from the linear relationship observed by Podreka et al. (1998). The relationship between the concentration of DDE applied and DDE penetrated would therefore need to be further investigated before the effects of DDE on *C. mydas* development at higher concentrations can be investigated.

The aim of this study was to investigate the response of *Chelonia mydas* eggs to the topical administration of DDE in ethanol in terms of the degree of penetration of these chemicals through the eggshells, their assimilation into the developing embryo and the effects of these chemicals on embryonic morphology and the rate of development.

Methods

Egg collection

In December 2005, 40 eggs were collected from a single nesting female *Chelonia mydas* at Heron Island, Queensland, Australia (23° 27' 26" S, 151° 57' 26" E). To avoid fungal contamination, the eggs were collected at the time of oviposition using a sterilised glove before they contacted the sand (Phillott 2002). The eggs were immediately placed into sterilised plastic bags, which were sealed and transferred a refrigerator (4 °C) within 30 minutes of being laid. This was done to suspend the onset of embryonic development and reduce movement-induced mortality of the eggs (Miller and Limpus 1983; Harry and Limpus 1989). The eggs were left in the refrigerator for 6 hours until they reached a temperature of approximately 8 °C and then placed in a small foam box, which was placed into a larger foam box and surrounded by frozen ice packs and foam beads. A digital thermometer was placed next to the eggs and the larger foam box was sealed with the thermometer digital

display unit on the outside to monitor temperature of the eggs throughout transport. The sealed foam box was left in the refrigerator until transport off the island.

The afternoon following collection, the eggs were transported back to Gladstone on the ferry and then immediately driven ~ 600 km back to Griffith University on the Gold Coast. Approximately 19 hours elapsed between oviposition and arrival at the Griffith University laboratories and the temperature inside the foam box did not exceed 14 °C during transport. Keeping sea turtle eggs below 16 °C for up to 72 hours has previously been used to successfully transport sea turtle eggs over long distances, while not affecting hatching success or sex ratio (Miller and Limpus 1983; Harry and Limpus 1989; Podreka et al. 1998).

Incubation

Once in the laboratory, three eggs were placed in a freezer (-20 °C) for analysis of background levels of DDE. The remaining eggs were removed from the foam boxes and placed on a layer of moistened grade three vermiculite (Ausperl, NSW, Australia) in 5 L rectangular plastic boxes (three parts vermiculite to four parts deionised water by weight). Up to 13 eggs were placed in each box and care was taken not to rotate the eggs to avoid movement-induced mortality (Limpus et al. 1979). The eggs were completely covered with the moist vermiculite and lids were placed on the boxes with one corner left open to allow gas exchange. The boxes were weighed to monitor water loss and placed in a RI390D incubator (Electrolux, Sweden) fitted with a UP150 program temperature controller (Yokogawa, Japan). The incubator was set to 26.5 °C and data loggers (Maxim, CA, USA) accurate to 0.5 °C were placed in the incubator to monitor temperature throughout incubation. A container of deionised water was placed at the bottom of the incubator to maintain humidity and was topped up as evaporation occurred. To determine moisture loss in the vermiculite, the egg boxes were weighed once a week and in cases of weight loss, deionised water was added to the vermiculite using a spray bottle to return each box to its original weight.

After 15 days, the eggs were inspected for egg whitening, an indicator of the onset of embryonic development (Miller 1985). Twenty-five of the 37 eggs showed no sign of development and were removed from the experiment. The remaining 12 eggs were placed evenly separated into a single 5 L container and the eggs were completely

covered with moist vermiculite. The lid of the container was placed on with one corner left open for gas exchange. The container was weighed to monitor water loss and placed back into the incubator.

Topical administration of DDE

Eggs were dosed on day 23, which marked the beginning of the sexual differentiation period for this population of *Chelonia mydas* incubating at ~ 26.5 $^{\circ}$C (Miller 1985; Booth and Astill 2001). A stock solution of DDE (20 mg mL^{-1}) was prepared by completely dissolving 100 mg of 99% 2,2-bis (4-chlorophenyl)-1,1-dichloroethylene (Aldrich, WI, USA) in 5 ml of 95% ethanol (Chem-Supply, S.A., Australia). A concentration of 20 mg mL^{-1} was the maximum concentration possible for DDE in 95% ethanol (O'Neil 2006). This stock solution was used to prepare dilutions of 16, 12, 6 and 2 mg mL^{-1}. The vermiculite was removed to expose the top of the incubating eggs and 25 μL of each solution was administered topically to eggs in duplicate using a 25 μL glass syringe (SGE, VIC, Australia). Concentrations were randomly assigned to the eggs and there were two levels of control: 1) an incubation control where eggs were left to incubate without any interference, and 2) a solvent control where eggs were dosed with 25 μL of ethanol only. After dosing, the eggs were returned to the incubator and allowed to incubate up to day 62, which indicated the end of the sexual differentiation period. Eggs were removed at this stage, weighed and immediately frozen (-20 $^{\circ}$C) to terminate development.

Egg dissections

Prior to analysis, the eggs were dissected and the embryos were carefully removed from the remaining yolk, albumin and chorioallantoic membrane using hexane rinsed stainless steel dissection scissors and a scalpel blade. The embryos were weighed (wet mass) and the scute pattern on the head and carapace were recorded. An abnormality index, calculated as the total number of deviations from a normal pattern (see Chapter 2), was assigned to each hatchling. Straight carapace length (SCL), straight carapace width (SCW), head length (HL) and head width (HW) were measured using vernier callipers (see Chapter 2).

Analysis of DDE

The embryos were homogenised using a stainless steel scalpel blade and the remaining egg contents and egg contents from the three eggs taken at the time of oviposition for background analysis were homogenised with a stainless steel blender at 11,000 rpm for 2 minutes. The embryo and egg contents were analysed for DDE separately using ASE extraction, GPC and Florisil™ column clean-up and quantification by GC-MS/MS (see Chapter 3 for details).

Statistical analysis

The concentration of DDE (mean ± range) that penetrated the eggs was plotted against the concentration of DDE topically administered to the eggs. Sigma Plot (Systat Software, CA, USA) was used to plot the regression of best fit to the data and to calculate the equation, R^2, Kolmogrov-Smirnov statistic (normality) and significance of the relationship for both eggs and embryos.

Linear regressions were performed between the total concentration of DDE that penetrated the incubating eggs and each of the mass and length measurements taken. A significant relationship was considered if $P < 0.05$ and variation was estimated by R^2 values.

Results

Following the topical administration of DDE in ethanol all controls and treated eggs survived to day 62 when the incubation was ceased. All dissected embryos showed stage 28-29 characteristics, which indicated they had reached the end of the sexual differentiation period when incubation was ceased (Miller 1985).

The concentration of DDE in the three eggs taken for analysis at the time of oviposition to determine background levels was 1.8 ± 1.1 ng g^{-1} wet mass. The concentration of DDE in the incubation control and solvent control were 2.5 ng g^{-1} wet mass and 15.2 ng g^{-1} wet mass, respectively, for embryos and 50.8 ng g^{-1} wet mass and 128.5 ng g^{-1} wet mass, respectively, for the remaining egg contents.

Percent recovery (%) of DDE was 80.3 ± 2.4 and 85.2 ± 9.6 for the embryos and eggs, respectively. The coefficient of variation of DDE for the three replicates of the pooled

egg sample was 4.3% and the concentration of DDE in the SRM was within 37% of the certified value.

The relationship between the concentration of DDE added and the amount of DDE penetrated showed significant non-linear regression for both the eggs ($R^2 = 0.90$, $P < 0.0001$) and embryos ($R^2 = 0.80$, $P < 0.0005$). This non-linear relationship was an exponential increase to a maximum, fitting the equation $y = a(1-b^x)$, where a and b are constants. The equations for the relationships between DDE added and penetration of DDE were $y = 370.52(1 - 0.86^x)$ for the embryos, and $y = 515.78(1 - 0.68^x)$ for the remaining egg contents (Figure C). All data satisfied the normality assumption (Kolmogrov-Smirnov: $P > 0.05$).

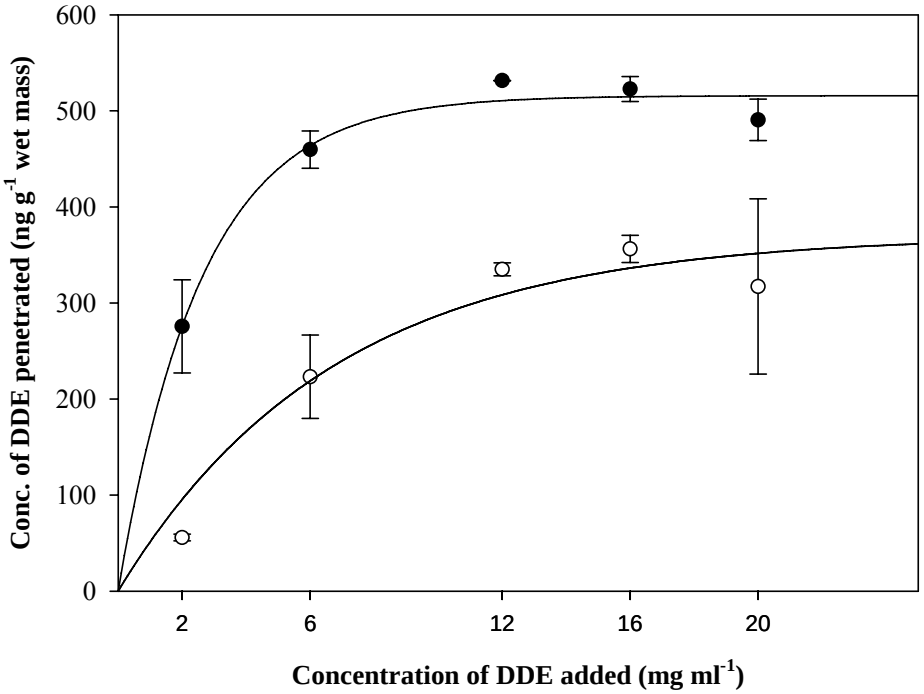

Figure C. Relationship between the concentration of DDE topically administered to *Chelonia mydas* eggs and the concentration (mean ± range) of DDE that penetrated the eggs (•) and embryos (o). N = 2 for each data point.

Embryo mass and head width were the only two parameters that had a significant relationship with the concentration of DDE topically administered (Table C; linear regression: $P < 0.05$).

Table C. Descriptive statistics of the *Chelonia mydas* eggs and hatchlings incubated with topical administration of DDE and the relationship of these parameters to the total concentration of DDE that penetrated the egg.

Egg/Embryo Paramater	Mean (± SE)	Range	Relationship to conc. DDE penetrated		
			F	P	R^2
Egg mass (g)	46.7 ± 0.5	44.3 - 50.6	1.70	0.22	0.15
Embryo mass (g)	7.0 ± 0.2	5.5 - 8.1	**20.94**	**0.001**	**0.68**
Straight carapace length (cm)	3.3 ± 0.1	2.9 - 3.7	2.81	0.13	0.22
Straight carapace width (cm)	2.6 ± 0.1	2.3 - 3.0	1.77	0.21	0.15
Head length (cm)	1.7 ± 0.02	1.6 - 1.8	2.96	0.12	0.23
Head width (cm)	1.1 ± 0.02	1.0 - 1.2	**14.92**	**0.003**	**0.60**
Abnormality index	1.6 ± 0.7	0 - 8	0.16	0.69	0.02

Discussion

The background concentration of DDE in *Chelonia mydas* eggs from Heron Island (1.8 ± 1.1 ng g^{-1} wet mass) was relatively low and similar to concentrations found in a previous study on this *C. mydas* population (Podreka et al. 1998). However, the relatively high levels of DDE in both controls, despite having no DDE added, indicated that some of the DDE added to the other eggs in the box may have leached across the vermiculite into these eggs. This is supported by the fact that the solvent control egg, which had higher concentration of DDE than the incubation control, was adjacent to three dosed eggs, while the incubation control was adjacent to two dosed eggs. Furthermore, the ethanol on the surface of the solvent control may have facilitated the penetration of any DDE in the vermiculite resulting in the higher concentration in this egg relative to the incubation control. The hypothesised movement of DDE through the vermiculite may also affect the DDE concentrations in the dosed eggs, contributing to the variation observed in some of the concentration treatments. The potential cross-contamination of adjacent eggs is therefore an important consideration in designing this type of dosing study on sea turtles.

The equations representing the relationships between the concentration of DDE topically administered and the concentration that penetrated the eggs showed an exponential increase to a theoretical maximum of 370.5 and 515.8 ng g^{-1} for the embryo and the remaining egg contents, respectively. The nature of these relationships was an initial rapid increase in DDE penetration that gradually decreased in rate as the concentration of DDE added increased. The rate of increase was slower for the embryos and computation of these equations indicated that the concentration

of DDE required to reach 99% of the maximum penetration of DDE into the egg contents and embryos is 12 and 30.5 mg mL^{-1} respectively. It is therefore not possible to reach the maximum penetration of DDE into the embryo using this method as 30.5 mg mL^{-1} is above the maximum solubility of DDE in 95% ethanol (20 mg mL^{-1}). The majority of DDE penetration occurs with the addition of the lower concentrations of DDE, with 99% and 84% of DDE penetrating the egg contents and embryo, respectively, following the topical administration of DDE in ethanol at 12 mg mL^{-1}. There is therefore minimal increase in the penetration of DDE into the egg contents and the developing embryos above the administration of 12 mg mL^{-1} DDE.

The exponential increase in DDE penetration to a maximum observed in the present study is an important consideration when designing and making conclusions about experiments using the topical administration of eco-estrogens. Most previous studies of this kind have not investigated the degree of penetration of eco-estrogens across the eggshell to the embryo and egg contents, simply reporting sex reversal in relation to the concentrations of eco-estrogens applied. In the only study to date on *C. mydas,* a linear relationship was found between the concentration of DDE applied and the concentration of DDE penetrating to the embryo and egg contents (Podreka et al. 1998). However, this study applied DDE at concentrations of up to 2.66 mg mL^{-1}. This is nearly 10 times less than the maximum concentration used in the present study. It is therefore likely that the responses observed by Podreka et al. (1998) reflected the initial rapid increase in DDE penetration, as observed in the initial relationship between DDE administered and penetrated in the present study. If this linear relation is assumed to continue past these lower concentrations, the effect of increasing the concentration of DDE administered could be significantly overestimated.

There was no relationship between the total egg mass and the concentration of DDE that penetrated the eggshell of these incubating *C. mydas* eggs. However, embryo mass ($R^2 = 0.68$, $P = 0.001$) and head width ($R^2 = 0.60$, $P = 0.003$) showed strong relationships with the concentration of DDE that penetrated the eggshell during the sexual differentiation period. More specifically, the embryo mass increased by 47% from control eggs to eggs with the highest concentration of DDE. The concentration dependent increase in embryo mass indicated that DDE may have an influence on the

rate of embryonic development. This is further supported by the increase in head width with penetration of DDE, as at this stage of development the head is the major contributor to overall embryo size and mass (Miller 1985). Furthermore, the changes in embryonic development past stage 29 are generally subtle increases in size and changes in pigmentation (Miller 1985). It is therefore possible that the larger embryos observed in this study had developed past the end of the sexual differentiation period.

In freshwater and marine turtles, the temperature of incubation influences the rate of embryonic development (Booth 1998) and the sex of developing embryos (Mrosovsky and Yntema 1980; Ewert et al. 1994; Georges et al. 1994). Higher temperatures increase the rate of development through increased tissue synthesis and maintenance (Booth 1998) and produce female hatchlings through temperature dependent synthesis and activity of specific enzymes, the presence of heat shock proteins or temperature sensitive gene expression (Mrosovsky 1994). Although the specific mechanisms of these processes are still largely unknown, it is likely that increased rate of development and feminisation at warmer temperatures are related and may influence each other. The penetration of DDE into the *C. mydas* eggs in the present study may therefore have contributed to the increased embryonic development observed by the concentration dependent increase in embryo mass and head width. This artificial change in the rate of embryonic development would slightly shift the period of sexual differentiation and skew estimates determined by the incubation temperature. This could result in eggs dosed with higher concentrations of eco-estrogens being influenced by these chemicals for shorter periods within the incubation period. This would have subtle but important influences on studies investigating the effects of eco-estrogens on sex determination.

The effect of DDE on embryonic development was further investigated through embryo survival and scute abnormalities. Although all of the controls and treated eggs survived until incubation had ceased, there was a relatively high occurrence of scute abnormalities in the embryos. Developmental abnormalities in sea turtle embryos are generally very low (0.6% of 5666 eggs, McGehee 1979; < 1% of 2811 unhatched eggs, Blanck and Sawyer 1981; 0.17% of 90,000 eggs, Miller 1982). However, high incidence of embryonic abnormalities have been observed in freshwater snapping turtle hatchlings with high levels of PCBs and organochlorine pesticides (Bishop et al.

1991; Bishop et al. 1998). In the present study, both of the controls had normal scute patterns, while six of the ten dosed embryos exhibited scute abnormalities, ranging from one to eight deviations from normal. It is therefore possible that the penetration of DDE into the eggs has affected the development of the embryos, as reflected by these observed abnormalities. However, abnormalities did not increase with increasing penetration of DDE, indicating that this effect may not be concentration dependent. Furthermore, due to the low sample numbers at each DDE concentration, care must be taken in interpreting any effects of DDE on *C. mydas* hatchling development. This study would need to be repeated on a larger scale to determine if a relationship between concentration of DDE and degree of developmental abnormality existed.

Conclusions

The results from the present study indicate that there are a number of important factors to consider when using the topical administration of DDE in ethanol to investigate sex reversal and other developmental influences in *C. mydas* eggs. The penetration of DDE into the eggs and embryos increased exponentially with the concentration of DDE administered. The majority of penetration of DDE into the egg and embryo occurred within the lower concentrations of DDE topically administered, with very little increase in penetration into the egg contents and embryos above the administration of 12 mg mL^{-1}. There was also evidence to suggest that the DDE can leach across the incubation matrix (moist vermiculite) and contaminate adjacent eggs. This may affect the sex ratio and embryonic development of the controls and cause cross contamination between treatment groups. This information is important in the design and interpretation of experiments using topical administration of eco-estrogens to determine the effects of these chemicals on sex determination and embryonic development.

The penetration of DDE through the eggshell may also influence the rate of development and cause morphological abnormalities in the embryos. Artificially increasing the rate of development may shift the sexual differentiation period slightly and alter the time of embryonic exposure to these chemicals during the sex determination period. Furthermore, developmental abnormalities observed externally may reflect internal abnormalities, which could interfere with correct identification of

the gonads. However, care must be taken in interpreting the effects on hatchling development due to the small sample sizes used.

Although these findings are for *C. mydas*, it is expected that similar patterns and factors would apply to experimental incubations using other oviparous reptiles. However, many incubation and developmental characteristics are species-specific and different estrogenic compounds may act differently. It is therefore suggested that similar pilot studies should be carried out for individual species of interest to determine the most effective experimental design and interpretation of results.

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