Health risk assessment and molecular biological characteristics associated with organochlorine insecticides sprayed for control of pests and vector-borne diseases: One Health aspects

(有機塩素系殺虫剤散布による病原性媒介生物および疾患のコントロールが及ぼす分子生物学的特徴と健康リスク評価に関する研究)

A Dissertation Submitted for the Degree of Doctor of Philosophy

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Abbreviations

∑	sum
∑DDTs	summed DDTs
ABCC2	ATP binding cassette subfamily C member 2
ACHE	acetylcholinesterase
AFFA	Agriculture, Fisheries and Forestry-Australia
AHR	aryl hydrocarbon receptor
ANOVA	analysis of variance
ATSDR	Agency for Toxic Substances and Disease Registry
AvBD	avian beta-defensin(s)
BW	body weight
C	concentration
CBC	cancer benchmark concentration
CCK-8	Cell Counting Kit-8
cDNA	complementary DNA
CHLs	chlordanes
CREM/CBI	Consultancy and research for environmental management/ centre for the promotion of imports from developing countries
CSF	cancer slope factor
CXCL8	C-X-C motif chemokine ligand 8
CYP	cytochrome P450
CYP1A1	cytochrome P450 family 1 subfamily A member 1
CYP1A2	cytochrome P450 family 1 subfamily A member 2
CYP3A5	cytochrome P450 family 3 subfamily A member 5
DDD	dichloro-diphenyl-dichloroethane
DDE	dichloro-diphenyl-dichloroethylene
DDT	dichloro-diphenyl-trichloroethane
DDTs	DDT and metabolites
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DR	average daily consumption
EDCs	endocrine-disrupting chemicals
EDI	estimated daily intake
ELOVL	elongation of very long-chain fatty acids elongase
ESR1	estrogen receptor 1
EU	European Union
FAO	Food and Agriculture Organization (of the United Nations)
FBS	fetal bovine serum
FGF2	fibroblast growth factor 2
fw	fresh weight
GAL	gallinacin(s)

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC-ECD	gas chromatography with electron capture detector
gDNA	genomic DNA
GFP	green fluorescent protein
GPC	gel permeation chromatography
GST	glutathione S-transferase
HCB	hexachlorobenzene
HCH	hexachlorocyclohexanes
HepG2	liver hepatocellular carcinoma (cell line)
HMOX-1	heme oxygenase
HPTs	heptachlors
HR	hazard ratio
HSD	honest significant difference
IARC	International Agency for Research on Cancer
IGFBP	insulin-like growth factor-binding protein
IL10	interleukin 10
IL8	interleukin 8
IRIS	Integrated Risk Information System
IRS	indoor residual spraying
ITN(s)	insecticide-treated net(s)
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KZN	KwaZulu-Natal
LOD	limit of detection
LOQ	limit of quantification
lw	lipid weight
MCF-7	Michigan Cancer Foundation-7 (human breast adenocarcinoma cell line)
mf	milk fat
MRL	maximum residue limit
MRP2	multidrug resistance-associated protein 2
n	number of samples
N/K	not known
ND	below limit of detection
NFE2L2	nuclear factor, erythroid 2 like 2
Ni	nickel
NQO1	NAD(P)H quinone dehydrogenase 1
NR1I2	nuclear receptor subfamily 1 group I member 2
NR1I3	nuclear receptor subfamily 1 group I member 3
NRF2	nuclear factor erythroid 2-related factor 2
NWU	North-West University
OCPs	organochlorine pesticides
PCA	principal component analysis
PCB	polychlorinated biphenyl

PCR	polymerase chain reaction
POPs	persistent organic pollutants
PTDI	provisional tolerable daily intake
PUFA	polyunsaturated fatty acid
PXR	pregnane X receptor
QA/QC	quality assurance/quality control
qPCR	quantitative PCR
R^2	R-squared, square of correlation coefficient
RfD	reference dose
RNA	ribonucleic acid
RT ACE	reverse transcriptase
RT buffer	reverse transcriptase buffer
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SOD	superoxide dismutase
SQLE	squalene epoxidase
SULT1A1	sulfotransferase family 1A member 1
TH-1	leukemic monocyte (cell line)
T*m*X	2,4,5,6-tetrachloro-*m*-xylene
TNF	tumor necrosis factor
UGT	UDP glucuronosyltransferase
UNEP	United Nations Environment Programme
US EPA	U.S. Environmental Protection Agency
US FDA	U.S. Food and Drug Administration
VEGFA	vascular endothelial growth factor A
WHO	World Health Organization
ww	wet weight

Preface

For this PhD thesis, contamination levels of biota (chicken and fish products) by DDTs in areas using indoor residual spraying for malaria control were analysed, enabling an assessment of the human health risk from consumption of these. Molecular biological changes associated with exposure in chickens (chronic field exposure in liver samples from free-range chickens in South Africa) and humans (acute *in vitro* exposure of a human cell line) were investigated.

The organochlorine pesticide DDT was commonly used globally in agriculture in the middle of the 20th century, but following the discovery of toxic effects in non-target species was banned in many countries. The Stockholm Convention formalised this ban in 2001, but use of the pesticide to control disease vectors has been permitted under a specific exemption and the World Health Organization (WHO) guidelines since 2006. Toxicity includes effects on reproductive, neurological and endocrine systems in both avian and mammalian species. DDT has been classified by the International Agency for Research on Cancer as probably carcinogenic to humans (Group 2A). However, the chemical is an effective and cheap method of controlling vector-borne diseases such as malaria in many countries without suitable alternatives.

To ascertain the risks posed by DDT use, a review[a] was conducted of organochlorine pesticide contamination of foods in Africa. Subsequently samples were collected from areas using DDT in indoor residual spraying programs for malaria control: free-ranging chicken[b] (48 muscle samples, 13 eggs and 39 liver samples) in KwaKulu-Natal Province, South Africa, and fish (23 muscle samples from 6 species) in Maputo Bay, Mozambique. Animals were subjected to lifetime (chronic) exposure to DDT and its metabolites in the environment. A human breast cancer cell line, MCF-7, was purchased for the acute exposure study in the laboratory.

[a] Thompson et al. 2017. Organochlorine pesticide contamination of foods in Africa: incidence and public health significance. *Journal of Veterinary Medical Science*, 79(4), pp.751-764.

[b] Thompson et al. 2017. Concentrations and human health risk assessment of DDT and its metabolites in free-range and commercial chicken products from KwaZulu-Natal, South Africa. *Food Additives & Contaminants: Part A*, 34(11), pp.1959-1969.

Chapter 1

General introduction

Background

Dichloro-diphenyl-trichloroethane's (DDT) insecticidal properties were first discovered by Paul Hermann Müller in 1939, and the chemical was used extensively both for disease control (including typhus during World War II) and in agriculture. The parent compound is relatively rapidly degraded in the natural environment to dichloro-diphenyl-dichloroethylene (DDE) and dichloro-diphenyl-dichloroethane (DDD), and these are more persistent than DDT itself.

DDT is an organochlorine pesticide, and a member of the group of toxic chemicals known as persistent organic pollutants (POPs). Between 1950 and 1963, the United Nations Environment Programme estimated that 175,000 tons of DDT were used worldwide annually, with peak use in 1970 (Mansouri et al., 2017). These chemicals are highly persistent in the environment, and are known to bioaccumulate and biomagnify within the food chain. Although initially purported to be toxic only to insects, a number of adverse effects have been demonstrated by and linked epidemiologically to POPs, including DDTs, across taxonomic groups. After the discovery of toxic effects in the environment, in particular effects on wildlife such as birds, was widely publicised by Rachel Carson's book "Silent Spring" in 1962, use of DDT was restricted (Carson, 1962). The first country to withdraw DDT use from agriculture was Hungary in 1968. The Stockholm Convention on POPs identified DDT amongst the "dirty dozen" in 2001, and signatories now ban the use of DDT in agriculture (UNEP, 2001). Other OCPs identified by the Stockholm Convention include: aldrin, chlordane, dieldrin, endrin, heptachlor, hexachlorobenzene (HCB), mirex and toxaphene.

The major breakdown metabolites of DDT are DDE and DDD (Figure 1.1.1). In people, the half-life of *p,p'*-DDE is estimated to be greater than seven years (Axmon and Rignell-Hydbom, 2006). DDE appears to be the most persistent metabolite in many vertebrate species, and high levels have been linked to pathological conditions such as eggshell thinning in birds, and prostate cancer and sperm abnormalities in people (Brokken et al., 2014; Khan and Cutkomp,

1982; Kumar et al., 2010; Messaros et al., 2009). The *o,p'*-DDT congener appears to be linked with human breast cancer (Cohn et al., 2015). Thus, DDT and its metabolites (*p,p'*-DDT and *o,p'*-DDT, *p,p'*-DDD and *p,p'*-DDE) were the focus in this thesis.

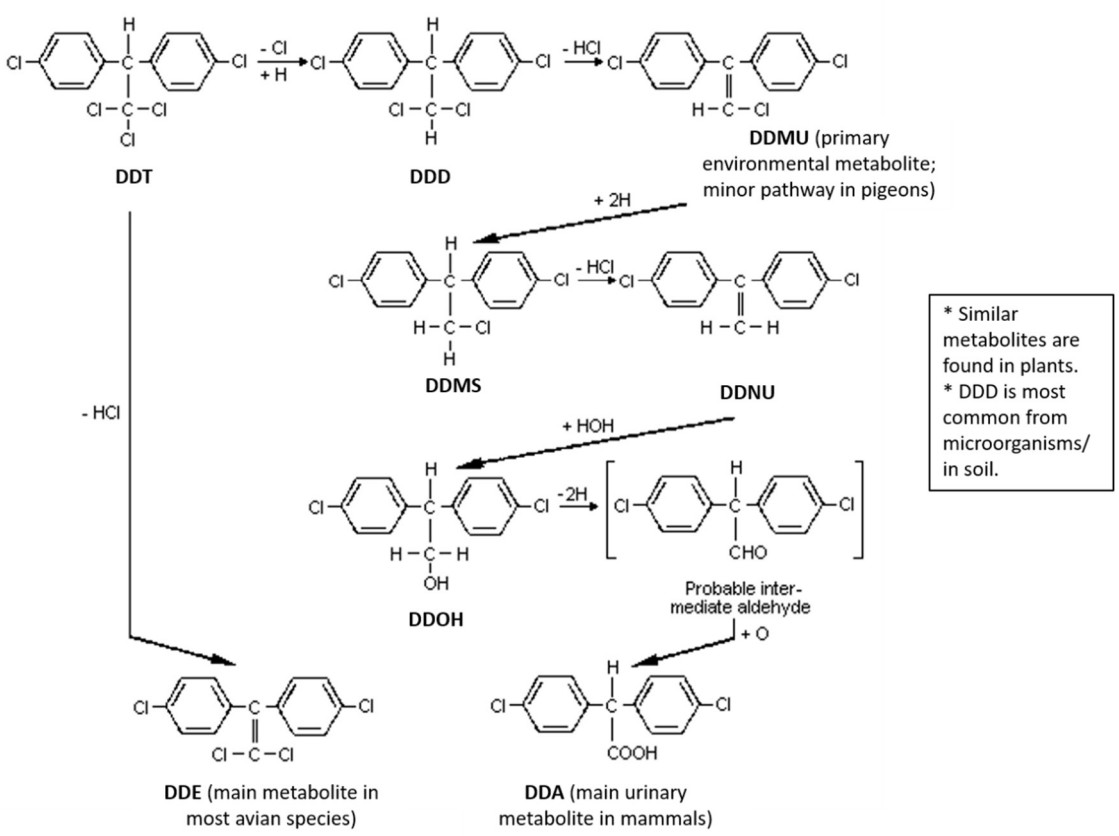


Figure 1.1.1: Chemical structures and biotransformation of the main DDTs analysed in this thesis (adapted from Peterson and Robison 1964).

The World Health Organization (WHO) has reported a steady reduction in cases of malaria worldwide, estimating 212 million cases in 2015 compared to 245 million in 2010 (WHO, 2016a). This corresponds to a reduction in annual deaths due to the disease, by 22% over this five year period. However, this still equates to an estimated 429,000 deaths in 2015. Worryingly, 303,000 of these deaths were estimated to occur in children under 5 years old, or 70% of the global total. Malaria during pregnancy is also a cause of maternal mortality, anaemia and low birth weight, which is linked to infant mortality.

Some 90% of malaria cases occur in the WHO African Region (WHO, 2016a). In South Africa, 10% of the population lives in an area where malaria is endemic; the disease is endemic in three provinces: KwaZulu-Natal (KZN), Limpopo and Mpumalanga (Maharaj et al., 2013, 2012). The WHO estimated there were over 35,000 malaria cases in South Africa in 2015, resulting in 110 reported deaths (WHO, 2016a).The hotspot for malaria in KZN Province is uMkhanyakude, with almost 700 new cases (fatality rate 1.7%) in the 2013-14 period, an incidence of 1.09 per 1000 population at risk (KwaZulu-Natal Department of Health, 2014). The KZN area includes significant poorly developed rural areas, with high levels of poverty and poor service provision (Morgenthal et al., 2006). South Africa has a campaign to eliminate endemic malaria in all of its affected provinces by 2018 (Blumberg et al., 2014).

The mainstay of malaria control is insecticide use in indoor residual spraying (IRS) programs and/or insecticide-treated nets (ITNs) (Figure 1.1.2). For many malaria endemic countries, especially those in Africa, Asia and Latin America, DDT is a cheap and effective insecticide to use for IRS. Its persistence means that application once or twice annually will provide long-lasting protection to cover peak mosquito seasons which coincide with malaria occurrence.

As outlined above, many countries banned the use of DDT in the late 20th century and signatories to the Stockholm Convention banned use in 2001, but it was reintroduced in September 2006 to help control malaria (WHO, 2006a). Thus, since 2006, under an exemption from the Stockholm Convention, countries may use DDT under guidance from the WHO, for control of vector-borne diseases where no suitable alternatives exist. Although resistance to DDT is developing in insects in some areas, it is still a highly effective method of controlling malaria vectors in many endemic areas. Used in IRS programs, the chemical is sprayed once or twice annually onto walls and roof eaves of houses. DDT both kills mosquitoes and deters them, reducing contact with human inhabitants and thereby reducing malaria transmission. Although exposure to DDT for workers administering IRS is primarily by dermal contact and inhalation of aerosolized

spray, ingestion is a more significant exposure route for other people and non-target animals (Mrema et al., 2013; Ortelee, 1958; Sereda et al., 2009).

After administration of DDT to the environment, the chemical in soil can evaporate into the air and be deposited on surface water. DDT has a low solubility in water and a strong affinity for suspended particulate matter, which therefore provides a substrate for stability and persistence of the chemical in the environment (Sanger et al., 1999). The half-life of DDTs in soil is 4–30 years (ATSDR, 2002). Concurrent transport in waterways and air permits long-range transport, and DDTs are redistributed globally, even to areas such as Antarctica where the insecticide has never been used (Klánová et al., 2008).

One of the largest concerns about DDTs in the environment is aquatic contamination. Although the half-life of DDTs in the atmosphere is merely 1.5–3 days and a number of days (26–56) in water, the chemicals can accumulate in sediment for 1–4 years and thereafter in sediment-dwelling organisms (ATSDR, 2002). Biomagnification occurs, and higher trophic levels have increasingly high levels of contamination (Bettinetti et al., 2010; Deribe et al., 2013; Yohannes et al., 2014a). Contamination at high levels may result in toxic effects in aquatic species. At the same time, contamination of fish species poses a potential threat to human health through consumption. Livestock living in close association with people are exposed to similar levels of contamination in the environment as their human carers. Previous studies report contamination levels of DDTs in human foodstuffs, including fish, cattle and chicken products in several African countries (Bouwman et al., 2015; Gebremichael et al., 2013; Ndengerio-Ndossi and Cram, 2005; Van Dyk et al., 2010a; Yohannes et al., 2014b). Contamination has also been reported in humans, and high levels of DDTs in human breast milk are a concern for developing infants (Luzardo et al., 2014; Manaca et al., 2011).

Eggshell thinning in wild birds is the most notorious toxic effect of DDTs, but they also have other effects on birds – for example reduced post-hatch survival, altered sexual behaviour, and neurotoxicity (Gómez-Ramírez et al., 2012; Iwaniuk et al., 2006; Kamata et al., 2013; Lundholm, 1988). Recently, the

International Agency for Research on Cancer classified DDT as Group 2A, that is to say, probably carcinogenic to humans (IARC, 2016). Other epidemiological studies have linked DDTs exposure with a variety of reproductive, neurological and metabolic disorders in people (Al-Othman et al., 2015; Bretveld et al., 2008; Weiss, 2011; Windham et al., 2005; Zaganas et al., 2013). *In vivo* laboratory studies in rodents in particular have shown clear links to cancer, and also epigenetic transgenerational disease linked to metabolism (ATSDR, 2002; Skinner et al., 2013). Exposures of cell cultures to various environmental pollutants have confirmed dysregulation of many genes involving several pathways, for example glucose metabolism, estrogen receptors and growth (Aubé et al., 2008; Bratton et al., 2012; Norberto et al., 2017; Qin et al., 2011). Molecular studies have identified some genes as potential biomarkers to sense persistent organic pollutants (Sakai et al., 2006).

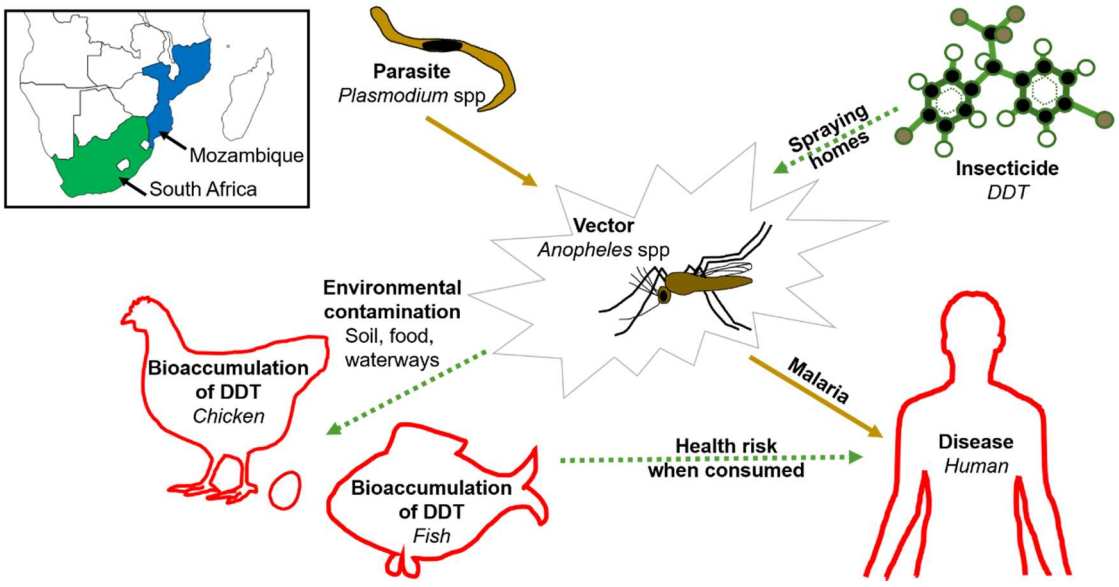


Figure 1.1.2: Use of DDT to control malaria-carrying mosquitoes results in environmental contamination, livestock contamination and human health risk.

Objectives of the thesis

DDTs affect many body systems, with apparent species-specific differences both in sensitivity and resultant effects. Although previous works have identified contamination of people and livestock food sources in many countries, human health risk assessments are infrequently performed. Also, there are large gaps in our knowledge regarding the mechanisms of toxic effects relating to DDTs – including how changes in inflammation, reproduction and lipid metabolism are effected. If we can understand these more fully, we may be able to reduce the toxic effects produced.

The main objectives of this doctoral thesis were therefore to:

- Review current knowledge of food contamination by organochlorine pesticides, including DDTs, in Africa.
- Ascertain contamination levels of DDTs in food products in an area where DDT is currently used for vector control, and perform a risk assessment for human health risk from consumption of these products.
- Elucidate mechanisms involved in metabolism and toxicological effects of DDTs in both chickens and humans.

Chapter 2

Contamination of food products and assessment of human health risk

2.1 Organochlorine pesticide contamination of foods in Africa: incidence and public health significance

2.2 Human health risk from consumption of marine fish contaminated with DDT and its metabolites in Maputo Bay, Mozambique

2.3 Concentrations and human health risk assessment of DDT and its metabolites in free-range and commercial chicken products from KwaZulu-Natal, South Africa

2.1 Organochlorine pesticide contamination of foods in Africa: incidence and public health

Abstract

Organochlorine pesticides (OCPs) have been used worldwide, particularly in Africa, for several decades. Although many are banned, several African countries still use OCPs especially for the prevention and control of malaria. OCPs are characterized by their bio-accumulation in the environment, especially in the food chain, where they find their way into the human body. Despite no clear epidemiological studies confirming hazardous effects of these chemicals on human health, many studies have reported positive associations between the use of OCPs and neurological and reproductive disorders, and cancer risk. There is a clear gap in published reports on OCPs in Africa and their potential health hazards. Thus, the aim of this review is to summarize the incidence of OCP contamination in various foods in Africa, to demonstrate the potential transmission of these chemicals to people, and to discuss their possible health hazards.

Introduction

Organochlorine pesticides (OCPs) have been extensively used worldwide for several decades because of their prolonged period of action, low cost and toxicity against various pests (Pirsaheb et al., 2015). Several OCPs—including aldrin, dieldrin and dichlorodiphenyltrichloroethane (DDT)—became widely used in agriculture in the 1950s. Initially DDT was thought to be unsafe only for insects, but toxicity and extensive biomagnification were soon highlighted in other species, notably wild birds, many of which had dramatic population declines related to use of this chemical (Ames, 1966). Lipophilic chlorine residues from OCPs accumulate within animals, and biomagnification is also seen in species at the top of the food chain. An international environmental treaty, the Stockholm Convention on Persistent Organic Pollutants (POPs), was adopted in May 2001 (UNEP, 2002) and currently has 179 parties. The 12 initial POP chemicals banned under this treaty included the following OCPs: aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene (HCB), mirex and toxaphene.

Although a number of OCPs have been banned for use in agriculture, others are still currently used in many countries as agricultural pesticides. DDT use continues under an exemption for approved disease vector control in many African countries. In this situation, chemicals are applied to bed nets (insecticide treated nets) or sprayed in homes (indoor residual spraying, IRS). Stockpiled or obsolete chemicals are likely to exist in some locations, with unmonitored and potentially inappropriate storage conditions. It is only possible to detect these by assessment of environmental samples for contamination.

Humans can be exposed to OCPs via several routes including breathing polluted air, dermal penetration, or ingestion of contaminated foods and drinking water. OCP-contaminated foods (fruit, vegetables, cereals and various meats) are considered the main source of human exposure to pesticides (Hassal, 1990). Maternal transfer is also possible across the placenta to the foetus or via breast milk to infants. Residue levels of these compounds in living organisms depend on each organism’s habitat and position in the food chain (Zhou et al., 2007). OCPs contamination of food and their public health implications attracted the

attention of many researchers and scientists to report intensive information about this worldwide problem (Kalantzi et al., 2001; Kannan et al., 1997, 1992; Pirsaheb et al., 2015; Tariq et al., 2007; Toft, 2014). Despite suspected adverse human health effects due to OCP exposure in Africa, available information is scarce. Thus, based on a literature search of peer-reviewed manuscripts published between 1st January 2000 and 11th August 2015, this review aims to highlight and summarise the incidence of OCP contamination of food in different African countries. Furthermore, the presence of OCP residues in human fluids and related health effects were ascertained.

OCP contamination of foods in African countries

Ingestion of contaminated foods is a major source of human exposure to chemical residues. Although no complete diets have been assessed, several publications describe detection of OCPs in foods destined for human consumption (Fig 2.1.1, Table 2.1.1, Table 2.1.2). In this section, we will summarize the incidence of OCP contamination of foods in some African countries.

Figure 2.1.1: A declarative map for the reported OCPs in some African countries

Table 2.1.1: Reported levels of OCPs in various foods in African countries

Country	Foodstuff	Pesticide concentration (ng/g dw, except where specified)		Reference
		DDTs	Other	
Benin	Fish (6 species)	3.88-11.3	α-endosulfan (0.50-3.39), Σaldrin (0.12-0.95), lindane (γ-HCH, ND-0.532)	(Pazou et al. 2014)
	Shellfish	0.20-18.5	α-endosulfan (ND-0.90), Σaldrin (ND-0.371), lindane (ND)	
	Fish	74-1,185 ng/g lw	α-endosulfan (23-7,926), aldrin (<1-24), dieldrin (2-57), endrin (<13), lindane (<13) ng/g lw	(Pazou, Azehoun, Aléodjrodo, et al. 2013)
	Fish	242-1,239 ng/g lw	Σendosulfan (9-215), dieldrin (0-10) ng/g lw	(Pazou, Azehoun, Aléodjrodo, et al. 2013)
	Vegetables	647-1,578 µg/kg dw	Σdrins (15-57), lindane (7-444) µg/kg dw	
	Fish	129-1,642 ng/g lw	Σendosulfan (0-215), lindane (0-6.5), dieldrin (0-9) ng/g lw	(Pazou et al. 2006)
	Fish	30 µg/g	Lindane (105), dieldrin (77), heptachlor (40) µg/g	(Okoumassoun et al. 2002)
Cameroon	Maize	-	Lindane (9.53), endosulfan (0.06)	(Sonchieu et al. 2010)
	Millet	-	Lindane (0.08), α-endosulfan (0.05), β-endosulfan (0.02)	
	Cowpea	-	Lindane (0.24), α-endosulfan (0.08)	
	Ndole/keleng keleng (local leaves)	-	<LOQ	(Gimou et al. 2008)
	Tomatoes	-	Endosulfan 0.02	
Egypt	Raw buffalo milk (local vendors, dairy farms and shops)	-	Alachlor (<0.001), dieldrin (<15), HCB (<0.200), lindane (<0.192), mehoxychlor (<0.200)	(Shaker & Elsharkawy 2015)
	African catfish (*Claria gariepinus*)	*p,p'*-DDE (0.70-0.90)	Alachlor (0.23-0.25), lindane (1.38-2.10), dieldrin (0.72-1.20), aldrin (ND), heptachlor (0.30-0.50), HCB (ND) µg/kg fw	(Yahia & Elsharkawy 2014)
	Nile tilapia (*Oreochromis niloticus*)	*p,p'*-DDE (ND)	Alachlor (ND), lindane (0.41-0.57), dieldrin (ND), aldrin (0.17-0.28), heptachlor (0.18-0.19), HCB (3.04-3.10) µg/kg fw	
	Nile tilapia	*p,p'*-DDE (0.012-0.084), *p,p'*-DDD (ND-0.087), *p,p'*-DDT (ND-0.010) µg/kg	α-HCH (ND-0.059), heptachlor (ND-0.003), aldrin (ND-0.060), heptachlor epoxide (0.021-0.113), endrin (ND-0.026)	(Azab et al. 2013)
	Buffalo liver	ND-18.41 ng/g lw	ΣHCHs (34.97-89.21), Σdrins (14.92-30.27), HCB (ND-10.15), ΣCHLs (ND-19.08) ng/g lw	(Mahmoud et al. 2013)
	Buffalo kidney	ND-35.67 ng/g lw	ΣHCHs (41.97-247.73), Σdrins (11.19-68.13), HCB (ND-96.47), ΣCHLs (ND-45.09) ng/g lw	
	Buffalo tongue	ND-62.83 ng/g lw	ΣHCHs (59.24-351.57), Σdrins (22.92-84.00), HCB (ND-23.18), ΣCHLs (ND) ng/g lw	
	Mussels	0.94-31 µg/g dw	ΣHCHs (<LOD-11), ΣCHLs (<LOD-35), dieldrin (<LOD-12), endosulfan (<LOD-9.3), methoxychlor (<LOD-4.0), mirex (<LOD-0.89), ΣHCBs (<LOD-0.50) µg/g dw	(Khairy et al. 2012)
	Cucumbers	-	ΣOCPs 0.0-1.628 µg/g	(Mansour, Belal, A. A K Abou-Arab, et al. 2009)
	Potato tubers: conventionally farmed	-	ΣOCPs 0.685 µg/g	(Mansour, Belal, Asem A K Abou-Arab, et al. 2009)
	Potato tubers: organically farmed	-	ΣOCPs 0.308 µg/g	
	Cucumbers	-	Dicofol 0.021 µg/g	(Loutfy et al. 2008)
	Vegetables (pepper highest)	-	Dicofol <3.40 µg/g	(Dogheim et al. 2002)
	Fruits (strawberry highest)	-	Dicofol <3.30 µg/g	
	Potato tubers (skin and pulp)	0.537 µg/g	HCB (0.014, <0.026), lindane (0.141, <0.221) µg/g	(Soliman 2001)
	Pommes frites	0.061 (<0.094) µg/g	HCB (0.006, <0.011), lindane (0.021, <0.121) µg/g	
	Potato chips	0.022 (<0.033) µg/g	HCB (0.003, <0.006), lindane (0.006, <0.0084) µg/g	
Ethiopia	Fish	Means 19-56 ng/g ww (range 1.65-409.6)	ΣCHLs (0.85-2.15, range 0.75-3.56), Σendosulfan (ND-25.90, range ND-42.5) ng/g ww	(Deribe et al. 2014)
	Fish (4 species)	0.77-61.9 ng/g ww	ΣHCHs (0.16-5.10), ΣCHLs (0.17-4.00), ΣHPTs (0.19-2.27) ng/g ww	(Yared B. Yohannes et al. 2014)
	Various foods (pepper, maize, teff, coffee pulp/beans)	*p,p'*-DDE (0.00-0.086), *p,p'*-DDD (0.049-0.128), *o,p'*-DDT (0.085-0.193), *p,p'*-DDT (0.099-0.461) µg/g	α-endosulfan (0.0042-0.0332), β-endosulfan (0.002-0.063) µg/g	(Mekonen et al. 2014)
	Milk: cow	<1,230 µg/kg	Aldrin (<11.6), α-endosulfan (<77.6), β-endosulfan (ND) µg/kg	(Deti et al. 2014)
	Milk: goat	<874.4 µg/kg	Aldrin (ND), α-endosulfan (<142.1), β-endosulfan (<87.0) µg/kg	
	Cow milk	0.389 µg/kg	-	(Gebremichael et al. 2013)
	Beef	Means *o,p'*-DDT (ND-3.23), *p,p'*-DDT (0.37-4.32) µg/g	Means endosulfan-I (ND-0.06), aldrin (ND-0.012), dieldrin (ND-0.04), endrin (ND-0.011), lindane (ND-0.05) µg/g	(Letta & Attah 2013)
	Khat	*p,p'*-DDT (ND-1,223.8) µg/kg	-	(Daba et al. 2011)

Table 2.1.1: Continued

Country	Foodstuff	Pesticide concentration (ng/g dw, except where specified)		Reference
		DDTs	Other	
Ghana	Cereal-based complementary foods (locally produced)	-	Means β-HCH (<0.017), lindane (<0.022), δ-HCH (<0.008), heptachlor (<0.006), γ-chlordane (<0.013), α-endosulfan (<0.008), β-endosulfan (<0.021) µg/g	(Akoto et al. 2015)
	Cowpea	-	Total OCPs (including HCHs, heptachlor, drins, γ-chlordane, endosulfans, DDTs, methoxychlor) 0.314 µg/g	(Akoto et al. 2013)
	Maize	-	Total OCPs (including HCHs, heptachlor, drins, γ-chlordane, endosulfans, DDTs, methoxychlor) 0.354 µg/g	
	Okra	-	OCPs (including lindane, heptachlor, drins, DDTs, endosulfan, methoxychlor) 3.10-7.60 µg/kg	(Essumang et al. 2013)
	Fish	*p,p'*-DDT (ND-0.93), *p,p'*-DDE (ND-0.50) ng/g	Lindane (0.02-0.54), δ-HCH (0.01-0.98), aldrin (0.29-4.26), dieldrin (0.01-0.06), α-endosulfan (0.01-7.52), endosulfan sulfate (0.01-2.76), endrin (ND-0.67), endrin aldehyde (ND-0.77), endrin ketone (ND-0.26) ng/g	(Kuranchie-Mensah et al. 2013)
	Fish (redbelly tilapia and catfish)	Average 440.90 ng/g lw	Average HCB (2.10), ΣHCHs (0.72), ΣCHLs (7.19) ng/g lw	(Adu-Kumi et al. 2010)
	Fruits (locally produced pawpaw, locally produced tomato, imported apples)	*p,p'*-DDE (0.05, <LOD, 0.01), *o,p'*-DDD (ND, <LOD, <LOD), *o,p'*-DDT (0.03, ND, ND), *p,p'*-DDT (0.02, 0.01, 0.09)	Lindane (0.04, 0.02, 0.02), δ-HCH (0.06, 0.02, 0.04), heptachlor (0.02, 0.02, 0.05), aldrin (<LOD, ND, <LOD), heptachlor epoxide (<LOD, 0.06, <LOD), γ-chlordane (ND, <LOD, <LOD), α-endosulfan (0.02, <LOD, <LOD), endrin (0.01, <LOD, <LOD), β-endosulfan (0.02, ND, ND), endrin aldehyde (0.02, 0.01, 0.11), endrin ketone (0.02, 0.02, 0.01), methoxychlor (0.03, <LOD, <LOD)	(Bempah & Donkor 2011)
	Milk	*p,p'*-DDE (0.01-4.62), *p,p'*-DDT (0.44-28.62)	Range lindane (<LOD), aldrin (<LOD-0.05), endosulfan (0.02-0.12), dieldrin (0.04-4.62)	(Darko & Acquaah 2008)
	Yoghurt	*p,p'*-DDE (0.01-2.05), *p,p'*-DDT (0.71-19.20)	Range lindane (<LOD-0.05), aldrin (<LOD-0.15), endosulfan (<LOD-0.34), dieldrin (<LOD-0.34)	
	Cheese	*p,p'*-DDE (0.16-485.76), *p,p'*-DDT (1.33-119.0)	Range lindane (<LOD-4.41), aldrin (0.01-7.88), endosulfan (0.14-9.06), dieldrin (0.88-30.49)	
	Lettuce	0.4 (0.02-0.9) µg/g	Lindane (average 0.3, range 0.03-0.9), endosulfan (0.4, 0.04-1.3) µg/g	(Amoah et al. 2006)
	Tomatoes	-	Heptachlor epoxide 1.65 ng/g fw	(Ntow 2001)
Morocco	Tomatoes	-	Dicofol (0.001-0.400), endosulfan (0.003-1.123) ng/g	(Salghi et al. 2012)
	Clams and eels	<2,000 ng/g lw	-	(Mehdaoui et al. 2000)
Nigeria	White maize	-	OCPs (means 7.9-52.0) µg/kg	(Ogah et al. 2011)
Sene-Gambia	Bivalves and gastropods	0.8-16.6	∑14 OCPs 1.9-17.8	(Bodin et al. 2011)
	Fish and shrimps	11.1-199.2 ng/g fat	∑HCHs (ND-13.3), ∑endosulfans (ND-49.7) ng/g fat	(Manirakiza et al. 2002)
South Africa	Chicken eggs	Median 11,000 (range 5,200-48,000) ng/g ww	-	(Bouwman et al. 2015)
	Fish (fat)	0.322-81.491 mg/kg fat	-	(Barnhoorn et al. 2009)
	Bovine milk	0.15 µg/kg mf	-	(Sereda et al. 2009b)
	Leafy vegetables	43.0 µg/kg	-	(J C Van Dyk et al. 2010)
	Chicken meat	700 µg/kg	-	
Tanzania	Tilapia fish (*Oreochromis* sp)	7.2-319 ng/g lw	HCB (0.6-4.0), ∑HCHs (0.6-4.0), ∑CHLs (<LOD-2.1), ∑CHBs (<LOD-0.9), ∑endosulfan (<LOD-405) ng/g lw	(Polder et al. 2014)
	Fish fillet (Nile tilapia and Nile perch)	0.03 mg/kg fw	Endosulfan 0.2 mg/kg fw	(Henry & Kishimba 2006)
	Spinach	2.89 µg/kg	Lindane 0.08 µg/kg	(Ndengerio-Ndossi & Cram 2005)
	Rice	0.76 µg/kg	Lindane 0.08 µg/kg	
	Beef meat	0.76 µg/kg	Lindane 0.14 µg/kg	
	Stiff porridge	0.03 µg/kg	Lindane 0.06 µg/kg	
	Beans	0.13 µg/kg	Lindane 0.04 µg/kg	
	Fish fillet (Nile tilapia and Nile perch)	-	Lindane 0.05 µg/kg	
	Various aquatic biota (fish and crab species)	6.6-54 µg/kg fw	-	(Mwevura et al. 2002)
Togo	Cowpea grains	-	OCPs (including dieldrin, endrin, heptachlor epoxide, endosulfan) 13.16-98.79 µg/kg	(Mawussi et al. 2009)
	Maize grains	-	OCPs (including dieldrin, endrin, heptachlor epoxide, endosulfan) 0.53-65.70 µg/kg	

Table 2.1.1: Continued

Country	Foodstuff	Pesticide concentration (ng/g dw, except where specified)		Reference
		DDTs	Other	
Tunisia	Dover sole (*Solea solea*)	54.2-512 ng/g lw	HCB (1.7-18.0), ∑HCHs (ND-58.0), ∑OCPs (65-752) ng/g lw	(Ben Ameur, El Megdiche, et al. 2013)
	Mullet (*Mugil cephalus*)	14.3-47.3 ng/g lw	HCB (1.27-15.1), ∑HCHs (0.57-20.5), ∑OCPs (19.6-157) ng/g lw	(Ben Ameur, Trabelsi, et al. 2013)
	Sea bass (*Dicentrarchus labrax*)	25.4-227 ng/g lw	HCB (1.62-28.5), ∑HCHs (2.69-33.6), ∑OCPs (47.9-265) ng/g lw	
Uganda	Nile perch and Nile tilapia	-	∑HCHs ND-73,000 pg/g lw	(Ssebugere et al. 2014)
	Fresh cow's milk	Mean 0.052 (range 0.018-0.152) mg/kg mf	Lindane (0.026, 0.001-0.086), aldrin (0.009, 0.002-0.018), dieldrin (0.007, 0.001-0.018), α-endosulfan (0.002, 0.001-0.004), β-endosulfan (<LOD)	(Kampire et al. 2011)
	Pasteurized cow's milk	Mean 0.041 (range 0.012-0.088) mg/kg mf	Lindane (0.022, <LOD-0.066), aldrin (0.006, 0.005-0.008), dieldrin (0.005, 0.001-0.021), α-endosulfan (<LOD), β-endosulfan (<LOD)	
	Fish	ND-68 µg/kg fw	-	(Ssebugere et al. 2009)
	Nile perch (belly flap oil)	Mean 43.74 µg/kg oil	-	(Ogwok et al. 2009)
	Nile tilapia and African catfish	*p,p'*-DDE (<0.01), *p,p'*-DDT (0.002) mg/kg ww	Endosulfan sulphate <0.002 mg/kg ww	(Bagumire et al. 2008)
	Nile tilapia	Mean *p,p'*-DDE (0.80), *p,p'*-DDT (0.59) µg/kg	Mean lindane (0.74), aldrin (0.28), α-endosulfan (1.70), dieldrin (0.30) µg/kg	(Kasozi et al. 2006)
	Nile perch	Mean *p,p'*-DDE (0.86), *p,p'*-DDT (0.81) µg/kg	Mean lindane (0.87), aldrin (0.48), α-endosulfan (1.45), dieldrin (0.18) µg/kg	

ND = below limit of detection, ww or fw = wet weight or fresh weight, dw = dry weight, lw = lipid weight, mf = milk fat, LOQ = limit of quantification, LOD = limit of detection.
CHLs = chlordanes, DDTs = DDT + DDE + DDD, HCB = hexachlorobenzene, HCH = hexachlorocyclohexanes, HPTs = heptachlor

Table 2.1.2: Maximum Residue limits (MRLs) recorded by different organizations and reported in this review

Organization	Pesticide	MRLs (ppb)	Reported in
Codex Alimentarius Commission	Lindane	200	(Yahia & Elsharkawy 2014)
US EPA	DDT	20	
EU	DDT	100	
EU	DDT in cereals	50	(Daba et al. 2011)
EU	Endosulfan in meat	50	(Polder et al. 2014)
FAO/WHO	DDT in fish	200	(Mwevura et al. 2002)
Canadian limits	DDT in fish	500	
WHO	DDT in water	2	(Mawussi et al. 2009)
German Food Law	Lindane	500	(Kasozi et al. 2006)
German Food Law	Aldrin + dieldrin	200	
German Food Law	Endosulfan + endosulfan sulphate	100	
German Food Law	ΣDDTs	500	
AFFA	ΣDDTs	1000	
U.S. FDA	ΣDDTs	5000	
Codex Alimentarius Commission	ΣDDTs	5000	(Ssebugere et al. 2014)
CREM/CBI	ΣDDTs	5000	(Ogwok et al. 2009)
CREM/CBI	Total endosulfan	10	

US EPA: U.S. Environmental Protection Agency
EU: European Union
WHO: World Health Organization
AFFA: Agriculture, Fisheries and Forestry-Australia
U.S. FDA: U.S. Food and Drug Administration
CREM/CBI: Consultancy and research for environmental management/centre for the promotion of imports from developing countries

Benin

Fish and shellfish

Studies conducted in Benin have focused mainly on fish species in the Ouémé River and Lake Nokoué. Pazou and colleagues detected several OCPs in fish with different concentrations (ng/g lipid weight (lw)), including DDTs (1,642), endosulfans (7,926), aldrin (24), dieldrin (57), endrin (13) and lindane (13), respectively (Pazou et al., 2014, 2013a, 2013b, 2006). Interestingly, an earlier study in the same river detected lindane as the most prevalent OCP (105 µg/g) (Okoumassoun et al., 2002). Shellfish in Lake Nokoué and Cotonou Lagoon contained DDTs (maximum 18.5 ng/g dw detected), α-endosulfan and aldrin (Table 2.1.1) (Pazou et al., 2014, 2013a, 2013b, 2006).

Vegetables

Vegetables grown in the river's floodplains in Benin also contained several OCPs in variable concentrations (µg/g dry weight (dw)), DDTs (1,578), drins (57) and lindane (444), respectively (Table 2.1.1) (Pazou et al., 2014, 2013a, 2013b, 2006).

Cameroon

Leaf crops and tomatoes

In Cameroon, assessment of pesticide levels in local leaf crops (ndole/keleng keleng) and tomatoes detected only endosulfan in the tomatoes and even then at a level below the acceptable daily intake (ADI), at 0.02 mg/kg (Gimou et al., 2008).

Cereal crops

A study on maize, millet and cowpea detected lindane, at 9.53 mg/kg in maize, above the maximum residue limit (MRL) (Tables 2.1.1, 2.1.2) (Sonchieu et al., 2010).

Egypt

Agricultural crops

Although use has been banned in Egypt since the 1980s, OCPS are still detected in various foods in the country. For farmed cucumbers, the method of farming has been shown to affect pesticide contamination levels (greenhouse cucumbers >

conventional > organic farming), as has season (winter/spring range of OCPs 0.0-0.362 mg/kg compared to summer/fall range 0.219-1.628 mg/kg) (Mansour et al., 2009b). A similar study also showed organic potatoes to contain lower levels of contaminants than conventionally farmed specimens (Mansour et al., 2009a). Further elucidation of potato contamination compared different washing and cooking methods, and showed that deep-fried chips contained lower OCP contaminant levels than pommes frites, with both lower than uncooked potatoes (Soliman, 2001). In addition, potato skin samples contained the highest levels. Many locally produced foods in Ismailia city were assessed for various contaminants, but dicofol was found only in cucumbers, and then at a level (0.021 mg/kg) well below recommended safe limits (Table 2.1.1) (Loutfy et al., 2008). Another study on samples from several Egyptian markets ascertained dicofol as the most frequently occurring pesticide residue (5.1% of samples), notably in peppers and strawberries (Dogheim et al., 2002).

Fish and shellfish

Mussels from Abu Qir Bay contained several OCPs, with DDT concentrations up to 31 µg/g dw (Table 2.1.1), but a risk assessment showed no expected adverse effects on people through mussel consumption (Kasozi et al., 2006). Study of fish in Assiut city region found higher levels of various OCPs in catfish compared to tilapia, though all were below recommended MRLs (Table 2.1.2) (Yahia and Elsharkawy, 2014). Tilapia fish from the Manzala Lake on the north eastern edge of the Nile Delta contained several OCPs, including DDTs, although levels were below FAO/WHO maximum permissible limits (Azab et al., 2013).

Edible offal

Even within a single animal species, OCP levels may vary by tissues; for example, buffalo tongue has been shown to contain higher concentrations than liver or kidney (Mahmoud et al., 2013). In this study, HCHs were the predominant OCP detected (up to 351.57 ng/g lw in tongue samples) (Table 2.1.1).

Milk

Assessment of raw buffalo milk in the Egyptian city of Assiut showed some variation in pesticide level between sources, with the number of OCPs detected lower in milk samples from local vendors, although this was combined with high

mean values for HCH (88% of samples) and pesticide types other than OCPs (Table 2.1.1) (Shaker and Elsharkawy, 2015).

Ethiopia

Milk

As in Egypt, milk samples in Ethiopia were found to contain several pesticides, with highest concentrations seen in malaria areas, and overall cow and goat samples contained an average DDT level of 328.5 µg/kg (Table 2.1.1) (Deti et al., 2014). Analysis of cow milk samples focusing on the south west of the country also detected DDTs, with *p,p'*-DDT predominating (Gebremichael et al., 2013).

Edible offal

Cattle samples from the West Shoa Zone contained several OCPs, with levels reducing by tissue: liver > kidney > meat (Letta and Attah, 2013). Heat treatment (boiling for 90 minutes) was shown to reduce contamination levels by 29–62.2%, depending on the OCP.

Fish

In the Ethiopian Rift Valley lakes, various fish species have been assessed for OCP contamination. In Lake Awassa, DDTs were the most predominant OCP, with the greatest degree of contamination (up to 56 ng/g ww) seen in fish at the highest trophic level, the African big barb (Deribe et al., 2014). Levels in this region were shown to exceed safety limits published by the US EPA for children under 1 year of age. In a similar study in Lake Ziway fish, DDTs again predominated (up to 61.9 ng/g ww), and calculated cancer risk estimates and hazard ratios indicated a potential cancer risk from consumption of the fish (Table 2.1.1) (Yohannes et al., 2014b).

Other food substrates

Samples of various foods from a local market in the Jimma Zone showed a third to be contaminated with pesticides at levels above recommended MRLs, predominantly DDTs and endosulfans, with red peppers and green coffee beans the most contaminated (Mekonen et al., 2014). Khat leaves (chewed locally) were found to have levels of DDTs over 1,000 times the EU MRL in some locations (Tables 2.1.1, 2.1.2) (Daba et al., 2011).

Ghana

Cereal crops

In Ghana, assessment of 10 brands of processed cereal-based complementary foods detected levels of several OCPs exceeding recommended MRLs, and indicated possible adverse health effects particularly on infants and young children (Akoto et al., 2013). Cowpea and maize samples from farms in Ejura contained several OCPs at levels exceeding MRLs (Table 2.1.2) and again showed potential for chronic toxicity when consumed (Akoto et al., 2015).

Fruits and vegetables

Fruits purchased in Accra Metropolis were analysed, and both locally-produced fruits (pawpaw and tomato) and imported apples were found to have pesticide contamination, mainly with OCPs, with 32.8% of samples exceeding MRLs (Table 2.1.2) (Bempah and Donkor, 2011). Okra grown on a farm adjoining one using pesticides, though not directly subjected to pesticide application, was shown to be contaminated by several OCPs (Essumang et al., 2013). Lettuce from various markets and sellers in three Metropolitan Districts contained lindane, endosulfan and DDT in over 30% of samples, exceeding MRLs (Amoah et al., 2006). Half of tomatoes sampled from a vegetable-farming community in Offinso District contained heptachlor epoxide (Ndengerio-Ndossi and Cram, 2005).

Fish

Although fish from the Densu river basin contained several OCPs, with α-endosulfan predominating, MRLs were not exceeded (Khairy et al., 2012). A study sampling fish from several lakes in Ghana determined DDTs to have the highest concentration, with the highest level found in a catfish (2,205.50 ng/g lw) (Table 2.1.1) (Adu-Kumi et al., 2010).

Dairy products

Examination of dairy products in the Kumasi metropolis showed pesticide contamination, with 75% containing aldrin (Darko and Acquaah, 2008). Of note in these samples was the high ratio of DDT to DDE concentrations, suggesting recent exposure to DDT, although regional variations were present (Table 2.1.1).

Morocco

Vegetables

Tomatoes cultivated in greenhouses in the Souss Massa Valley of Morocco showed endosulfan as the predominant OCP (1.2 mg/kg), but only 2 of 120 samples exceeded MRLs established by European Union legislation (Table 2.1.2) (Ntow, 2001). Dicofol was also detected in samples.

Fish and shellfish

A separate study focused on clams and eels sampled from the Moulay Bousselham lagoon on the west coast of the country (Mehdaoui et al., 2000). DDTs were shown to accumulate, particularly in samples taken from near channels draining agricultural land. Notably, higher concentration was observed in eels, particularly in large specimens.

Nigeria

Cereal crops

White maize samples from markets in the Lagos State were analysed for OCP residues (Ogah et al., 2011). 96% of samples contained at least one OCP, with the mean OCP concentration up to 52.0 µg/kg; this resulted in MRLs being exceeded in up to 7% of the samples. The study highlighted particular concern for levels of aldrin and dieldrin in the diet (Table 2.1.1).

The Gambia and Senegal

Fish and shellfish

Sampling of fish and shrimps from local markets in The Gambia and Senegal showed contamination of OCPs, mostly DDTs (Manirakiza et al., 2002). DDT levels in shrimp (199.2 ng/g fat) exceeded those detected in fish (95.2 ng/g fat) respectively, with both less than the European Union MRL of 1,000 ng/g edible fat. Analysis of molluscs from southwestern Senegal also confirmed contamination with OCPs, including lindane, HCB, cyclodienes (heptachlor and *trans*-nonachlor) and DDTs (Bodin et al., 2011). In these molluscs, OCP levels were similar to those found in surface sediments. DDTs were the most abundant OCP (up to 15.6 ng/g dw) (Table 2.1.1).

South Africa

Chicken eggs

In the Limpopo Province of South Africa where DDT is used for IRS, chicken eggs were found to contain DDTs up to 48,000 ng/g ww (Bouwman et al., 2015).

Fish

In the same region, DDTs were detected in 2 fish species, with concentrations and pollutant profile varying depending on sampling location (Barnhoorn et al., 2009).

Vegetables

Leafy vegetables in the same region were shown to have a predominance of *o,p'*-DDT and *o,p'*-DDD while *p,p'*-DDE predominated in chicken samples; both food types exceeded safe consumption limits by WHO guidelines (Van Dyk et al., 2010b).

Milk

KwaZulu-Natal Province is another malaria control area, but DDTs detected in cow milk samples in this region were lower than FAO-stipulated MRLs (Table 2.1.1) (Mwevura et al., 2002).

Tanzania

Fish and shellfish

Tilapia fish from 4 lakes in Tanzania showed geographical variation in pollutants, with endosulfanes highest in Lake Victoria (mean 94 ng/g lw) and DDTs highest in Lake Tanganyika (mean 274 ng/g lw); levels were below EU MRLs (Polder et al., 2014). Assessment of various OCPs in Nile tilapia and Nile perch from Lake Victoria detected only DDTs and endosulfans, mostly less than calculated ADI limits (Henry and Kishimba, 2006). Crustaceans collected from coastal and estuarine sites near Dar es Salaam contained DDTs at levels deemed safe for human consumption, with levels depending on mode of feeding and age of the specimen (Table 2.1.1) (Mwevura et al., 2002).

Vegetables

An interesting study analysed "table-ready" foods (ready for consumption), and although levels did not pose a health risk according to recommended limits, there

was concern over the presence of DDTs in foods, particularly spinach (2.89 µg/kg) (Ndengerio-Ndossi and Cram, 2005).

Togo

Cereal crops

Assessment of cowpea grains and maize grains in Togo detected several OCPs, including dieldrin, endrin, heptachlor epoxide and endosulfan (Mawussi et al., 2009). Although levels in maize (up to 65.70 µg/g) were below the MRL set by the World Health Organization (WHO) (Table 2.1.2), those in cowpea grains exceeded this level (up to 98.79 µg/g) (Table 2.1.1).

Tunisia

Fish

Fish contaminated by OCPs in Tunisia have been investigated in a number of species—Dover sole, mullet and sea bass—in the Bizerte Lagoon in the northern part of the country (Amoah et al., 2006; Aneck-Hahn et al., 2007). In the Dover sole study, the dominant chemicals were HCB, *p,p'*-DDE and *o,p'*-DDD. The report suggests many sources for the pollution, including surface run-off and wastewater discharges from intensively cultivated areas. DDTs were present in greater levels than HCHs or HCB. The distribution pattern of pesticide accumulation differed between fish species, with Dover sole containing the highest levels overall (752 ng/g lw for all OCPs, compared to 265 ng/g lw in sea bass and 157 ng/g lw in mullet) (Table 2.1.1).

Uganda

Fish

Nile perch and Nile tilapia sampled from the northern shore of Lake Victoria in Uganda contained HCHs, at levels considered safe for human consumption (Ssebugere et al., 2014). Assessment of other OCPs in these fish also showed residues below recommended MRLs (Kasozi et al., 2006). In southwestern Uganda, 5 fish species from Lake Edward were analysed for DDT, and a maximum level of 68 µg/kg fw was detected; most samples were below

FAO/WHO MRLs (Table 2.1.2) (Ssebugere et al., 2009). Fish farms in Uganda export internationally, and analysis of such farmed Nile tilapia and African catfish detected OCPs, although within prescribed limits (Bagumire et al., 2008). Interestingly, DDT and endosulfan were only detected in catfish, suggesting this species is more prone to contamination than tilapia. Belly flap oil from Nile perch in Lake Victoria was found to contain several OCPs, predominantly DDTs (Ogwok et al., 2009). Concentrations of these increased with fish size, and notably levels of endosulfan in the group of largest fish exceeded MRLs.

Milk

Cow's milk from Kampala markets showed pasteurized samples to contain lower levels of OCPs than fresh samples, however most were above international residue limits and thus likely pose a risk to human health from consumption (Table 2.1.1) (Kampire et al., 2011).

Comparing the OCP situation in African countries with that outside Africa, it notes worthy that DDT use has been banned since the 1970s/1980s in Europe, North America and the temperate industrial regions of the northern hemisphere. DDT use has been continuous in some parts of Asia and Africa as well as in central and south America (Kalantzi et al., 2001). For instance, approximately a 100-fold reduction in the concentration of DDT and HCH is recorded in farm products during the last two decades in India (Kannan et al., 1992). Correspondingly, in Japan, DDT and HCH concentrations were less than in developing countries, as concentrations in most foodstuffs were less than 0.05% of the recommended MRLs (Matsumoto et al., 1988).

In general, analysis of the cited literature in this mini-review showed a clear bias in reporting of pesticide contamination in peer-reviewed publications, with some African countries highly represented such as Egypt, Ethiopia, Ghana and South Africa. Data about national pesticide usage—current and historical—are not always readily available, and it is unlikely that published reports accurately reflect the extent of pesticide contamination in each country. Of note was the detection in foods of certain pesticide residues in areas where use of specific pesticides has been banned. Some of these may be due to persistence in the environment,

but it is suspected that some instances relate to inappropriate or illegal use of pesticides (Soliman, 2001).

Public health importance of OCPs

It is well-established that some OCPs are still used in African countries for various reasons including disease control, malaria in particular (WHO, 2003). These and obsolete pesticide stocks can contaminate food, water, soil and air, and pose serious health threats to Africa's rural and urban populations (UNEP, 2006). Thus, OCP exposure is a public health concern among African populations. It is not surprising that, according to WHO, one-third of disease burden in Africa is attributable to environmental hazards (Prüss-Üstün and Corvalán, 2006). However, there is a lack of clear epidemiologic data relating specific pesticide exposures to adverse health effects among African populations. In this section, we will highlight recent studies (published between 1st January 2000 and 11th August 2015) confirming human exposure to OCPs with serum and breast milk concentrations and possible related adverse health effects (Table 2.1.3 and Table 2.1.4).

Detection of OCP residues in human serum in African countries

Benin

Azandjeme et al. (Azandjeme et al., 2014) measured the distribution of serum concentrations of 14 OCPs in 118 diabetic subjects (54.2% men and 45.8% women; 43% lived in urban areas, 14.4% were obese and 39.8% had high economic status) in Benin. The four detected OCPs were *p,p'*-DDT (497.1 ± 4.5 ng/g of total serum lipids), *p,p'*-DDE (20.6 ± 7.9), β-HCH (2.9 ± 3.4) and *trans*-nonachlor (2.0 ± 2.3). OCP levels were significantly higher in obese, wealthier and more educated subjects, and in those living in urban areas as compared to the other groups, particularly for *p,p'*-DDE, *p,p'*-DDT and β-HCH (Table 2.1.3).

Table 2.1.3: Reported levels of OCPs in human serum samples in African countries

Country	Pesticide	Concentration detected (ng/g lw)	Reference
Benin	*p,p'*-DDT	497	(Azandjeme et al. 2014)
	p,p'-DDE	21	
	β-HCH	3	
	Trans-nonachlor	2	
Congo Republic	ΣOCPs	660	(Luzardo et al. 2014)
Egypt	DDE	40	(Ahmed et al. 2002)
Gambia	ΣDDTs	6,920	(Manirakiza et al. 2002)
Ghana	HCB (ng/g)	30	(Ntow 2001)
	p,p'-DDE (ng/g)	380	
Guinea Bissau	ΣOCPs	134	(Luzardo et al. 2014)
Senegal	ΣOCPs	124	(Luzardo et al. 2014)
Sierra Leone	ΣOCPs	574	(Luzardo et al. 2014)
South Africa	HCH	956	(Channa et al. 2012)
Sudan	Heptachlor (ng/g)	170	(Elbashir et al. 2015)
	ΣDDE (ng/g)	618	
	α-HCH (ng/g)	92	
	Dieldrin (ng/g)	82	
Tunisia	*p,p'*-DDE	169	(Ben Hassine et al. 2014)
	HCB	49	
	p,p'-DDE	128	(Artacho-Cordón et al. 2015)
	HCB	20	

lw = lipid weight. DDTs = DDT + DDE + DDD, HCB = hexachlorobenzene, HCH = hexachlorocyclohexanes

Egypt

Ahmed et al. (Ahmed et al., 2002) conducted an investigation to detect residues of DDE in blood serum samples collected from fasting females in Port Said, Egypt between July 1999 and July 2000. Included in the study were 43 women diagnosed with invasive adenocarcinoma of the breast, 21 suffering benign breast disease, and 11 healthy individuals. Mean residues of DDE detected in the three groups examined were 41 ± 5.2, 48 ± 6.2 and 31 ± 2.5 ng/g for breast cancer cases, benign breast disease cases and controls, respectively, indicating significantly lower residues in blood serum from control females (Table 2.1.3). In addition, Elserougy et al. (Elserougy et al., 2013) found a high odds ratio (8.3) in *o,p'*-DDD between maternal and umbilical sera of mothers, suggesting potential placental transfer of OCPs between mothers and their children during pregnancy.

Sene-Gambian region

Manirakiza et al. (Manirakiza et al., 2002) measured the OCP concentrations in human serum samples from the Sene-Gambian region. α-HCH, *p,p′*-DDE, *o,p′*-DDT and *p,p′*-DDT were detected in all 16 pooled serum samples, whereas endosulfansulfate, methoxychlor, mirex, heptachlorepoxide and endrin were detected in 15 samples (Table 2.1.3).

Ghana

Ntow (Ntow, 2001) collected serum samples from inhabitants of Akumadan, a prominent vegetable-farming community in Ghana. High HCB and *p,p′*-DDE residues were found in serum; mean values were 30 ng/g and 380 ng/g, respectively. Additionally, DDTs and dieldrin residues were significantly higher ($p < 0.05$) in males' than in females' pooled samples of human serum (n = 115) from vegetable farmers in Ghana, during 2005 (Ntow et al., 2008) (Table 2.1.3).

Guinea-Bissau

Linderholm et al. (Linderholm et al., 2010) collected serum samples from an open cohort of police officers in Guinea-Bissau, (n = 33) at five time points between 1990 and 2007, totaling 147 samples. They observed that the major OCP in all samples was *p,p′*-DDE followed by *p,p′*-DDT. Levels of *p,p′*-DDE, *p,p′*-DDT, β- and γ-HCH significantly decreased over time.

South Africa

Channa et al. (Channa et al., 2012) reported on the concentrations of α-, β- and γ-HCH and HCB detected in maternal blood plasma from delivering women (n = 241) in three coastal sites of the KwaZulu-Natal Province, South Africa. γ-HCH was the most dominant pesticide at all three sites. Significantly higher levels of γ-HCH (mean 956 ng/g lipids) were found in site 3 (Empangeni town vicinity) compared to the other two sites. HCB, α-HCH and β-HCH were detected in less than 31% of the samples from all sites. Additionally, investigators concluded that the high levels of γ-HCH in maternal plasma samples at site 3 indicate current

and on-going exposure, which is of great concern for reproductive health and perinatal exposure (Table 2.1.3).

Sudan

Elbashir et al. (Elbashir et al., 2015) collected 96 human blood samples from six locations representing areas of intensive pesticide use, including irrigated cotton schemes (Wad Medani, Hasaheesa, Elmanagil and Elfaw) and sugarcane schemes (Kenana and Gunaid) in Sudan. Residues of *p,p′*-DDE, heptachlor epoxide, γ-HCH and dieldrin were detected in blood from all locations surveyed. The levels of total organochlorine burden detected were higher in blood from people in the irrigated cotton schemes (mean 261 ng/ml) than from the irrigated sugarcane schemes (mean 204 ng/ml). The highest levels of heptachlor epoxide (170 ng/ml) and γ-HCH (92 ng/ml) were observed in blood samples from Hasaheesa, while the highest levels of DDE (618 ng/ml) and dieldrin (82 ng/ml) were from Wad Medani and Kenana, respectively (Table 2.1.3).

Tunisia

Ben Hassine et al. (Ben Hassine et al., 2014) detected *p,p′*-DDE and HCB in > 95% of human serum samples (n = 113) from Bizerte, northern Tunisia, collected between 2011 and 2012. The mean levels of *p,p′*-DDE and HCB in serum were 168.8 and 49.1 ng/g lipid, respectively. However, in another study, slightly lower concentrations (127.59 and 19.98 ng/g lipid) were recorded for *p,p′*-DDE and HCB in serum samples from 54 Tunisian women (Artacho-Cordón et al., 2015). These authors observed that age, working outside the home and cereal consumption were positively correlated with serum levels of *p,p′*-DDE (Table 2.1.3).

Luzardo et al. (Luzardo et al., 2014), measured levels of 36 POPs in the serum of recent immigrants (n = 575) from 19 Sub-Saharan countries entering the Canary Islands, Spain. OCP levels increased with age. The most frequently detected compound was *p,p'*-DDE (100% of the samples); its parent compound (*p,p'*-DDT) was detected in 72.2% of the samples. Participants from the Republic of the Congo and Sierra Leone had the highest levels of OCP contamination

(median 660 ng/g lipid and 574 ng/g lipid, respectively). Those from Guinea Bissau and Senegal had the lowest levels (median 134 ng/g lipid and 124 ng/g lipid, respectively).

Detection of OCP residues in human breast milk in African countries

During pregnancy, the placenta appears to allow transport of OCPs to the developing foetus (Elserougy et al., 2013). Maternal breast milk is also a potentially significant source of some pesticides for breast-fed infants (Mishra and Sharma, 2011). Conversely, pregnancy and lactation are routes by which OCPs can be decreased in the maternal body (by vertical transmission), and levels in maternal serum have been shown to reduce with each parity. Due to concerns regarding susceptibility to toxic effects of DDTs in young children, the focus of OCPs in breast milk samples has been mainly on assessment of levels of DDT and its metabolites (Manaca et al., 2011).

Egypt

Elserougy et al. (Elserougy et al., 2013) detected lindane in breast milk of 38 healthy participants submitted to cesarean delivery with a mean value of 90 ng/g. DDTs were also detected in about 65% of breast milk specimens (Table 2.1.4).

Ghana

Both DDE and HCB were detected with mean concentrations of 490 ng/g lw and 40 ng/g lw, respectively, in breast milk samples taken from residents of a farming community in Ghana (Ntow, 2001). Additionally, Ntow et al. (Ntow et al., 2008) determined OCP concentrations in pooled samples of human breast milk (n = 109) from vegetable farmers during 2005. The mean concentrations of ΣDDTs, ΣHCHs, dieldrin and HCB were 78.3, 46.4, 122.8 and 4.9 ng/g lw, respectively (Table 2.1.4).

Mozambique

Concentrations of DDTs in breast milk were higher in samples collected in 2006 (930 ng/g lw) compared with those from 2002 (370 ng/g lw) in two populations of mothers in Manhiça, Mozambique. The 2006 samples were obtained several months after implementation of indoor residual spraying with DDT for malaria vector control in dwellings, while the earlier samples were taken for reference prior to DDT use (Manaca et al., 2014) (Table 2.1.4).

South Africa

A recent study investigated the levels of DDT in 163 breast milk samples from four South African villages, three of which use DDT in IRS to control malaria. Mean ΣDDT levels in breast milk were 18, 11, and 9.5 mg/kg mf from the DDT-sprayed villages, respectively, including the highest ΣDDT level ever reported for breast milk from South Africa (140 mg/kg mf) (Bouwman et al., 2012).

Tunisia

Ennaceur et al. (Ennaceur et al., 2007) measured the levels of 13 OCPs in breast milk from 87 Tunisian mothers throughout their lactation periods. All samples contained detectable residues of DDT, the mean concentrations of ΣDDTs, HCB, ΣHCHs and dieldrin were 3.863, 0.260, 0.067 and 0.059 ng/g lw, respectively. Additionally, OCPs were determined in breast milk samples (n = 36) of primipara and multipara mothers from Bizerte in 2010 (Ben Hassine et al., 2012). The mean concentrations of ∑DDTs and HCB in breast milk were 1,163.9 and 286.8 ng/g lw respectively (Table 2.1.4).

It is clear from the previously mentioned information in this section that breast milk is a major source for OCPs by infants in areas where the pesticides are used. Infants depend on breast milk as their main source of nutrition, and are therefore at high risk of adverse effects related to OCPs, such as neurological and reproductive disorders (Balali-Mood and Balali-Mood, 2008).

Table 2.1.4: Reported levels of OCPs in human breast milk samples in African countries

Country	Pesticide	Concentration detected (ng/g lw, except where specified)	Reference
Egypt	Lindane	90	(Elserougy et al. 2013)
Ghana	HCB	40	(Ntow 2001)
	p,p'-DDE	490	
	ΣDDTs	78	(Ntow et al. 2008)
	ΣHCHs	46	
	Dieldrin	123	
	HCB	5	
Mozambique	ΣDDTs	930	(Manaca et al. 2011)
South Africa	ΣDDT	ND– 8,540 ng/g	(Okonkwo et al. 2008)
	ΣDDE	1–14,580	
	ΣDDD	ND–5,910	
	ΣDDTs	8-140 mg/kg mf	(Bouwman et al. 2012)
	ΣDDTs	1.3 – 10 µg/g mf	(Sereda et al. 2009b)
Tunisia	ΣDDTs	1,164	(Ben Hassine et al. 2012)
	HCB	289	
	p,p'-DDE[a]	661	(Ennaceur & Driss 2013)
	p,p'-DDT[a]	438	
	p,p'-DDE[b]	77	
	p,p'-DDT[b]	106	
	ΣDDTs	1,931	(Ennaceur et al. 2008)
	ΣHCHs	65	
	HCB	85	
	Dieldrin	25	
	HCB	260	(Ennaceur et al. 2007)
	ΣHCHs	67	
	Dieldrin	59	
	ΣDDTs	3,863	

ND = below limit of detection, lw = lipid weight, mf = milk fat, LOQ = limit of quantification, LOD = limit of detection.
DDTs = DDT + DDE + DDD, HCB = hexachlorobenzene, HCH = hexachlorocyclohexanes
[a] 3 d post-partum, [b] 8 m post-partum

Possible human health hazards from OCP exposure

In Africa, very few health effect studies of worker populations—including farm workers and those administering IRS—have directly assessed the adverse effects associated with single or multiple pesticide use and exposure among general African populations, and no studies have examined the toxicological consequences of interactions resulting from cumulative exposures to several

pesticides in these populations. Most of the published studies on farmworker populations rely on self-reported information to diagnose adverse effects and therefore may not provide the most objective data.

OCPs are suggested to be endocrine disrupting chemicals (McKinlay et al., 2008), believed to produce a wide variety of adverse health outcomes in people such as reduced fertility and fecundity, spontaneous abortion, skewed sex ratios within the offspring of exposed communities (Windham et al., 2005), and male and female reproductive tract abnormalities (Bretveld et al., 2008).

In South Africa, Aneck-Hahn et al. (Aneck-Hahn et al., 2007), conducted a cross-sectional study on healthy male subjects (n = 311) between 18 and 40 years of age in Limpopo Province, an endemic malaria area where DDT is sprayed annually. The results showed a significant positive association between percent sperm with cytoplasmic droplets, low ejaculate volume, and *p,p'*-DDT concentration. Additionally, 28% of the study group presented with oligozoospermia and 32% with asthenozoospermia. In another study, de Jager et al. (de Jager et al., 2009) suggested a weak link between non-occupational environmental DDT exposure and a negative impact on sperm chromatin integrity in young South African males.

Positive associations of OCPs with a wide variety of human cancers have been reported (Hoyer et al., 2000). For instance, Ahmed et al. (Ahmed et al., 2002) reported higher residual levels of DDE in the sera of invasive adenocarcinoma cases compared with control subjects. In Tunisia, Arrebola et al. (Arrebola et al., 2015) found a positive association between breast cancer risk and β-HCH, HCB, heptachlor and *p,p'*-DDE.

From the aforementioned reports, it is clear that OCP exposure might have implications on public health. However, it notes worthy that analysis of public health implications of OCPs due to food consumption must be quoted carefully due to many reasons such as: 1) Most studies take a single sample from each food type for analysis, but the distribution pattern of contamination within a foodstuff may vary. An example is higher pesticide residues present in the skin compared to the pulp of potato tubers (Soliman, 2001). 2) Concentrations of

pesticides in soil samples can be important indicators as vegetables can accumulate these chemicals efficiently, with levels 4–45 times higher in the plant than in the soil (Gonzalez et al., 2005). 3) The method of farming and season also result in different residue concentrations in plants (Mansour et al., 2009a). 4) Food preparation, particularly cooking, alters pesticide content and therefore human risk from consumption (Letta and Attah, 2013; Soliman, 2001). 5) Another complicating factor in interpretation is a lack of standardisation of reporting concentrations. As OCPs are lipophilic, it is often useful to report results according to lipid weight of samples, but for human risk analysis dry weight or wet weight of foods are more relevant to estimate daily consumption values. Even within countries and sampling areas, there may be great variation in levels of residue concentrations in samples, making general risk assessments difficult.

In many instances, high percentages of foods tested contained pesticide residues, often above MRLs set either at national or international levels. This shows that a potential risk exists for people consuming such foodstuffs. Some reports state the “violation percentage” of foods, that is, those containing levels greater than permissible limits. These stated limits vary, for example European versus International (e.g. Codex Alimentarius Commission, or Joint FAO/WHO Meeting on Pesticide Residues) limits, making comparative interpretation difficult. Where multiple chemicals are analysed concurrently, samples are often shown to contain residues from many compounds (Mahmoud et al., 2013). The toxic effects of such chemical cocktails are difficult to ascertain. Age or life stage of the consumer may also be relevant to the exposure risk or toxic effects – for example, children may be exposed to higher levels of DDTs from breast milk and may also be more susceptible to the endocrine disruptive effects of such chemicals (Mishra and Sharma, 2011).

Conclusion

Food contamination is the main route of exposure to OCPs for people. However, levels of OCP residues in food and possible human health risk assessment must be analysed carefully for several reasons. These reasons include the sample size, the type of the plant, the consumed parts of the plant, the feeding habits of the people in each country, the method of farming and the season, food preparation methods, and the lack of standardisation of reporting concentrations, age and health conditions of the consumers.

In addition, this review declares without doubt that Africa's environmental health issues are complex and need more effort and collaborations between governmental authorities and research institutes for continuous surveillance programs in order to draw a clear map about the pesticide situation in Africa, in particular OCPs.

2.2 Human health risk from consumption of marine fish contaminated with DDT and its metabolites in Maputo Bay, Mozambique

Abstract

Many countries with malaria use DDT to reduce mosquitoes, and contaminated water from these areas enters the Indian Ocean at Maputo Bay. This study aimed to sample marine fish in the region to assess current contamination levels of DDTs, and the human health risk associated with their consumption. The median for ∑DDTs was 3.8 ng/g ww (maximum 280.9 ng/g ww). The overall hazard ratio (HR) for samples was 1.5 at the 75th percentile concentration and 28.2 at the 95th percentile. These calculations show a potential increased cancer risk due to contamination by DDTs, and local consumers should be advised against regularly eating fish meat.

Introduction

Some 90% of global malaria cases occur in the WHO African Region, necessitating control measures (WHO, 2016a). Indoor residual spraying (IRS) with pesticides such as dichloro-diphenyl-trichloroethane (DDT) is commonly used under WHO advisement to control the mosquito vectors of malaria in many countries. This pesticide contaminates the environment, both soil and waterways, and is persistent for many years. The ecological risk of DDTs on fish and other wildlife has been common knowledge for over seven decades (Cottam and Higgins, 1946; McHugh et al., 2011). Once ingested by biota such as invertebrates or fish, these lipophilic compounds bioaccumulate and biomagnify in the food chain (Yohannes et al., 2013). Although the mechanisms of toxicity are still unclear, DDT has now been classed by the International Agency for Research on Cancer as a Group 2A agent, probably carcinogenic to humans (IARC, 2016).

Maputo Bay is an important environmental site as water originating from the Phongolo/Maputo River Basin in three countries—Mozambique, Swaziland and South Africa—enters the Indian Ocean here. These countries use IRS as part of their malaria control strategies and thus the impact on the local people and wider environment should be assessed (Blumberg and Frean, 2007). The objectives of this study were therefore to assess the levels of DDT and its metabolites in muscle samples from different fish species, and to investigate possible human health risks from DDTs through consumption of contaminated fish in Maputo Bay.

Materials and Methods

Marine species caught by fishermen in Maputo Bay were purchased from local markets in the Maputo Province, southern Mozambique (Figure 2.2.1). The species were mainly reef fish, with various dietary behaviours (Heemstra and

Heemstra 2004). Marine species included: rockcod (*Epinephelus* spp, n = 7), blacktip kingfish (*Caranx heberi*, n = 5), spadefish (*Tripterodon orbis*, n = 4), delagoa threadfin bream (*Nemipterus bipunctatus*, n = 3), blue-lined barenose (*Gymnocranius grandoculis*, n = 2) and great barracuda (*Sphyraena barracuda*, n = 2). Muscle samples were collected from each fish, placed into clean plastic containers, and transported to the Laboratory of Toxicology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan. They were stored at -20°C in a deep freezer until analysis.

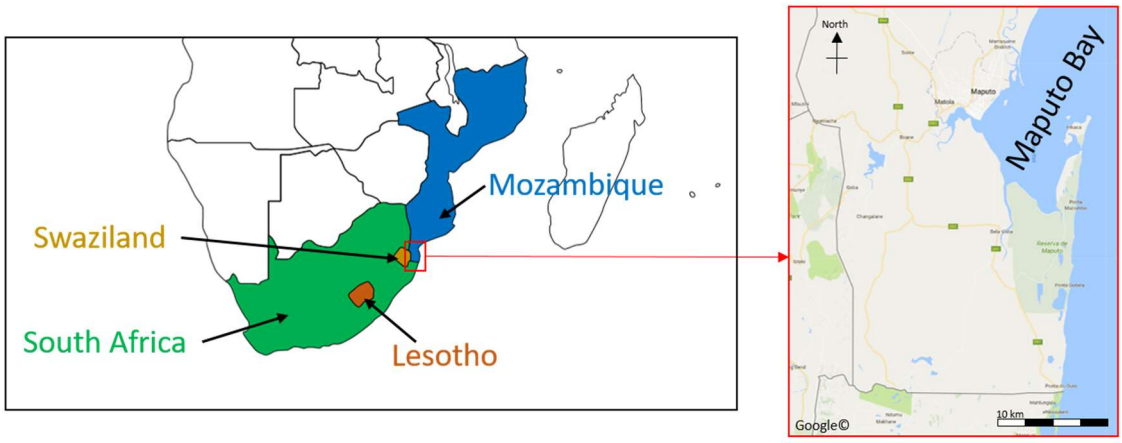


Figure 2.2.1: Map showing Maputo Bay sampling region in southern Mozambique.

DDTs were extracted and analysed using a modified protocol (Yohannes et al., 2014b). Approximately 5 g muscle sample was homogenized with anhydrous sodium sulfate, before extraction with hexane:acetone (3:1 *v/v*) in a Soxhlet extractor (SOX416 macro SOXTHERM unit, Gerhardt, Germany). An aliquot of the extract was used for gravimetric lipid determination. The surrogate standard 3,3',4,4'-tetrachlorobiphenyl (PCB 77) was used to spike the sample; then the extract was concentrated prior to clean-up in a glass column packed with activated florisil and eluted with hexane:dichloromethane (7:3 *v/v*). After further concentration, 2,4,5,6-tetrachloro-*m*-xylene was added as a syringe spike. Final analysis was conducted using a gas-chromatograph with ^{63}Ni electron capture detector (GC-ECD: Shimadzu GC-2014, Kyoto, Japan). Chemical identification in samples was performed by comparison of retention times with those of

standards (Dr Ehrenstorfer GmbH, Germany), quantifying concentrations in samples from peak areas compared to the internal standard. Multi-level calibration curves had square of correlation coefficients (R^2) greater than 0.99. Detection limits were between 0.16 and 0.45 ng/g, based on a signal to noise ratio (S/N) of 3:1.

Potential human health risk from consumption of fish meat was assessed. Using detected concentrations (C, ng/g ww) of DDTs, the estimated daily intake (EDI) was calculated using the following equation:

$$EDI = (C \times DR) / BW$$

Where DR is the average daily consumption of fish (23.3 g/d), according to published values for national consumption (FAO, 2013). BW is body weight (kg), which was set at 60 kg. EDIs were calculated at 25th, 50th, 75th and 95th percentiles of DDT concentrations, expressed as nanogram per kilogram body weight per day (ng/kg/bw/d). Then cancer risk estimates and hazard ratios (HR) were calculated using US EPA guidelines. For an acceptable lifetime cancer risk set at one in a million, i.e. 10^{-6}, the cancer benchmark concentration (CBC) for carcinogenic effects represents the lifetime exposure concentration. A risk level greater than 10^{-4} is considered unacceptable, while the area of concern is set between 10^{-4} and 10^{-6}. The cancer slope factor (CSF) for DDTs is set according to the Integrated Risk Information System (IRIS) database to 0.34 per mg/kg/d (US EPA, 2015), and CBC calculated thus:

$$CBC = 10^{-6} / \text{slope factor}$$

The hazard ratio (HR) for cancer risks was calculated by comparing EDI with CBC:

$$HR = EDI / CBC$$

With this definition, an HR of greater than one implies a greater than one in a million lifetime cancer risk (Dougherty et al., 2000).

Statistical analysis was performed using JMP Pro software, Version 12 (SAS Institute). Concentration of DDTs data are shown as median and range values in ng/g wet weight (ww) of tissue.

Results and Discussion

Contamination levels of fish differed among fish species. The median ∑DDTs by species ranged from 2.4 ng/g ww in *T. orbis* to 11.6 ng/g ww in *Epinephelus* spp. (Table 2.2.1). The highest value of ∑DDTs detected in an *Epinephelus* sample was 280.9 ng/g ww. Previously it has been shown that biota at higher trophic levels have higher accumulation of DDTs due to bioaccumulation and biomagnification effects (Yohannes et al., 2013). There is a diet overlap in fish analysed for this study (Heemstra and Heemstra, 2004). Further fish and environmental samples should be analysed to investigate this relationship in the study area. Considering all samples, the median ∑DDTs was 3.77 ng/g ww. A previous study on freshwater tigerfish (*Hydrocynus vittatus*) from Lake Pongolapoort showed contamination by DDTs of 5,400–6,000 ng/g lipid weight (Wepener et al., 2012). Water from this lake travels via the Phongolo River to Maputo Bay. Although a few samples in this study from Maputo Bay exceeded that level of contamination, the median for all fish sampled was 922.7 ng/g lipid weight.

Table 2.2.1 ∑DDTs (ng/g wet weight) detected in muscle from marine fish in Maputo Bay.

Species (n)	Median	Minimum	Maximum
Epinephelus spp (7)	11.6	ND	280.9
Gymnocranius grandoculis (2)	9.0	6.8	11.2
Sphyraena barracuda (2)	3.3	2.9	3.8
Nemipterus bipunctatus (3)	7.8	1.5	13.0
Caranx heberi (5)	2.4	ND	95.1
Tripterodon orbis (4)	2.4	ND	11.5
All samples (23)	3.8	ND	280.9

n = number of samples, ND = below level of detection

Of the DDT congeners analysed (*o,p′*-DDT, *p,p′*-DDT, *o,p′*-DDE, *p,p′*-DDE, *o,p′*-DDD and *p,p′*-DDD), *o,p′*-DDD was detected in only two fish samples, and *o,p′*-DDE was not detected in any. The most common congeners detected were *p,p′*-DDT (in *N. bipunctatus* and *G. grandoculis*), *p,p′*-DDE (in *C. heberi*, *T. orbis* and *S. barracuda*), and *p,p′*-DDD (in *Epinephelus* spp) (Figure 2.2.2). The highest concentration of *p,p′*-DDD, 210.8 ng/g ww, was detected in an *Epinephelus* sp. sample. This species is a major predator, and thus relatively higher contamination levels would be expected. Based on these data, the order of magnitude for abundance of congeners detected is: DDE > DDT > DDD. DDT is rapidly degraded both biotically and abiotically (Boul 1995). DDE is the most common metabolite of DDT detected in many species, and has been linked to some toxic side effects (Mrema et al., 2013). The *p,p′*-DDT congener was present in all but two fish samples, and the DDT/DDE ratio greater than one in nine samples, suggesting recent exposure to the parent DDT compound (Hooper et al., 1997).

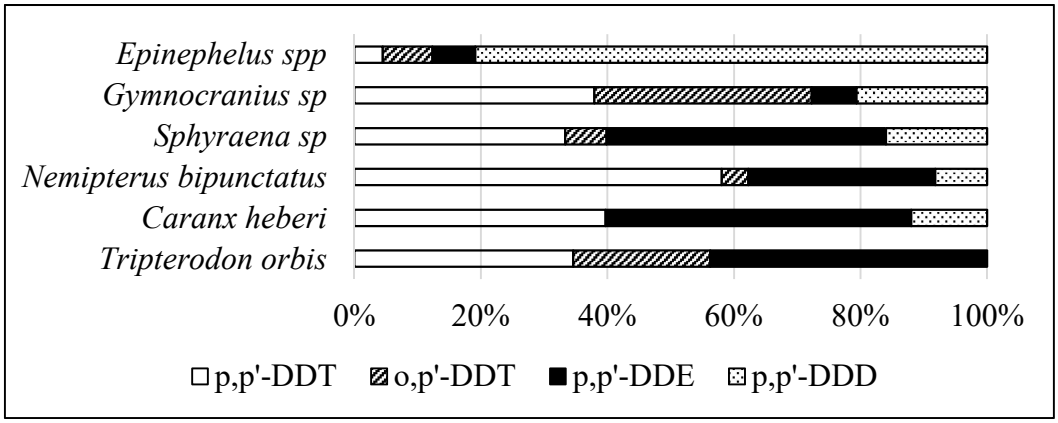


Figure 2.2.2: Relative abundance of DDT congeners in marine fish from Maputo Bay, Mozambique.

When all samples were considered and EDIs calculated, hazard ratios greater than one were found above the 75th percentile (HR of 1.5 at 75th and 28.2 at 95th percentile) (Table 2.2.2). These equate to 1.5 to 28.2 x 10^{-4} (1.5 to 28.2 chance in 10,000) risk of cancer associated with consumption of the fish. Calculations for *S. barracuda* alone did not show an increased risk. As expected, the greatest risk was associated with consumption of *Epinephelus* spp (HR of 1.5 at 50% and 34.9 at 95% percentile, or 1.5 to 34.9 chance in 10,000).

Fish are a very important part of the diet for many local people around Maputo Bay. As contamination concentrations and congener profiles vary between fish species, it is necessary to consider not only how much fish is consumed but also the species. All of the species sampled are fished for consumption, but discussions with local people in Inhaca at the time of sampling suggested that they were more likely to consume smaller fish species caught in shallow waters than larger species.

Table 2.2.2: Estimated daily intake values (EDI, ng/kg bw/d) of ∑DDTs in people from consumption of fish sampled, with corresponding cancer risk estimates (hazard ratio, HR). Values presented correspond to 25th, 50th, 75th and 95th percentile measured concentrations. An HR value greater than one indicates a potential health risk.

	EDI (ng/kg bw/d)				Cancer risk estimates (HR)			
Species	25th	50th	75th	95th	25th	50th	75th	95th
Epinephelus spp	0.6	4.5	46.9	102.7	0.2	1.5	16.0	34.9
Gymnocranius grandoculis	3.1	3.5	3.9	4.3	1.0	1.2	1.3	1.4
Sphyraena barracuda	1.2	1.3	1.4	1.4	0.4	0.4	0.5	0.5
Nemipterus bipunctatus	1.8	3.0	4.0	4.9	0.6	1.0	1.4	1.7
Caranx heberi	0.6	0.9	1.7	29.9	0.2	0.3	0.6	10.2
Tripterodon orbis	0.6	0.9	1.9	3.9	0.2	0.3	0.7	1.3
All species	0.7	1.5	4.5	82.9	0.2	0.5	1.5	28.2

In summary, historical and ongoing use of DDT in IRS programs results in contamination of the environment, including waterways. Thus, we investigated the concentrations of DDTs in muscle from marine fish species in Maputo Bay, Mozambique, and assessed the possible health risk through consumption of these fish. The results revealed that concentrations of DDTs ranged from ND to 280.9 ng/g ww. Contamination of aquatic species is a potential health risk not only for wildlife but also people, both locally and globally. Assessment of human health risk from consumption of fish meat shows that people eating *Epinephelus* spp. in particular should be made aware of the greater contamination levels and thus greater potential health risk from regular consumption of this species compared to others in the study. Future research should focus on alternatives to DDT use in vector control programs, as well as remediation methods for DDT and its metabolites in the environment and biota.

2.3 Concentrations and human health risk assessment of DDT and its metabolites in free-range and commercial chicken products from KwaZulu-Natal, South Africa

Abstract

Organochlorine pesticides such as dichloro-diphenyl-trichloroethane (DDT) have been used in agriculture and for disease control purposes over many decades. Reports suggest that DDT exposure may result in a number of adverse effects in humans. In the KwaZulu-Natal Province of South Africa, DDT is sprayed annually in homes (indoor residual spraying) to control the mosquito vector of malaria. In the northern part of the Province, samples of free-range chicken meat (n = 48) and eggs (n = 13), and commercially-produced chicken meat (n = 6) and eggs (n = 11) were collected and analysed. 94% (45/48) of free-range chicken meat samples contained DDTs (ΣDDTs median 6.1 ng/g wet weight (ww), maximum 79.1 ng/g ww). Chicken egg contents were also contaminated (ΣDDTs in free-range eggs median 9,544 ng/g ww, maximum 96,666 ng/g ww, and in commercial eggs median 1.3 ng/g ww, maximum 4.6 ng/g ww). The predominant DDT congener detected was *p,p'*-DDE in both free-range meat (> 63%) and eggs (> 66%), followed by *p,p'*-DDT and then *p,p'*-DDD. Based on estimated daily intake values, calculated human risk (carcinogenic) values were greater than one for DDTs detected in both free-range chicken products. Consumption of free-range eggs poses a particularly high health risk.

Introduction

In the 1950s, dichloro-diphenyl-trichloroethane (DDT) became popular as an agricultural pesticide, but associated toxic effects were soon seen, initially as population declines in wild avian species. DDT use is now permitted only under advisement by the World Health Organization (WHO) with special exemption from the Stockholm Convention for approved disease vector control (Secretariat of the Stockholm Convention, 2008). Although originally thought to be relatively safe in humans, reports have shown bioaccumulation to occur and suggested exposure may result in neurotoxic, carcinogenic, immunotoxic and reproductive effects (van den Berg, 2009). DDT is now classed by the International Agency for Research on Cancer as a Group 2A agent, probably carcinogenic to humans (IARC, 2016).

In South Africa, malaria is mostly caused by the *Plasmodium falciparum* parasite and spread mainly by *Anopheles arabiensis* and *An. funestus* mosquitoes (WHO, 2015a). According to WHO estimates there were over 35,000 cases of malaria in the country in 2015, with 110 deaths (WHO, 2016a). 10% of the population lives in a malaria-endemic area. Malaria is endemic in three provinces – KwaZulu-Natal (KZN), Limpopo and Mpumalanga (Maharaj et al., 2013, 2012). Health reports show that the uMkhanyakude district is the hotspot of malaria cases in the KZN Province, with 696 new cases (fatality rate of 1.7%) in the period 2013–2014, an incidence of 1.09 per 1000 population at risk (KwaZulu-Natal Department of Health, 2014). In the region, the mainstay of disease control is annual application of long-acting insecticides such as DDT in buildings (indoor residual spraying (IRS)) (Wepener et al., 2012). With interventions, KZN has shown the greatest reduction in both cases and number of deaths due to malaria since an outbreak in 2000 that resulted in over 40,000 cases and 100 deaths (KwaZulu-Natal Department of Health, 2016; Maharaj et al., 2013; Moonasar et al., 2012).

Although DDT is sanctioned for use only in malaria control programs, other possible sources of the pesticide exist. Previously-applied DDT residues may persist for up to 30 years in the environment, and obsolete chemicals accumulated prior to the ban may be being used without license (ATSDR, 2002). Exposure to DDT and its metabolites (DDTs) is a potential health risk for people in the area.

A common source of xenobiotic exposure is through ingestion, such as consumption of contaminated livestock products. The primary objective of this study was thus to investigate the presence of DDTs in chickens reared for consumption in an area of KZN where this chemical is currently used routinely to control malaria vectors, including an assessment of any ensuing potential health risk to people from consuming such free-range chicken meat or eggs.

Materials and Methods

Study area

The study area is located in the north-eastern corner of South Africa, bordered to the north by Mozambique and to the west by Swaziland (Figure 2.3.1). Sampling locations were within the Jozini (3,442 km^2 area) and uMhlabuyalingana (3,964 km^2) local municipalities, in uMkhanyakude District Municipality of KZN Province. Malaria is endemic in this district (KwaZulu-Natal Department of Health, 2014). Significant portions of this subtropical valley bushveld region are poorly developed rural areas, with a high level of poverty and poor service provision (Morgenthal et al., 2006). Industrial hubs in the province are in the more southerly urban areas, while the study region has no significant industry. The estimated population density in the two local municipalities from which samples were collected is a mere 46 people per km^2, with unemployment between 44 and 47% (Statistics South Africa, 2011a, 2011b). As such, products from home-reared

chickens offer a relatively cheap and accessible option for nutrition in the local population.

Malaria control in KZN is by IRS, predominantly using DDT. In the spraying season prior to sampling, 4.8 metric tons of DDT were applied to households by Jozini Health officials (official data). Spray coverage of households in KZN in 2013–2014 was 85%, down from the previous year due to factors such as use of temporary spray operators for spraying and surveillance, but still above the 80% coverage of premises as recommended by WHO (KwaZulu-Natal Department of Health, 2014; WHO, 2006b). Spraying of DDT for malaria control in the area is undertaken by staff who work under the guidance and central control of the Jozini Health Office. Living conditions for people and animals were similar in homesteads across the sampling area.

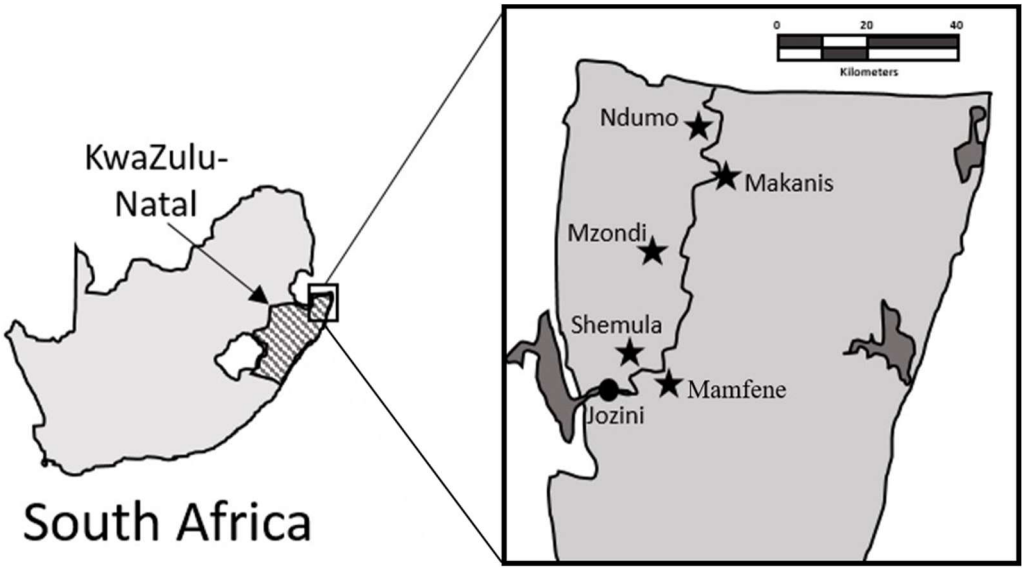


Figure 2.3.1: Map of region showing sampling sites in northern KwaZulu-Natal Province, South Africa

Sampling

Sampling was conducted in October 2014 during the dry season, just before spray teams commenced the annual IRS application. Representative rural

homesteads were selected within five areas covered by health centres under central control by the Jozini Health Office – namely Mamfene, Shemula, Mzondi (also known as Mlambongwenya), Makanis and Ndumo (Figure 2.3.1 and Table 2.3.1). Traditional buildings are thatch-roof huts with walls of mud/cement and floors of mud. Alternative buildings are constructed of concrete blocks with corrugated metal roofs. During IRS, inner walls are sprayed with pesticide, as are the outside eaves of thatched roofs, with DDT being used on un-painted surfaces. The surrounding ground that chickens inhabit within the homestead boundary is soil.

Table 2.3.1: Biometric data for samples in this study from KwaZulu-Natal.

	Free range	Commercially produced
Chickens		
Estimate age (months)[a]	7–30, mean 13 ± 7	N/K
Weight (kg)[b]	0.9–2.8, mean 1.5 ± 0.4	1.3–1.8, mean 1.5 ± 0.2
Body condition score (0-4)[c]	0–3, mean 2 ± 0.7	4 (in all)
Sex	22 male, 26 female	3 male, 3 N/K
Supplied diet[d]	Maize (home-grown or shop-bought), leftovers, rice, bread, fresh vegetables	Commercial feed
Lipid content of meat (%)	0.004–1.4, mean 0.3 ± 0.3	0.4–2.0, mean 1.5 ± 0.6
Source (*n*)[e]	Mamfene (8), Shemula (12), Mzondi (10), Makanis (10), Ndumo (8)	Jozini town, Jozini (n = 4), Ephondweni, uMhlabuyalingana (n = 2)
Eggs		
Supplied diet[d]	Maize (home-grown or shop-bought), leftovers, rice, bread, fresh vegetables	Commercial feed
Eggshell thickness (mm)	2.6–3.5, mean 3.0 ± 0.3	3.1–4.0, mean 3.6 ± 0.3
Lipid content of eggs (%)	7.8–13.1, mean 10.0 ± 1.5	7.9–21.2, mean 10.6 ± 3.5
Source (*n*)[f]	Mamfene (1), Shemula (1), Mzondi (7), Makanis (2), Ndumo (2)	Jozini (n = 8), Ephondweni (n = 3)

N/K, not known. Values are mean ± SD.
[a]Age estimation by owner at time of purchase.
[b]Body weights for free-ranging chickens are ante-mortem. Those for commercial chickens are as prepared for purchase, thus were eviscerated and excluded feathers/feet/head/neck.
[c]Based on Gregory and Robins (1998) 0–3 scale for layer hens, with an additional score of 4 for individuals with concave breast muscle development resulting in difficulty palpating the keel.
[d]Diet supplied by owner in addition to chickens foraging around the homestead.
[e]Commercial chickens were from three different companies (n = 2 of each). Source listed is purchase location.
[f]Commercial chicken eggs were from two different companies. Source listed is purchase location.

Muscle tissue of recently slaughtered free-ranging chickens living in the homesteads were purchased for DDT analysis (total n = 48, see Table 2.3.1 for biometric details). In the case of eggs, contents from free-range eggs (total n = 13) were analysed (Table 2.3.1). Commercially-produced and slaughtered chickens (n = 6) and chicken eggs (n = 11) were also purchased from local shops and sampled for comparative analysis. Each sample was individually stored in clean plastic vessels, transported to the laboratory in Hokkaido University, and maintained at -20°C until chemical analysis. After egg contents were collected,

shell thickness was measured using a micrometer at the equator of cleaned, dried eggs according to Kamata et al. (Kamata et al., 2013).

DDT analysis

A standard mixture of DDTs (Dr Ehrenstorfer GmbH, Germany), pesticide grade organic solvents and anhydrous sodium sulfate (Kanto Chemical Corp., Tokyo, Japan) were purchased. The DDTs analysed were: *o,p'*-DDT, *p,p'*-DDT, *o,p'*-DDD, *p,p'*-DDD, *o,p'*-DDE and *p,p'*-DDE.

Sample analysis was performed using a slightly modified version of the method previously described by Yohannes et al. (Yohannes et al., 2014b). In brief, a sample of approximately 5 g of muscle or 1 g of egg contents was homogenised with anhydrous sodium sulfate. After automatic extraction for 3.5 hours with a mixture of hexane:acetone (3:1 v/v) in a Soxhlet extractor (SOX416 macro SOXTHERM unit, Gerhardt, Germany), the sample was spiked with the surrogate standard 3,3',4,4'-tetrachlorobiphenyl (polychlorinated biphenyl, PCB, congener 77). The extract was concentrated and an aliquot separated for gravimetric lipid determination. Egg samples were then additionally subjected to gel permeation chromatography (GPC) packed with S-X3 Bio-Beads in a 500 mm x 25 mm glass column eluted with dichloromethane:hexane (1:1 v/v) for lipid removal. All samples underwent clean-up in a glass column packed with florisil (activated at 180°C for 8 h, and then 5% deactivated with distilled water) topped with anhydrous sodium sulfate, and eluted with hexane:dichloromethane (7:3 v/v). The resultant extract was concentrated to near dryness, before redissolution in n-decane. The internal standard 2,4,5,6-tetrachloro-*m*-xylene (TC*m*X) was added to the sample prior to instrumental analysis using a gas-chromatograph coupled with ^{63}Ni electron capture detector (GC-ECD: Shimadzu GC-2014, Kyoto, Japan).

Quality control and quality assurance

Chemicals were identified by comparing retention times with reference to corresponding standards, and concentrations quantified from sample peak area to that of the internal standard. Square of correlation coefficients (R^2) for multilevel calibration curves were all > 0.99. Based on a signal to noise ratio (S/N) of 3:1, detection limits were between 0.16 and 0.45 ng/g for all DDTs.

Risk assessment

Potential risk to human health through consumption of chicken products was assessed in two ways. Firstly, daily intake of DDTs in foods were calculated and compared with published guidelines for acceptable levels. Secondly, cancer risk due to consumption was estimated.

Estimated daily intake

The estimated daily intake (EDI) was calculated using the detected concentrations of DDTs in chicken meat and eggs. In samples with concentrations below the LOD, a value equivalent to half of the LOD was used for this calculation. As risk analysis is dependent on wet weight (ww) consumed, concentrations of DDTs detected are expressed on a wet weight (ww) basis.

$$EDI = (C \times DR) / BW$$

Where C is the measured concentration of DDTs (ng/g ww), DR is the average daily consumption rate of chicken product (g/d) and BW is body weight (kg), which was set at 60 kg. EDIs were calculated based on the DR derived from annual national poultry meat and egg consumption values (South African Poultry Association, 2015a, 2015b). In South Africa, the national reported daily chicken meat consumption is 103 g/d and chicken egg consumption is 24 g/d per person.

Estimation of the risk of non-carcinogenic effects from consumption of chicken meat was made by comparing these calculated EDIs with published reference doses: the WHO provisional tolerable daily intake (PTDI) of DDTs of 0.01 mg/kg bw/d, and the United States Environmental Protection Agency (US EPA) reference dose for chronic oral exposure (reference dose (RfD)) for *p,p'*-DDT of 0.0005 mg/kg bw/d (JECFA, 2010; US EPA, 2015).

Potential carcinogenic risks

US EPA guidelines were used to assess potential carcinogenic public health risks from consumption of chicken meat and eggs containing DDT residues. Cancer risk estimates and hazard ratios (HR) were calculated.

The cancer benchmark concentration (CBC) for carcinogenic effects represents the lifetime exposure concentration at which the acceptable lifetime cancer risk is set at one in a million, i.e. 10^{-6}. A risk level between 10^{-6} and 10^{-4} is considered to be of concern, while a level greater than 10^{-4} is considered an unacceptable risk. The CBC was calculated as the cancer risk divided by the cancer slope factor:

$$CBC = 10^{-6} / \text{slope factor}$$

The cancer slope factor (CSF) for DDTs was set to 0.34 per mg/kg/d, obtained from the IRIS database (US EPA, 2015). Comparison of EDI with the CBC for carcinogenic effects was used to assess the hazard ratio (HR) for cancer risks:

$$HR = EDI / CBC$$

Using the above definition of CBC, a one in a million lifetime cancer risk results in a HR of 1. Thus an HR value of greater than one indicates a potentially increased human health risk over that considered acceptable in the general population (Dougherty et al., 2000).

Statistical analysis

Data were processed and statistically analysed using Microsoft Excel 2014 and JMP® Pro 12 (SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) was used to produce descriptive statistics to characterise DDT levels in chicken samples. Tukey's HSD post hoc test was used for multiple comparisons among sample sites. The significance level was set at $p < 0.05$.

Results and Discussion

The fresh weight of the free-ranging chickens ranged from 0.9 to 2.8 kg, and the dead weight of commercial chicken carcasses from 1.3 to 1.8 kg (Table 2.3.1). The mean lipid (% ± standard deviation, SD) of chicken muscle was 0.3 ± 0.3 for free-range and 1.5 ± 0.6 for commercially reared chickens (Table 2.3.1). The mean lipid (%) of egg contents was 10.0 ± 1.5 for free-range and 10.6 ± 3.5 for commercially produced eggs (Table 2.3.1).

Levels of DDTs

Chicken meat

In muscle samples from free-ranging chickens in KwaZulu-Natal, varying concentrations of DDTs were detected. DDTs were not detected in muscle samples from commercially produced chickens.

Concentrations of DDTs (values of *p,p'*-DDE, *p,p'*-DDD and *p,p'*-DDT) detected in chicken meat in each area sampled in the region are shown in Table 2.3.2. The congeners *o,p'*-DDE, *o,p'*-DDD and *o,p'*-DDT were not detected in any meat samples. DDTs were detected in 93.8% (45/48) of free-range chicken muscle samples but were below the limit of detection (LOD) in all commercial samples. Summed DDT concentrations in free-range samples ranged up to a maximum of 79.1 ng/g ww, being highest in Makanis (median 29.6 ng/g ww) and lowest in Shemula (median 1.3 ng/g ww). In these samples, the predominant chemical

Table 2.3.2: Levels of DDT and metabolites in chicken products from KwaZulu-Natal.

	Chicken meat (uncooked breast meat), ng/g ww							
	Free range[a]							
	Mamfene	Shemula	Mzondi	Makanis	Ndumo	*Mean*	*Median (max)*	Commercially produced[a]
p,p'-DDE	6.9 ± 4.9 (7/8)	4.3 ± 6.2 (10/12)	6.8 ± 9.6 (10/10)	21.7 ± 16.3 (10/10)	11.7 ± 18.6 (8/8)	*10.1 ± 13.6 (45/48)*	*6.0 (60.2)*	< LOD
p,p'-DDD	2.4 ± 2.9 (5/8)	0.5 ± 0.8 (3/12)	0.2 (1/10)	1.6 ± 1.9 (8/10)	0.6 (1/8)	*1.0 ± 1.8 (18/48)*	*(6.7)*	< LOD
p,p'-DDT	1.4 ± 1.6 (6/8)	0.3 ± 0.5 (2/12)	0.18 (1/10)	9.4 ± 8.0 (10/10)	0.63 (1/8)	*2.4 ± 5.2 (20/48)*	*(26.2)*	< LOD
ΣDDTs	**10.8 ± 7.3 (7/8)**	**5.0 ± 7.0 (10/12)**	**7.2 ± 10.1 (10/10)**	**32.6 ± 25.5 (10/10)**	**12.9 ± 21.3 (8/8)**	***13.5 ± 18. 9 (45/48)***	***6.1 (79.1)***	**< LOD**

	Chicken egg contents (uncooked), ng/g ww			
	Free range[a]		Commercially produced[a]	
	Mean ± SD (number of samples)	***Median (maximum)***	**Mean ± SD (number of samples)**	***Median (maximum)***
o,p'-DDE	1.1 ± 1.8 (6/13)	*0.3 (5.1)*	0.8 ± 0.5 (5/11)	*0.7 (1.5)*
p,p'-DDE	12,079 ± 16,245 (13/13)	*6,819 (59,966)*	0.8 ± 0.7 (8/11)	*0.5 (2.2)*
o,p'-DDD	3.1 ± 3.9 (11/13)	*1.9 (11.3)*	<LOD	*<LOD*
o,p'-DDT	6.8 ± 8.5 (13/13)	*4.0 (32.4)*	1.0 (1/11)	*1*
p,p'-DDD	506.9 ± 760.3 (13/13)	*289.4 (2,870)*	0.3 ± 0.3 (8/11)	*0.2 (0.9)*
p,p'-DDT	5,485 ± 9,573 (13/13)	*1,918 (33,782)*	0.5 ± 0.2 (7/11)	*0.5 (0.9)*
ΣDDTs	**18,081 ± 25,879 (13/13)**	*9,544 (96,666)*	**1.5 ± 1.3 (11/11)**	***1.3 (4.6)***

LOD, limit of detection.

[a] Mean ± standard deviation (SD) (number of samples > LOD/total number of samples in group).

detected was the *p,p'*-DDE metabolite (median 6.0 ng/g ww, maximum 60.2 ng/g ww). The most likely reason for such high levels of DDTs is past and current use of DDT in IRS within homesteads, resulting in contamination of the local environment, although illegal usage or contamination from obsolete pesticides cannot be ruled out. These results indicate that free-ranging chickens in the sample area are subject to a high degree of DDT exposure, likely associated with the regular use of the chemical in IRS for malaria control in the region.

Composition profiles of DDTs in chicken meat are shown in Figure 2.3.2 (A). The predominant metabolite congener detected was *p,p'*-DDE, at 73.6% (range 29.5 to 98.3%), followed by *p,p'*-DDT (16.0%) and *p,p'*-DDD (10.3%). The predominant congener in technical grade DDT is *p,p'*-DDT, which is then degraded to the other two congeners, with *p,p'*-DDE the most persistent in biological organisms. In 50% (24/48) of free-range chicken samples, *p,p'*-DDE was the sole DDT congener detected (> LOD). Most samples contained either none or only a small percentage of the *p,p'*-DDD congener, but notably a higher proportion was detected in samples from Mamfene (maximum 37%). Between areas, Makanis contained the highest percentages of *p,p'*-DDT (14.1 to 61.0%). In this area, the ratio of *p,p'*-DDT to *p,p'*-DDE was > 1 in a single sample, suggesting the possibility of recent exposure to the parent chemical.

Comparison of different sampling sites showed a significant difference between concentrations of total DDTs at Makanis and Shemula ($p = 0.0036$) and Makanis and Mzondi ($p = 0.0126$). No significant association was shown between DDT

concentrations and parameters such as body weight, body condition score, estimated age, or sex. Concentrations of *p,p'*-DDE were significantly higher in Makanis (mean 21.6 ± 16.3 ng/g ww) than in Shemula (mean 4.3 ± 6.2 ng/g ww, p = 0.0212) and *p,p'*-DDT significantly higher in Makanis (mean 9.4 ± 8.0 ng/g ww) than in all other sites (p < 0.001 for Shemula and Mzondi, p = 0.0003 for Ndumo and p = 0.0011 for Mamfene).

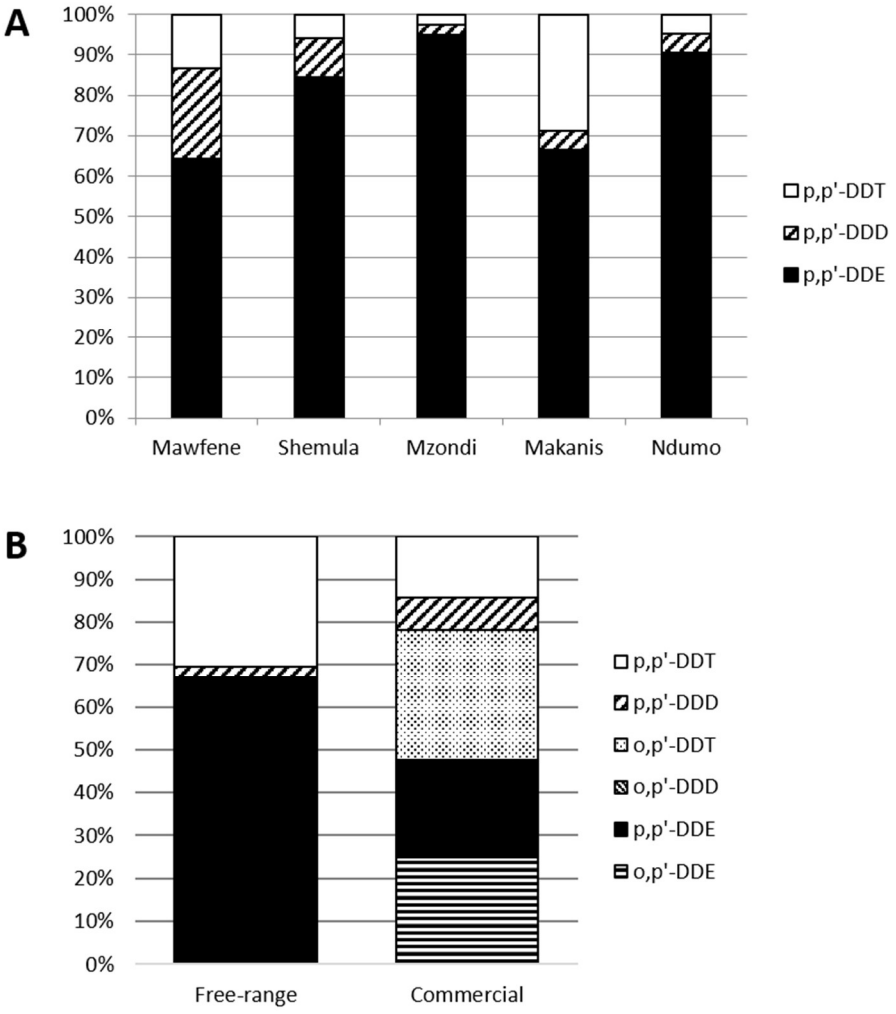


Figure 2.3.2: Relative abundance of individual DDT components in free-range chickens from KwaZulu-Natal. (A) Chicken meat samples; (B) Chicken egg contents.

Concentrations of DDTs in chicken meat in this study (mean 13.5 ± 18.9 ng/g ww, maximum 79.1 ng/g ww) were lower than those detected in a smaller study on chickens in the Limpopo Province of South Africa in 2008, another IRS-using province (mean 500 ± 400 ng/g ww, with a maximum of 1,400 ng/g ww meat) (Van Dyk et al., 2010b). This difference may in part be attributed to the fact that sampling was performed soon after IRS in the Limpopo study. In the current KZN study, sampling was performed just before IRS, with the most recent spraying in homesteads 9 months prior to sampling.

Chicken eggs

DDTs were also detected in chicken egg contents, although differences were seen between free-range and commercial samples.

Concentrations of DDTs (values of *o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDD, *p,p'*-DDD, *o,p'*-DDT and *p,p'*-DDT) detected in chicken egg contents sampled in the region are shown in Table 2.3.2. DDT and its metabolites were detected in 100% of free-range (13/13) and commercial (11/11) egg samples. Summed DDT concentrations in free-range samples ranged from 807.6 ng/g ww to 96,666 ng/g ww (median 9,544 ng/g ww). By comparison, summed DDTs in commercial samples ranged from <LOD to 4.6 ng/g ww (median 1.3 ng/g ww). In these samples, the predominant chemical detected was again the *p,p'*-DDE metabolite (median 6,819 ng/g ww in free-range samples and 0.5 ng/g ww in commercial samples). These results not only support the evidence for high levels of DDTs

exposure to free-range chickens, but show that commercially-reared birds are also exposed to, albeit much lower, levels of contamination.

Composition profiles of DDTs in chicken eggs are shown in Figure 2.3.2 (B). The predominant metabolite congener detected was *p,p'*-DDE, median presence in samples of 75.7% (range 48.0 to 92.1%), followed by *p,p'*-DDT (22.0%) and *p,p'*-DDD (3.0%) in free-range samples. Contribution by *o,p'* congeners was < 1% in total. However, in commercial eggs, *p,p'*-DDT predominated, at a median presence of 21.3% (range <LOD to 75.8%). The pattern of remaining congeners was of a varied composition: *p,p'*-DDE (19.4%) > *p,p'*-DDE (6.7%), and *o,p*'-congeners < 1.0%. This difference may indicate a different source of DDTs in these birds. Again, the ratio of *p,p'*-DDT to *p,p'*-DDE exceeded one in a single free-range sample from Makanis.

A study of DDT contamination in eggs collected in 2008 in Limpopo just after IRS application reported a median level of 11,000 ng/g ww and a maximum of 48,000 ng/g ww (Bouwman et al., 2015). Contamination levels in eggs obtained in this KZN study had a lower median value (9,544 ng/g ww) but the maximum was more than double that in Limpopo (96,666 ng/g ww). Another study in Limpopo detected a mean concentration of 1,600 ng/g in chicken liver samples, higher than levels in KZN chicken meat (mean 13.5 ng/g ww) but lower than those in KZN eggs (mean 18,081 ng/g ww) (Van Dyk et al., 2010b). There may be a temporal shift in body distribution pattern of DDTs in chickens, initially with higher

concentrations in the animal's organs but taking time for metabolites to pass to egg contents.

Mechanisms of DDT toxicity in humans are poorly understood. The most abundant DDT detected in this KZN study was *p,p'*-DDE. This congener is commonly associated with fertility problems in many wild avian species (Gómez-Ramírez et al., 2012). Laboratory studies also showed effects in chickens, with reduced fertility and hatchability when exposed to DDT in feed (Sauter and Steele, 1972). Reduced fertility in the chickens was not reported by owners, and post-mortem examination did not reveal any gross abnormalities. Eggshell thickness was not significantly reduced by DDTs in these samples (Table 2.3.1). The presence of *p,p'*-DDT is an indicator of recent release of DDT into the environment, although homestead IRS record cards indicated that the most recent application for properties was 9 months prior to sampling.

Human health risk assessment

The study area is a low economic region, and many homesteads rear free-range chickens to provide a readily available source of nutrition. Coetzee et al. reported that chicken is the most common form of protein consumption in the study area (Coetzee et al., 2015). Consumption of meat or eggs from these may pose a risk to human health if DDTs accumulate in livestock from contaminants in the environment. DDT concentrations from the current study were evaluated against existing international limits. In order to evaluate risk exposure through

consumption of chicken products, EDIs were calculated at 25th, 50th, 75th and 95th percentiles of DDT concentrations, expressed as nanogram per kilogram body weight per day (ng/kg/bw/d) (Table 2.3.3). EDI values were then compared with the WHO and US EPA published limits for non-cancer effects.

Further calculations assessed the carcinogenic risk of DDTs, using cancer risk estimates and hazard ratios (HRs) for the above percentile concentrations (Table 2.3.4). Based on published daily consumption values for chicken meat (103 g/person/d) and eggs (24 g/person/d) in South Africa, calculated HR values were much greater than 1 for DDTs detected in free-range chicken meat and eggs (Schonfeldt et al., 2013).

For chicken meat and commercial egg samples, these were at exposure levels below the WHO provisional tolerable daily intake (PTDI) of DDTs of 0.01 mg/kg bw/d, and the US EPA reference dose (RfD) of 0.0005 mg/kg bw/d (JECFA, 2010; US EPA, 2015). However, at the 75th percentile and above, these levels were breached in free-range egg samples, due to contributions from high levels of *p,p'*-DDE and *p,p'*-DDT.

Using the national consumption values, the calculated cancer risk for DDTs through consumption of free-range chicken meat ranged from 0.7 x 10^{-4} in the Shemula area to 17.2 x 10^{-4} in Makanis (at the 50th percentiles), suggesting a 0.7–17.2 chance in 10,000 of developing cancer due to the DDTs present in chicken meat. These risks increased, relatively, to 10.7 and 43.9 x 10^{-4} at the

95th exposure level (10.7–43.9 chance in 10,000) – risks considered unacceptable for human health. Considering concentrations of all free-range meat samples together, cancer risk estimates for DDTs ranged from 3.6 at 50th to 34.9 x 10^{-4} at 95th exposure levels (3.6–34.9 chance in 10,000). Again, compared to a target risk of less than 1 x 10^{-4}, these risk estimates are considered above an acceptable level and should be of concern.

Table 2.3.3: Estimated daily intake values (ng/kg bw/d) in people of DDTs from the studied chickens. Values are given at the 25th, 50th, 75th and 95th percentile measured concentrations.

Percentile	**25th**	**50th**	**75th**	**95th**
Chicken meat				
p,p'-DDE	2.3	11.2	21.1	72.8
p,p'-DDD	0.2	0.2	2.7	11.0
p,p'-DDT	0.2	0.4	3.5	19.6
ΣDDTs	**2.8**	**12.1**	**32.4**	**111.0**
Chicken egg contents				
Free range				
o,p'-DDE	0.003	0.004	0.1	0.9
p,p'-DDE	479.6	2,727	7,440	16,204
o,p'-DDD	0.1	0.7	0.9	4.4
o,p'-DDT	0.6	1.6	3.3	8.9
p,p'-DDD	18.8	115.7	177.0	756.9
p,p'-DDT	91.8	767.3	1,314	9945
ΣDDTs	**525.7**	**3,818**	**11,803**	**24,775**
Commercially produced				
o,p'-DDE	0.00	0.003	0.2	0.6
p,p'-DDE	0.02	0.1	0.3	0.8
o,p'-DDD	0.002	0.002	0.002	0.003
o,p'-DDT	0.002	0.002	0.002	0.2
p,p'-DDD	0.01	0.05	0.1	0.3
p,p'-DDT	0.002	0.1	0.2	0.3
ΣDDTs	**0.2**	**0.5**	**0.9**	**1.4**

In commercial egg samples, calculations resulted in HR values < 1 (0.5×10^{-4} at the 95th percentile) indicating no associated increase in cancer risk through consumption. Much higher concentrations of DDTs detected in free-range eggs samples contributed to higher HRs. Even at the 25th percentile, HR was 178.7×10^{-4}, ranging up to $8{,}423 \times 10^{-4}$ at the 95th percentile level (178.7–8,423 chance in 10,000). Of great concern is the finding in KZN that levels high enough to be potentially detrimental to human health were present in chicken eggs many months after DDT spraying occurred.

Calculated risk values for human health may be over-estimated in this study for two main reasons. In this area of lower economic status, consumption of meat products may be lower than the national average. Also, in the region, commercially-produced chicken products are eaten as well as free-range animal products. In this study, levels in commercially-reared chicken meat purchased locally were below the limit of detection, and levels in commercial eggs much lower than in free-range eggs. A study in Beijing, China, showed low levels of DDTs in farmed chicken meat (0.05 ng/g ww) and eggs (2.4 ng/g ww) (Tao et al., 2009). These concentrations were lower than samples from the KZN free-range chickens. Higher levels (30 ng/g ww) were detected in farmed chicken meat in India several decades ago (Kaphalia et al., 1981). The lower level of DDTs in recent commercial products gives a correspondingly lower adjusted risk value from consumption.

Table 2.3.4: Cancer risk estimates (hazard ratio[a]) for DDTs. Values are given at the 25th, 50th, 75th and 95th percentile measured concentrations (based on national consumption rates).

Percentile	25th	50th	75th	95th
Chicken meat				
p,p'-DDE	0.8	3.5	5.9	23.6
p,p'-DDD	0.1	0.1	0.5	3.4
p,p'-DDT	0.1	0.1	1.2	6.5
ΣDDTs	**0.9**	**3.6**	**10.5**	**34.9**
Chicken egg contents				
Free range				
o,p'-DDE	0.001	0.001	0.04	0.3
p,p'-DDE	163.1	927.3	2,529	5,509
o,p'-DDD	0.0	0.2	0.3	1.5
o,p'-DDT	0.2	0.5	1.1	3.0
p,p'-DDD	6.4	39.4	60.2	257.4
p,p'-DDT	31.2	260.9	446.8	3,381
ΣDDTs	**178.7**	**1,298.0**	**4013**	**8,423**
Commercially produced				
o,p'-DDE	0.001	0.001	0.1	0.2
p,p'-DDE	0.01	0.03	0.1	0.3
o,p'-DDD	0.001	0.001	0.001	0.001
o,p'-DDT	0.001	0.001	0.001	0.1
p,p'-DDD	0.002	0.02	0.03	0.1
p,p'-DDT	0.001	0.05	0.1	0.1
ΣDDTs	**0.1**	**0.2**	**0.3**	**0.5**

[a] A value >1 indicates a potential health risk.

Cumulative consumption of both free-range chicken meat and eggs at current detected contamination levels in the KZN study area have been shown by hazard risk analysis to yield a lifetime cancer risk of greater than one in a million, indicating a relatively high risk to human health, particularly from consumption of free-range eggs. WHO estimates cancer mortality in South Africa to be 6.8% of

all deaths (WHO, 2014). Concern is not only due to continued use of DDT in the region but also due to the prolonged persistence of the DDT and its metabolites in the environment. However, other studies have shown that cooking (including boiling) of chicken meat reduces total DDT compounds by approximately 25% (Morgan et al., 1972). Of the total DDT compounds remaining after cooking, 80% are found in the broth and 20% still remain in the meat (except for DDD isomers, of which 60% remain in the meat). As such, local people should be advised against regular consumption of free-range chicken products, particularly eggs, even if they are cooked. Discarding the broth after boiling meat appears to significantly reduce available DDTs and may be a simple option to reduce exposure. Based on discussions with local residents about their diet, the local consumption of chicken products is likely lower than the national per capita level (Schonfeldt et al., 2013). The risks therefore reported may be overestimations, but if local chicken consumption increased in line with the average South African, the associated risk from DDTs may become greater. These risk estimates do not include contamination in other food sources. For example, DDTs in leafy vegetables from the IRS-using Limpopo area in South Africa contained 43.0 ng/g of DDTs (Van Dyk et al., 2010b). Although ingestion is the main route of DDTs exposure in people, other additional sources of exposure are inhalation and dermal contact (Yu et al., 2012). These are a concern particularly for the family members who spend most time in the home – usually women and infants.

KZN Province's Department of Health has a strategic objective to maintain preventative strategies to reduce and maintain malaria incidence at less than 1

per 1000 population, but cites various reasons for deviation from this planned target including difficulties in performing IRS due to furniture in homes, and poor acceptance of spraying. Resistance by vectors to pesticides (including DDT) and therapeutic agents have been recorded in many malaria areas. It is also reported that DDT has recently become more expensive to purchase in South Africa (KwaZulu-Natal Department of Health, 2014). The benefits of using this chemical may soon be outweighed by the costs—financially, to health, and to the environment—and research developing viable alternatives to DDT for malaria control is ongoing but requires validation and implementation. It is likely that the solution will be a combination of different methods (WHO, 2016a, 2016b). The South African government has a goal of eliminating malaria by 2018 (WHO, 2015b).

This study shows that free-range chickens in homesteads are contaminated with DDTs. It would be useful for future studies to analyse feed for both free-range and commercially-reared chickens in the region to clarify the source of contamination. Although they do not live so intimately with people as chickens, other livestock such as goats and cattle are also susceptible to exposure from environmental contamination and therefore are a source of DDTs if consumed. The Jozini and uMhlabuyalingana Local Municipalities encompass the Ndumo Game Reserve, with rich biodiversity and home to over 400 bird species. Except for research on fish (McHugh et al., 2011), the potential risks from DDTs to wildlife in this region have not yet been fully ascertained, but there is concern that environmental contamination by DDT near such an area may affect wildlife,

particularly the abundant bird species for which the reserve is renowned (Smit et al., 2016). The effects of chronic DDT exposure on people are still uncertain. Several studies have suggested effects on body systems including immunological function, neurodevelopment and reproductive success, but others have shown weak or no statistically proven correlation (Bornman et al., 2010; Cohn et al., 2015; Gaspar et al., 2015; Jusko et al., 2016; Ouyang et al., 2014; Perry et al., 2016).

Conclusion

This is the first study reporting levels and human risk assessment of DDTs in food products from free-ranging chickens in the northern area of KwaZulu-Natal. Chemicals used on buildings as part of malaria vector-control strategies make their way into the adjacent environment and thence to livestock living there. This study based on recent sampling confirms the presence of high levels of the parent DDT compound and its metabolites in products from free-ranging chickens. The persistence of DDT in the environment ensures that its detrimental effects will be seen for some time to come on the environment and organisms, including humans, their livestock and wildlife.

- Organochlorine pesticides are reported to contaminate many foods in African countries. Foods are the most likely source of pesticides in people, and studies have confirmed contamination in both human serum and breast milk.

- Assessment of levels of DDT and its metabolites (collectively, DDTs) in biota collected from two locations, South Africa and Mozambique, where DDT use is ongoing in malaria control programs, confirmed contamination of fish and chicken products. Levels were sufficiently high as to be a potential risk for human health through consumption, with chicken eggs a much greater risk than chicken meat, which in turn carried a greater risk than fish meat.

- It is difficult to ascertain the clinical effects of DDTs, particularly with field-exposed biota, and so further investigations were conducted at the molecular level.

Chapter 3

Molecular changes in chickens and humans associated with exposure to DDTs

3.1 Investigation of genetic changes associated with field exposure to DDTs in chickens from KwaZulu-Natal, South Africa

S3.1.1 Supplemental data: Microarray analysis of mRNA extracted from free-range chicken livers sampled from KwaZulu-Natal, South Africa

3.2 Effects of the organochlorine *p,p'*-DDT on MCF-7 cells: investigating metabolic and immune modulatory transcriptomic changes

3.1 Investigation of genetic changes associated with field exposure to DDTs in chickens from KwaZulu-Natal, South Africa

Abstract

The objective of this study was to identify potential genetic changes in chickens associated with environmental exposure to dichloro-diphenyl-trichloroethane (DDT) and its metabolites (DDTs), screening expression of mRNA extracted from liver tissue. In particular, we focused on genes relating to the immune system and metabolism.

We analysed liver samples from free-ranging chickens in KwaZulu-Natal, South Africa, for contamination by DDTs. This area predominantly uses DDT in its malaria control program, and homes are sprayed annually with the pesticide. Genes relating to the immune system and metabolism were selected as potential genetic biomarkers that could be linked to higher contamination with DDTs. RT-qPCR analysis on 39 samples showed strong correlations between DDTs contamination and gene expression for the following genes: *AvBD1*, *AvBD2*, *AvBD6* and *AvBD7* (down-regulated), and *CYP17A1*, *ELOVL2* and *SQLE* (up-regulated).

This study shows for the first time interesting and significant correlations between genetic material collected from environmentally-exposed chickens and several

genes involved in immunity and metabolism. These findings show the usefulness of genetic analysis on field samples from a region with high levels of environmental contamination in detecting subclinical effects. In particular, we observed clear effects from DDT contamination on genes involved in immune suppression, endocrine-disrupting effects, and lipid dysregulation. These results are of interest in guiding future studies to further elucidate the pathways involved in toxicity associated with DDT exposure from contaminated environments, to ascertain the health risk to livestock and any subsequent risks to food security for people.

Introduction

The KwaZulu-Natal Province of South Africa is currently considered an endemic area for malaria and the mainstay of malaria control is the use of dichloro-diphenyl-trichloroethane (DDT) in indoor residual spraying (IRS) programs (Maharaj et al., 2012; Wepener et al., 2012). Under guidance from the World Health Organization (WHO), local health centers annually spray the pesticide inside homes on walls and outside under roof eaves to reduce mosquito populations which transmit the disease. DDT and its breakdown metabolites (collectively known as DDTs) enter the environment as dust contamination and are a source of contamination via inhalation, contact and ingestion (Mansouri et al., 2017; Sereda et al., 2009). While exposure to DDTs is primarily via dermal contact and inhalation of aerosolized spray for workers administering DDT, ingestion is thought to be a more significant exposure route for other people and non-target species such as livestock (Mrema et al., 2013; Ortelee, 1958). Chickens in the region have been shown to be contaminated (Thompson et al., 2017b).

When DDT was initially popular as an agricultural pesticide in the 1940s, it was thought to be toxic only to insects. However, by the 1960s it became apparent that non-target species were susceptible to toxic effects, notably DDT affecting reproduction in birds of prey populations, publicized widely in Rachel Carson's book "Silent Spring" (Carson, 1962). Eggshell thinning is the most well-known effect of DDT in birds, but other effects include reduced post-hatch survival,

altered sexual behavior, neurotoxicity and smaller brain size (Gómez-Ramírez et al., 2012; Iwaniuk et al., 2006; Kamata et al., 2013; Lundholm, 1988). Field sampling of avian species with lifelong environmental exposure has identified correlations between DDTs contamination and various hormonal and immune responses (Bustnes et al., 2004; Verreault et al., 2007, 2006, 2004). Previous studies have shown that chickens are relatively insensitive to the toxic effects of DDT and its breakdown products (predominantly dichloro-diphenyl-dichloroethylene (DDE) and dichloro-diphenyl-dichloroethane (DDD)) (Heath et al., 1969; Kamata et al., 2009; Waibel et al., 1972). Despite the growing list of toxic effects of DDTs, our understanding of the mechanisms of action is still lacking.

Plasma DDE levels have been linked to immune suppression (Vine et al., 2001). Host defense peptides are conserved across a wide range of organisms, and play an important role in the innate immune system (Lehrer and Ganz, 2002). In birds, only beta-defensins (avian beta-defensins, AvBD, also known as gallinacins, GAL) have been described, with over 25 detected (Hellgren and Ekblom, 2010). Although expressed in a wide range of tissues, expression of most *AvBD* genes is usually low in the liver (Cuperus et al., 2013). Factors which affect expression include estrogen in the female reproductive tract, dietary Vitamin D_3 concentration, inflammatory stimuli, and infections such as *Salmonella spp* and viruses (Akbari et al., 2008; Cuperus et al., 2013; Derache et al., 2009; Ma et al., 2011; Subedi et al., 2007; Zhang et al., 2011). Effects seen with infections depend on the organ affected, and also the chicken breed or age. No studies have previously linked these genes to DDTs exposure.

Organochlorine pesticides like DDT are known to interfere with hormone signaling and metabolic pathways (Bradlow et al., 1995; Hayes et al., 1996; Mrema et al., 2013). Levels of DDTs have been linked to type 2 diabetes and metabolic syndromes in people (Al-Othman et al., 2015; Lee et al., 2011). Involvement of the insulin-like growth factor-binding protein 1 (IGFBP1) has been demonstrated in insulin signaling in chickens (Dupont et al., 2004). DDTs are lipophilic, with highest concentrations detected in high-lipid organs such as the liver. The liver is also a key location for whole body lipid metabolism, including fatty acid metabolism. Elongation of very long chain fatty acids elongase 2 (ELOVL2) is one of the two fatty acid elongase subtypes involved in polyunsaturated fatty acid (PUFA) biosynthesis in the chicken liver (Gregory et al., 2013; Jing et al., 2013). Squalene epoxidase (*SQLE*) is differentially expressed in fat tissues from fast-growing versus slow-growing chickens, and is involved in endogenous cellular cholesterol synthesis (Claire D'Andre et al., 2013). DDT exposure is associated with reproductive effects and gender alteration in ovo, and cytochrome P450 Family 17 Subfamily A Member 1 (CYP17A1) is involved in sex differentiation (Fry and Toone, 1981).

Chickens are an important food source for people, and thus any clinical or subclinical toxic effects could impact food security for local people where DDT is used to control vector-borne disease such as malaria. Chickens as livestock are relatively easy to sample and may be useful as a sentinel species for contamination by DDTs in wild birds in the region.

The objective of this study was therefore to investigate genetic changes associated with contamination by DDTs in free-ranging chickens from environmental exposure in an area where DDT is sprayed as part of a malaria control program. A number of genes were identified as biomarkers for DDTs exposure. RT-qPCR was used to confirm statistical significance of dose-related changes in gene expression in a large number of samples across a broad range of contamination levels.

Materials and methods

Chemicals and reagents

Test reagents (oligo(dT) primers, reverse transcriptase (RT) buffer, and RT ACE) were purchased from Toyobo Co. (Osaka, Japan), TRI reagent from Sigma Chemical Co. (St. Louis, MO, USA), dNTP mix from Takara (Takara Bio Inc Japan), primer sets from Invitrogen (Carlsbad, CA, USA), and RNAlater® from Sigma-Aldrich (St Louis, MO). A standard mixture of DDTs (Dr Ehrenstorfer GmbH, Germany) was purchased. Pesticide grade organic solvents and anhydrous sodium sulfate were purchased from Kanto Chemical Corp. (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical Industries (Tokyo, Japan).

Sampling sites and sample collection

The sampling area was within the Jozini and uMhlabuyalingana local municipalities, in uMkhanyakude District Municipality of KwaZulu-Natal Province

of South Africa (Figure 3.1.1). At the time of sampling in October 2014, malaria was endemic in the region and DDT applied annually to homes in the IRS program. Chickens live free-range in homesteads where DDT is applied. Liver samples were collected (n = 39) immediately after slaughter and aliquots stored in clean plastic vessels (for chemical analysis) and Eppendorf tubes containing RNAlater® preservative (for genetic analyses) (see Table 3.1.1 for details).

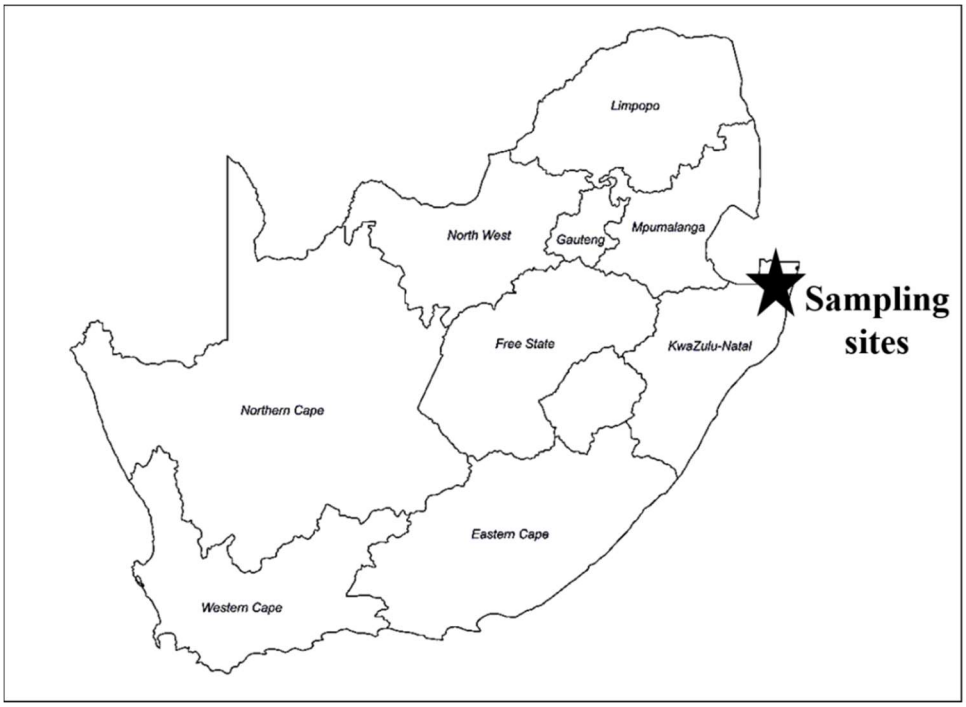


Figure 3.1.1: Map of sampling area in KwaZulu-Natal, South Africa.

Table 3.1.1: Biometric data for chickens sampled in KwaZulu-Natal for this study.

	Mean	Range
Estimated age (months)[1]	13 ± 7	7–30
Weight (kg)[2]	1.5 ± 0.4	0.9–2.8
Body condition score[3]	2 ± 0.7	0–3
Lipid % in liver samples	3.8 ± 2.2	0.3–7.9
Supplied diet[4]	Maize (home-grown or shop-bought), leftovers, rice, bread, fresh vegetables	
Sex	Male (16), female (23)	
Source	Shemula (11), Makanis (9), Ndumo (8), Mzondi (6), Mamfene (5)	

[1]Estimation by owner at time of purchase.
[2]Body weights for chickens are ante-mortem.
[3]Based on Gregory and Robins 0—3 scale for layer hens (Gregory & Robins 1998).
[4]Diet supplied by owner in addition to chickens foraging around the homestead.

This study was carried out in strict accordance with Hokkaido University guidelines, with veterinary certificates obtained from the agricultural office in Japan (Certificate number: 26 douken 523) and the veterinary office in Ndumo. Necessary approvals and international laws were adhered to regarding transfer of samples from South Africa to Japan.

Sample preparation and storage

Liver was selected as the organ of interest for two main reasons. Firstly, DDT is lipophilic and is stored in body compartments with high lipid content, such as the liver. This organ is thus a good representation of contamination within the animal, and many studies analyse concentration of DDTs in the liver. Secondly, this organ is important in detoxification of chemicals and has many metabolic functions.

Liver samples were collected from freshly slaughtered chickens and stored via two methods. Samples for chemical analysis were frozen to -20°C shortly after collection. Samples for genetic analysis were placed into RNAlater® tissue storage reagent to stabilize and protect cellular RNA, and frozen. Samples were then transported to the Laboratory of Toxicology in Hokkaido University, and maintained at -80°C until analysis.

Organochlorine extraction and analysis (DDTs)

Frozen samples were defrosted and analysed to measure levels of DDT and its metabolites (*o,p'*-DDT, *p,p'*-DDT, *o,p'*-DDD, *p,p'*-DDD, *o,p'*-DDE and *p,p'*-DDE – collectively termed "DDTs") using a slightly modified version of Yohannes et al.'s method (Yohannes et al., 2014b). Briefly, 1 g of liver was homogenized with anhydrous sodium sulfate before automatic extraction for 3.5 hours with a mixture of hexane:acetone (3:1 *v/v*) in a Soxhlet extractor (SOX416 macro SOXTHERM unit, Gerhardt, Germany). Each sample extract was spiked with 3,3',4,4'-tetrachlorobiphenyl (PCB 77) surrogate standard, then concentrated prior to clean-up in a glass column packed with activated florisil and eluted with hexane:dichloromethane (7:3 *v/v*). After further concentration, 2,4,5,6-tetrachloro-*m*-xylene (TC*m*X) was added as a syringe spike, before analysis using a gas-chromatograph with ^{63}Ni electron capture detector (GC-ECD: Shimadzu GC-2014, Kyoto, Japan). The machine condition parameters and QA/QC analysis were as in Thompson et al (Thompson et al., 2017b).

RNA extraction and cDNA synthesis

Total RNA was extracted using TRI reagent (Sigma-Aldrich) from the RNAlater®-preserved samples, following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized according to Darwish et al.'s method (Darwish et al., 2010).

Selection of genes of interest

A preliminary study using microarray analysis on liver samples from chickens exposed environmentally to DDTs identified a number of functional areas and possible genes of interest (Supplemental Data S3.1.1). These genes fell into two categories: the innate immune system (avian beta-defensins), and metabolism of steroid hormones and lipids (insulin-like growth factor-binding protein 1, elongation of very long chain fatty acids elongase 2, squalene epoxidase, and cytochrome P450 Family 17 Subfamily A Member 1).

Quantitative real-time polymerase chain reaction

Chicken liver mRNA levels were determined by quantitative real-time RT-PCR using SYBR® qPCR mix (Toyobo) and a StepOne® real-time PCR system (Applied Biosystems). Primer sets for specific genes tested are described in Table 3.1.2. The method was performed according to Mureithi et al. (Mureithi et al., 2012). In brief, PCRs were run with a final volume of 10µl, containing SYBR® qPCR Mix (Toyobo), 10 µM of each primer, 600 ng cDNA, 50X ROX reference

dye and RNase-free water. Cycle conditions were as follows: 95°C for 20 s initial holding stage, 40 denaturation cycles of 95°C for 3 s and 62°C annealing for 30 s, and 95°C extension for 15 s. Amplification of a single amplicon of the expected size was confirmed using melting curve analysis and agarose gel electrophoresis. Experiments were repeated at least three times on different occasions. The sample containing the lowest contamination level of DDTs was assigned as a relative reference sample. Gene expressions were normalized with respect to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression and calculated relative to the nominal reference level using the comparative threshold cycle (Ct) method.

Table 3.1.2: qRT-PCR primer sequence information used in this study.

Gene	Sequence		Accession number	Product size (bp)
	Forward	Reverse		
GAPDH (housekeeping)	ACACAGAAGACGGTGGATGG	GGCAGGTCAGGTCAACAACA	NM_204305.1	193
AvBD1	CCTGTGAAAACCCGGGACA	GCACAGAAGCCACTCTTTCG	NM_204993.1	145
AvBD2	ACTGCCTGCCACATACATTTC	AGACAACCCTGGAGAAGCCT	NM_001201399.1	127
AvBD6	TTGCAGGTCAGCCCTACTTT	CCGGTAATATGGCCACCGAC	NM_001001193.1	95
AvBD7	ATTTCACATCCCAGCCGTGG	AGGCCTAGGAATGAAGGGCT	NM_001001194.1	103
IGFBP1	TCACTGGATGGAGATTCCGC	AAGCTCCACAGAGAACCTGG	NM_001001294.1	164
ELOVL2	CATGTGGGTTTCCCTTTGGC	GACTTCTGTTGTGACGGGGG	NM_001197308.1	146
SQLE	CCATTTTTGGAGCGTCAGCC	GATGCCCAGGAAAGTCCACA	NM_001194927.1	71
CYP17A1	CCCTACCTGGAGGCTACCAT	CGGACCAGAGGTTGATGACC	NM_001001901.2	145

Key: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), avian beta-defensin 1 (*AvBD1*), avian beta-defensin 2 *(AvBD2*), avian beta-defensin 6 (*AvBD6*), avian beta-defensin 7 (*AvBD7*), insulin-like growth factor-binding protein 1 (*IGFBP1*), elongation of very long chain fatty acids elongase 2 (*ELOVL2*), squalene epoxidase (*SQLE*), cytochrome P450 Family 17 Subfamily A Member 1 (*CYP17A1*); adenine (A), cytosine (C), guanine (G), thymine (T)

Statistical analysis

Data analysis was conducted using Microsoft Excel® 2014 and JMP® Pro 12 (SAS Institute Inc., Cary, NC, USA). Contamination levels of DDTs are shown as median and range values in ng/g wet weight and ng/g lipid weight of tissue. Linear regression analysis was used to evaluate statistical significance. A *p*-value of <0.05 was considered significant. Principal components analysis was performed to assess correlations.

Results and discussion

DDTs concentrations

Contamination by DDT and its metabolites was detected in liver samples assessed (Table 3.1.3). The median of summed DDTs was 919 ng/g wet weight (ww), with a maximum of 14,398 ng/g ww. Concentrations of DDTs were comparable to those detected in chicken livers from Limpopo Province in another IRS-treated area of South Africa (Van Dyk et al., 2010b). In the Limpopo study, the median sum of DDTs was 1,100 ng/g ww compared to 919 ng/g ww in this KwaZulu-Natal study. A study sampling chicken livers from an electrical and electronic waste (e-waste) site in China detected a much lower contamination level of 200 ng/g lw (Labunska et al., 2015), compared to 29,235 ng/g lw in KwaZulu-Natal. DDT is not currently applied at the e-waste recycling area but legacy contamination is present.

Table 3.1.3: Levels of DDT and metabolites detected in liver from free-ranging chickens in KwaZulu-Natal. <LOD = below limit of detection

	ng/g wet weight		ng/g lipid weight	
	Median	Range	Median	Range
p,p'-DDE	692	18 - 10,537	20,186	289 - 227,891
o,p'-DDD	10	<LOD - 166	246	<LOD - 5,919
p,p'-DDD	89	<LOD - 1,840	2,929	<LOD - 47,273
o,p'-DDT	11	<LOD - 302	458	<LOD - 8,280
p,p'-DDT	54	<LOD - 1,923	1,333	<LOD - 18,756
Sum of DDTs	919	36 - 14,398	29,235	555 - 288,928

The predominant congener was *p,p'*-DDE, and comprised approximately 75% of the DDTs. This congener has been linked to many toxic effects in birds, including eggshell thinning (Lundholm, 1990; Lundholm and Bartonek, 1992). Estrogenic effects of DDTs are thought to affect avian embryos more than those of mammals, and phase I metabolites (DDE and DDD) may be more estrogenic than parent compounds (Bulger et al., 1978; Fry, 1995; Korach et al., 1988).

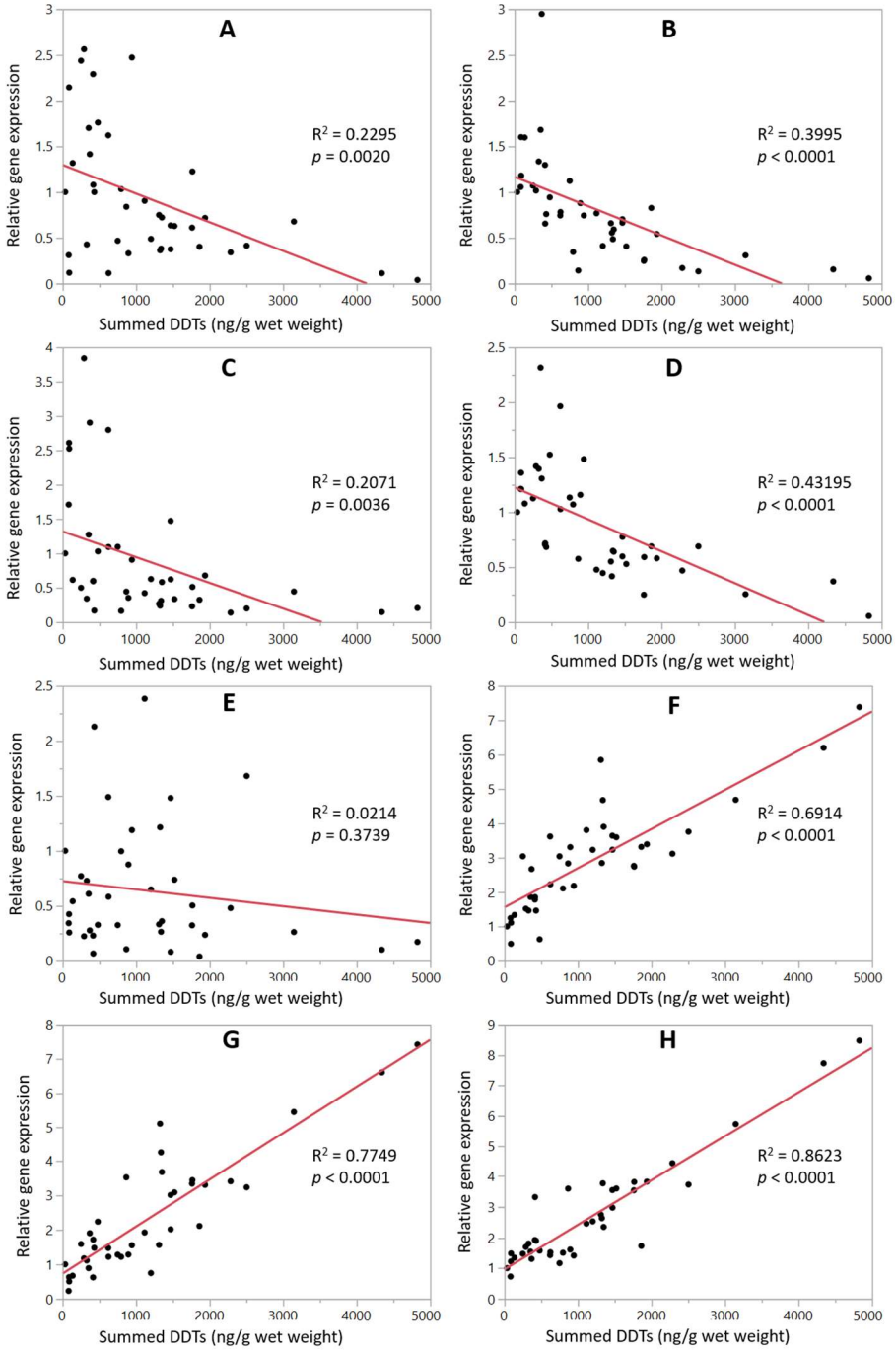
Several factors may confound results from samples collected under field conditions – for example, difference in chicken breed, age, diet, body condition and health. As far as was possible, chickens selected from the study site were comparable. Husbandry methods resembled other households in the region. Adult birds of similar weight and body condition, without clinical signs of disease, were analysed. No pathological conditions were detected on gross post-mortem examination of the birds. Chronicity of exposure may affect contamination levels,

as bioaccumulation of DDTs occurs (Li et al., 2008). Statistical analysis did not show any significant correlation between concentrations of DDTs and biometric data collected (Figure 3.1.3).

qPCR gene expression results

Samples were all obtained from an area in KwaZulu-Natal Province where the Jozini Health Center administers DDT annually for IRS as part of their malaria control program. As this region is endemic for malaria, all homes are treated. In this scenario, it was not possible to obtain negative control samples from untreated homesteads and so statistical analyses were conducted by setting the reference sample for relative comparisons as that with the lowest concentration of summed DDTs.

Genes of interest were selected based on consideration of reported effects of DDTs and results from microarray analysis in a preliminary study (Supplemental Data S3.1.1). In particular, we focused on the innate immune system and on metabolism, particularly that of steroid hormones and lipids. A number of genes examined were significantly down-regulated (Figure 3.1.2): *AvBD1*, *AvBD2*, *AvBD6*, and *AvBD7*. Although there was a trend for down-regulation of *IGFBP1* with increasing DDTs, it was not a statistically significant association. The following genes were significantly up-regulated in samples with higher contamination levels of DDTs: *ELOVL2*, *SQLE* and *CYP17A1*. There were minor differences in statistical significance between male and female samples, but



Key: A) Avian beta-defensin 1 (*AvBD1*), B) avian beta-defensin 2 *(AvBD2*), C) avian beta-defensin 6 (*AvBD6*), D) avian beta-defensin 7 (*AvBD7*), E) insulin-like growth factor-binding protein 1 (*IGFBP1*), F) elongation of very long chain fatty acids elongase 2 (*ELOVL2*), G) squalene epoxidase (*SQLE*), H) cytochrome P450 Family 17 Subfamily A Member 1 (*CYP17A1*)

Figure 3.1.2: Correlation between gene expression and contamination levels (summed DDTs by wet weight). Summed DDTs (ng/g wet weight, x-axis) are plotted against relative gene expression (y-axis). R^2 values and *p*-values are shown.

these did not impact the overall results. Statistical analysis did not show any correlation between gene expression and other biometric data collected or difference in sampling location within the study area.

Defensins have antimicrobial activity against many bacteria and fungi (reviewed in Cuperus et al (Cuperus et al., 2013)). They also play a role in immune regulation, binding to chemokine receptors, inducing pro-inflammatory cytokine expression, having anti-inflammatory properties and enhancing wound healing (Ganz, 2003; Semple et al., 2011; Semple and Dorin, 2012). Expression of a range of *AvBD* genes showed a negative correlation with summed DDTs contamination: AvBD1 ($p = 0.0020$), *AvBD2* ($p < 0.0001$), *AvBD6* ($p = 0.0036$) and *AvBD7* ($p < 0.0001$). Reduction in expression levels of these important innate immunity genes may lead to increased susceptibility to disease with DDTs exposure. In this study, no signs of clinical disease were noted in the chickens ante-mortem or during post-mortem examination, but other investigations such as histopathology or bacterial culture were not performed.

IGFBP1 is a regulator of somatic growth and has been identified as a biomarker for visceral adiposity (Lim et al., 2012). *IGFBP1* gene expression is involved in signaling between growth hormone, thyroid hormone and body fat regulation in chickens but has not previously been linked to DDTs exposure (Wang et al., 2007). Insulin signaling is affected by IGFBP1 in chickens (Dupont et al., 2004). There was a slight downward trend for *IGFBP1* expression with higher summed

DDTs concentrations in the chicken livers but it was not statistically significant (p = 0.3739). Adipose tissue has been shown to be the primary tissue for storage of DDTs, and future work may elucidate a link between DDTs contamination and adipose production (de Los Reyes and Mora, 1979).

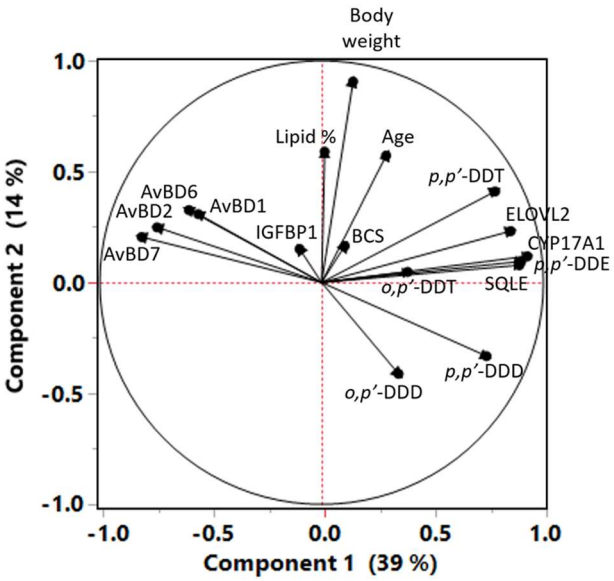
DDTs have been linked in mammalian species to obesity and metabolic syndromes (Skinner et al., 2013). Long chain PUFAs are necessary in vertebrates for normal growth and development, synthesized via either desaturation or elongation from dietary linoleic acid and a-linolenic acid. The *ELOVL2* gene plays a role in the fatty acid elongation pathway, and is essential for converting plant-derived α-linolenic acid (ALA) to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Gregory et al., 2013; Gregory and James, 2014). *ELOVL2* expression was also significantly up-regulated with increasing DDTs contamination ($p < 0.0001$). There are no reports of metabolic syndromes such as diabetes occurring in birds due to DDT. However, this change in metabolism is important as poultry are an important source of long-chain PUFAs for people, particularly in countries where fish consumption is low (Gregory and James, 2014).

Little is yet known about the function of SQLE. Administration of GH in rapidly-growing chickens down regulated expression of hepatic *SQLE*, which is also involved in lipid metabolism (Wang et al., 2007). SQLE is involved in cholesterol synthesis in cells and also peripheral clock genes (delta 2 crystallin, Cry, and aryl

hydrocarbon receptor nuclear translocator-like protein, Bmal) (Nakamura et al., 1996). Cry and Bmal are involved in regulation of corticosteroid synthesis pathways, and their expression in broiler chicken adrenal glands is affected by ACTH treatment (Bureau et al., 2009). Again, expression of this gene, *SQLE*, was significantly up-regulated with increasing concentration of DDTs ($p < 0.0001$). Many xenobiotics are known to affect hormones, and this potential effect from DDTs on corticosteroid synthesis and cholesterol synthesis could affect many areas of metabolism. In livestock species, successful and rapid growth is particularly important, and any imbalances caused by xenobiotics are likely to affect food production. This could be significant in areas like KwaZulu-Natal where poverty and food security are problematic (Morgenthal et al., 2006).

Studies on mammalian species have shown induction of cytochrome P450 (CYP) enzymes in rat liver microsomes after exposure with technical grade DDT (Sierra-Santoyo et al., 2000). CYP enzymes are important in phase I metabolism of xenobiotics (Kitamura et al., 2002). Concentration responses to *p,p'*-DDT have been shown to vary between avian species (Davison and Sell, 1972). CYP17A1 is also involved in androgen hormone synthesis and sex differentiation of birds (Akazome et al., 2002; Cruze et al., 2017; Freking et al., 2000; Yoshida et al., 1996). The association between up-regulation of *CYP17A1* gene expression and DDTs concentration in sampled livers was highly significant ($p < 0.0001$). This strong correlation supports evidence linking DDTs exposure to biased sex ratios in gull embryos (Fry and Toone, 1981).

No association was detected between gene expression and chicken biometric data. Regression analysis (Figure 3.1.3 and Table 3.1.4) showed strong positive correlation between concentrations of *p,p'*-DDE, *p,p'*-DDD and *p,p'*-DDT with gene expression of *ELOVL2*, *SQLE* and *CYP17A1*. There was strong negative correlation between DDT congeners and expression of the *AvBD* genes assessed (*AvBD1*, *AvBD2*, *AvBD6* and *AvBD7*). For the *AvBD* genes, *p,p'*-DDE and *p,p'*-DDD were most significantly associated. *AvBD7* gene showed significant (*p*-value <0.0001–0.0391) negative correlation with all of the DDT congeners detected, and thus may be a sensitive biomarker for DDTs contamination. These data support the hypothesis that the *p,p'*-DDE congener, the most abundant contaminant and known endocrine disruptor, is the cause of many adverse effects associated with DDTs exposure. However, in light of the comparatively low concentrations of *p,p'*-DDD and *p,p'*-DDT in chickens sampled, it is interesting to note that these also significantly affect most of the genes analysed.



Key: AvBD1 (avian beta-defensin 1), AvBD2 (avian beta-defensin 2), AvBD6 (avian beta-defensin 6), AvBD7 (avian beta-defensin 7), IGFBP1 (insulin-like growth factor-binding protein 1), ELOVL2 (elongation of very long chain fatty acids elongase 2), SQLE (squalene epoxidase), CYP17A1 (cytochrome P450 Family 17 Subfamily A Member 1), BCS (body condition score)

Figure 3.1.3: Principal components analysis of gene expression in chickens by DDT congener contamination concentrations.

Table 3.1.4: Comparison between DDT congeners and gene expression in KwaZulu-Natal chickens. *A *p*-value of <0.05 was considered significant.

	Regression statistics (R-squared (*p*-value))				
Gene	*p,p′*-DDE	*o,p′*-DDD	*p,p′*-DDD	*o,p′*-DDT	*p,p′*-DDT
AvBD1	**0.2473 (0.0013*)**	0.0033 (0.7264)	0.0920 (0.0604)	0.0187 (0.4060)	0.0975 (0.0529)
AvBD2	**0.3719 (<0.0001*)**	0.1154 (0.0344)	**0.3260 (0.0001*)**	0.0550 (0.1507)	**0.1478 (0.0157*)**
AvBD6	**0.1877 (0.0059*)**	0.0522 (0.1617)	**0.1711 (0.0089*)**	0.0680 (0.1089)	0.0761 (0.0891)
AvBD7	**0.3730 (<0.0001*)**	**0.1096 (0.0395*)**	**0.3866 (<0.0001*)**	**0.1101 (0.0391*)**	**0.2197 (0.0026*)**
IGFBP1	0.0210 (0.3789)	0.0078 (0.5939)	0.0059 (0.6412)	0.0045 (0.6838)	0.0475 (0.1825)
CYP17A1	**0.8298 (<0.0001*)**	0.0589 (0.1367)	**0.4179 (<0.0001*)**	0.0420 (0.2105)	**0.6816 (<0.0001*)**
ELOVL2	**0.6495 (<0.0001*)**	0.0363 (0.2451)	**0.2654 (0.0008*)**	**0.1928 (0.0052*)**	**0.6128 (<0.0001*)**
SQLE	**0.7318 (<0.0001*)**	0.0570 (0.1432)	**0.4516 (<0.0001*)**	0.0240 (0.3464)	**0.5894 (<0.0001*)**

Conclusions

This study shows for the first time interesting and significant correlations between genetic material collected from environmentally-exposed chickens and several genes involved in immunity and metabolism. During malaria control programs, DDT has been applied to the study area in KwaZulu-Natal for more than a decade. This has led to a high level of environmental contamination and a source of DDTs for local livestock. The study clearly shows a link between this contamination of free-ranging chickens and genetic changes that may have significant health impacts on both the chickens and the local human population.

Of particular interest are the genes involved in steroid synthesis, *CYP17A1* and *SQLE*. These are potential targets for the mechanism of estrogen-mimicry by DDTs. This endocrine disruption is well documented in several species. Up-regulation of the *ELOVL2* gene involved in fatty acid elongation is a strong link to lipid metabolism, and may help explain the connection between DDTs and metabolic syndromes reported in people. Down-regulation of several *AvBD* genes involved in the innate immune system are a serious concern for the health of poultry livestock, where lowered immunity linked to increased infectious disease will impact not only bird health but also may be a problem for food security in people.

Ideally samples would be matched for confounding factors. It would also be useful to perform further chemical analyses to ascertain co-contamination with other

xenobiotics in both the chickens and environment. An *in vivo* exposure study using environmental level concentrations of contaminants needs to be performed to remove potential confounding factors and bias such as age, breed, period of exposure, and concomitant exposure with other contaminants. It would be useful to consider DDTs across multiple generations to ascertain the full gamut of effects on the birds as embryos, developing young, and reproducing adults. Assessment of other closely related genes will also further elucidate the mechanisms involved and aid our understanding of the wide range of toxic effects of DDT and its metabolites.

S3.1.1 Supplemental Data: Microarray analysis of mRNA extracted from free-range chicken livers sampled from KwaZulu-Natal, South Africa

Introduction

DDTs cause many toxic effects, but the underlying mechanisms at the genetic level are not well understood. To this end, we screened environmentally-exposed chickens using microarray analysis to identify potential genes of interest before further analysis using RT-qPCR on a greater number of samples across a range of contamination levels.

Materials and methods

Sample collection and preparation

Samples (n = 2) of total RNA isolated from free-range chicken livers in KwaZulu-Natal, South Africa were selected to perform microarray analysis. One sample had a relatively low (1,115 ng/g ww; 22,319 ng/g lw) total DDTs concentration compared to the other (1,938 ng/g ww; 31,259 ng/g lw).

Microarray analysis

This analysis was performed by Hokkaido Systems Science Co., Ltd., Sapporo, Japan. First, RNA sample quality testing was performed using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA) and Agilent 2100 Bioanalyzer series II

(Agilent Technologies, Palo Alto, CA) according to manufacturers' protocols. Subsequently, gene expression analysis was performed using the Gallus (Chicken) 4x44K ver. 2.0 Gene Expression Microarray, 4x44K (Agilent Technologies). Data was normalized using GeneSpring GX (Agilent) and further interpreted using ArrayStar (DNASTAR Inc., Madison, WI).

Results

RNA concentration and quality were sufficiently high to proceed with microarray analysis (RIN values > 8).

Initial data analysis identified 444 genes with a greater than or equal to 5-fold change between the samples (Figure S3.1.1). Of these, 343 genes had 5-fold higher expression and an expression level/signal (absolute value signal) of log scale > 5 in the sample with higher DDTs concentration. 101 genes had at least a 5-fold lower expression and an expression level/signal of log scale > 5 in the sample with higher DDTs concentration. However, none of this group had a p-value < 0.05 when comparing the samples. Gene ontology identified many biological processes to be involved in altered gene expression, including metabolic processes, the immune system, biological regulation and cellular component organization.

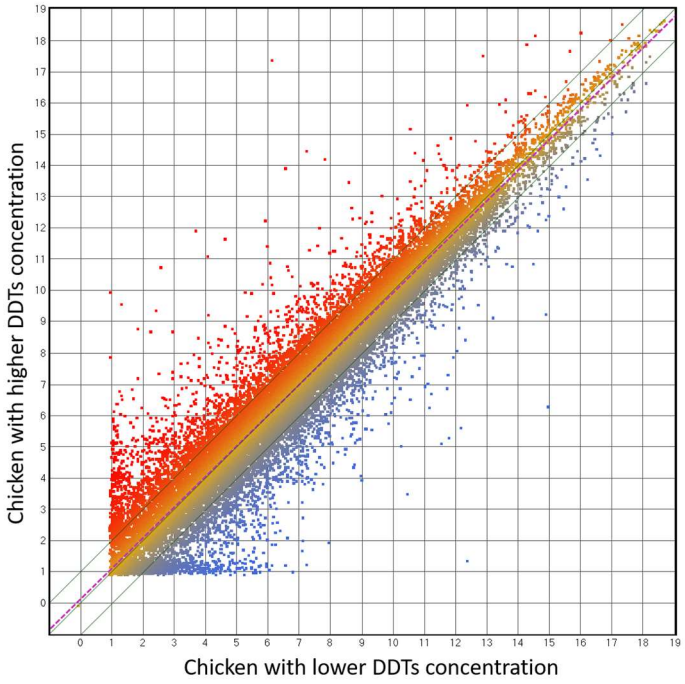


Fig S3.1.1: A scatter plot of mRNA expression values in livers isolated from free-ranging chickens in KwaZulu-Natal, South Africa. Points between green lines denote genes with < 2 fold change.

DDTs have been linked to immune suppression and altered metabolic pathways (Bradlow et al., 1995; Hayes et al., 1996; Mrema et al., 2013; Vine et al., 2001). Further data mining of the set of 444 genes into those relating specifically to the immune system or metabolism identified a number of genes of interest. Details including fold changes observed on microarray analysis are shown in Table S3.1.1.

Table S3.1.1: Selected gene expression results from microarray analysis of chicken liver samples from KwaZulu-Natal, South Africa.

Gene of interest	Function	Expression up- or down-regulated in sample with higher DDTs	Fold change
Avian beta-defensin 1 (*AvBD1*, *GAL1*)	Immune	Down	8.3
Avian beta-defensin 2 (*AvBD2*, *GAL2*)	Immune	Down	21.5
Avian beta-defensin 6 (*AvBD6*, *GAL6*)	Immune	Down	11.8
Avian beta-defensin 7 (*AvBD7*, *GAL7*)	Immune	Down	16.1
Insulin-like growth factor-binding protein 1 (*IGFBP1*)	Metabolism	Up	8.4
Elongation of very long chain fatty acids elongase 2 (*ELOVL2*)	Metabolism	Up	10.6
Squalene epoxidase (*SQLE*)	Metabolism	Up	13.8
Cytochrome P450 Family 17 Subfamily A Member 1 (*CYP17A1*)	Metabolism	Up	16.7

Key: GAL = gallinacin

Conclusion

Microarray analysis of gene expression in liver samples from free-range chickens exposed to DDTs in the environment identified several genes relating to the immune system and metabolism which may be affected by DDTs. These were further analyzed using RT-qPCR and are reported in Chapter 3.1.

3.2 Effects of the organochlorine *p,p'*-DDT on MCF-7 cells: investigating metabolic and immune modulatory transcriptomic changes

Abstract

The organochlorine pesticide dichloro-diphenyl-trichloroethane (DDT) is persistent in the environment and leads to adverse human health effects. High levels in breast milk pose a threat to both breast tissue and nursing infants. The objectives of this study were to investigate DDT-induced transcriptomic alterations in enzymes and transporters involved in xenobiotic metabolism, immune responses, oxidative stress markers, and cell growth in a human breast cancer cell line. MCF-7 cells were exposed to both environmentally-relevant and previously-tested concentrations of *p,p'*-DDT in a short-term experiment. Significant up-regulation of xenobiotic metabolizing enzymes and transporters (*ACHE*, *NQO1* and *ABCC2*) and oxidative stress markers (*CXCL8*, *HMOX-1*, *NFE2L2* and *TNF*) was clearly observed. On the other hand, aryl hydrocarbon receptor (AHR) and cell growth genes (FGF2 and VEGFA) were severely down-regulated. Identification of these genes helps to identify mechanisms of *p,p'*-DDT action within cells and may be considered as useful biomarkers for exposure to DDT contamination.

Introduction

Over several decades, the organochlorine pesticide dichloro-diphenyl-trichloroethane (DDT) was used for agricultural and disease vector control purposes. DDT and its metabolites (most commonly dichloro-diphenyl-dichloroethylene (DDE) and dichloro-diphenyl-dichloroethane (DDD)) are persistent in the environment, bioaccumulate, and show toxicity in many species. They are known to be endocrine-disrupting chemicals (EDCs). Use of DDT is now strictly regulated, with the main use to control insect vectors such as mosquitoes which transmit diseases like malaria and dengue fever (Weiss, 2011). Usually the pesticide is sprayed in and around homes to kill and/or deter the insects.

Residues of DDT and its metabolites (collectively known as DDTs) in foods have been described in many countries (Thompson et al., 2017a). Although levels in foods are declining, contamination levels in people are still a concern. In particular, high levels of lipophilic DDTs in human breast milk are a potential risk for infant health. Mean levels of DDTs in breast milk from malaria-endemic villages in South Africa using DDT regularly were 9.5-18 mg/kg milk fat, sufficiently high to exceed the provisional tolerable daily intake (PTDI) for infants and the maximum residue limit (MRL) set by FAO and the WHO (Bouwman et al., 2012; JMPR, 2010). The postnatal period is considered a critical phase of development, and exposure at this time may have significant impact on infants (Desaulniers et al., 2005). Concurrently, high levels of DDTs in lipid-rich breast milk may result in toxic effects within breast tissue. DDT is classed by the International Agency for

Research on Cancer (IARC) as a group 2A carcinogen, a “probable cause of cancer in humans”.

Exposure of human breast cells to high levels of DDTs contamination from the environment *in vivo* mean that *in vitro* exposure studies using human breast cell lines, such as MCF-7, are useful to investigate molecular changes at the cellular level. DDT is a known endocrine disruptor chemical, with some estrogen-like properties, and this cell line expresses estrogen receptor α (Zhong et al., 2013).

Xenobiotic exposure has been linked to various biochemical changes, including acetylcholinesterase (ACHE) activity which has been used as a biomarker for neurotoxic substances in vertebrate and invertebrate species (Binelli et al., 2006; Vieira et al., 2016). Exposure to DDT induced cytochrome P450 family 1 subfamily A member 1 (*CYP1A1*) expression in rat livers and ovaries (Chanyshev et al., 2014). Cytochrome P450 family 3 subfamily A member 5 (*CYP3A5*) is another enzyme involved in phase I metabolism of xenobiotics, and is associated with DNA damage in workers exposed to organophosphate pesticides (Singh et al., 2011). NAD(P)H quinone dehydrogenase 1 (*NQO1*), sulfotransferase family 1A member 1 (*SULT1A1*) and ATP binding cassette subfamily C member 2 (*ABCC2*, also known as *MRP2*) have been linked to metabolism of other xenobiotics (Hockley et al., 2006; Pascussi et al., 2008; Saengtienchai et al., 2014). The nuclear factor, erythroid 2 like 2 (*NFE2L2*, also known as *Nrf2*) gene is involved in regulation of the *ABCC2* gene, and is inhibited by *p,p’*-DDE (Jin et

al., 2014; Vollrath et al., 2006). C-X-C motif chemokine ligand 8 (*CXCL8*, also known as interleukin 8, *IL8*), tumor necrosis factor (*TNF*), and aryl hydrocarbon receptor (AHR) inflammatory changes have been linked to persistent organic pollutants (Buoso et al., 2017; Kim et al., 2012). AHR also plays a major role in regulation of xenobiotic metabolizing enzymes such as CYP1A1 and NQO1. Heme oxygenase (*HMOX-1*) is a biomarker for oxidative stress, and its expression has been linked to environmental contamination by xenobiotics such as PCBs in fish and *in vivo* exposure of mice to *p,p'*-DDE (Morales-Prieto et al., 2017; Schlenk et al., 2002). *p,p'*-DDT is known to induce adipocyte differentiation, and vascular endothelial growth factor A (*VEGFA*) and fibroblast growth factor 2 (*FGF2*) were included in the genes of interest due to their involvement in adipose tissue angiogenesis (Cao, 2010; Moreno-Aliaga and Matsumura, 2002). Various regulatory elements have been implicated in effects of endocrine disruptor pesticides, including aryl hydrocarbon receptor (*AHR*), nuclear receptor subfamily 1 group I member 2 (*NR1I2*, also known as *PXR*) and estrogen receptors (including estrogen receptor 1, *ESR1*) (Chanyshev et al., 2014; Mnif et al., 2011).

Previous studies on the MCF-7 human breast cancer cell line have investigated various effects of DDTs such as cell viability and proliferation, invasiveness and glucose metabolism (He et al., 2015; Norberto et al., 2017; Pestana et al., 2015). However, genes related to metabolism of xenobiotics, cellular stress, immunity and cell growth have not yet been investigated. Therefore, the objective of this study was to assess a selection of such genes to elucidate some of the

mechanisms by which *p,p'*-DDT, the main component of technical grade DDT, exposure affects cells.

Materials & Methods

Chemicals and reagents

We obtained the MCF-7/GFP cell line from Cell Biolabs, Inc. (distributed by Funakoshi Co. Ltd., Tokyo, Japan). For culture, Dulbecco's modified Eagle's medium (DMEM)-high glucose with L-Glutamine and Phenol Red (Wako, Tokyo, Japan), penicillin-streptomycin solution (Wako, Tokyo, Japan) and fetal bovine serum (FBS, Biowest, France) were purchased. Treatments were the solvent dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO, USA) and dichloro-diphenyl-trichloroethane (*p,p'*-DDT, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was purchased for cellular viability assessment. TRI reagent (Sigma, St. Louis, MO, USA), chloroform (Kanto Chemical Co., Inc., Tokyo, Japan), Nucleospin® RNA (Machery-Nagel, Germany), ReverTraAce® qPCR RT Master Mix with gDNA remover (Toyobo Co., Osaka, Japan), Fast SYBR Green master mix (Applied Biosystems, Life Technologies Japan Ltd., Tokyo, Japan) and primer sets (Invitrogen, Carlsbad, CA, USA) were purchased for RNA isolation, cDNA synthesis and qPCR analysis.

Cell culture conditions

MCF-7 cells were grown in DMEM (high glucose) medium, supplemented with 10% FBS and 5% antibiotic (penicillin-streptomycin). They were maintained in a fully humidified atmosphere with 5% CO_2 at 37°C. Culture medium was changed every 2–3 days and subcultured when confluent (every 5–7 days).

Cell treatments

Once cell confluency was reached, treatments were added to the medium. DMSO was used as the carrier for *p,p'*-DDT, prepared by mixing powdered *p,p'*-DDT ultrasonically with DMSO. The final concentration of DMSO was the same for all treatment wells (0.1%). Concentrations used for *p,p'*-DDT were 5 µM, 10 µM, 20 µM, 50 µM and 100 µM. These lie between environmental exposure doses and those used in previous *in vitro* exposure studies (Ballard and Morrow, 2013; Bratton et al., 2012; Desaulniers et al., 2005; Ennaceur and Driss, 2013; Gregoraszczuk et al., 2008; He et al., 2015). Cellular RNA was collected 24 h after addition of the treatment, and stored at -80°C until further analysis.

Cell viability

Cellular viability was determined by CCK-8 assay as per the manufacturer's instructions. Cell suspension (100 µl) was inoculated in a 96-well plate (collagen-coated microplate, Iwaki, Japan), and once confluency was attained, treatments were added. Previous studies have reported no effect on cell viability at 0.1%

DMSO concentration. Medium with DMSO only served as the solvent carrier negative control. Cells were pre-incubated with the treatments for 24 h before performing the assay to determine cell number. Absorbance was measured at 450 nm using a Thermo Scientific Multiskan® GO microplate spectrophotometer (Thermo Scientific, Japan). The assay was repeated three times. Cultures were also visually assessed by light microscopy (Olympus CK40, Tokyo, Japan).

RNA isolation and quantitative RT-PCR

For treatment prior to RNA isolation, cells were inoculated into 6-well collagen-coated microplates (Iwaki, Japan). Total RNA was isolated using a modified protocol for NucleoSpin® RNA (Macherey-Nagel GMbH & Co. KG, Düren, Germany). The first steps were replaced by TRI reagent (Sigma-Aldrich) used according to the manufacturer's instructions to lyse cells, and chloroform (Kanto Chemical Co., Inc., Tokyo, Japan) for phase separation. At this point, the aqueous phase was mixed with 70% ethanol and transferred to NucleoSpin® columns for DNA binding, desalting, DNA digestion, membrane washing, and RNA elution according to the NucleoSpin® protocol. For cDNA synthesis, ReverTraAce® qPCR RT Master Mix with gDNA remover (Toyobo Co. Ltd., Osaka, Japan) was used as described in the manufacturer's instructions. cDNA samples were stored at -20°C pending further analysis.

The mRNA expression levels were determined using real-time reverse transcriptase-PCR (RT-PCR), carried out using the Step One Plus Real-Time PCR system (Applied Biosystems, Foster, CA). The PCR mixture contained

600 ng of cDNA, Fast SYBR® Master mix, 10 µM of each primer, with RNase-free water added to a final volume of 10 µL. The reaction cycle comprised a holding stage for 20 s at 95°C, followed by 40 denaturation cycles of 3 s at 95°C and 30 s at 60°C (*GAPDH, ACHE, CYP1A1, CYP3A5, NQO1, ABCC2, CXCL8, HMOX-1, NFE2L2, TNF, FGF2, VEGFA, AHR, ESR1,* and *NR1I2*) or 62°C (*GAPDH and SULT1A1*), and 15 s extension at 95°C. Single amplicon amplification was confirmed using melting curve analysis, and absence of primer dimers and genomic DNA amplification by agarose gel electrophoresis. GAPDH was used for normalization by the comparative Ct method, and each experiment repeated at least three times. Genes of interest were selected from a range of genes shown to be involved in exposure to xenobiotics, including various stages of xenobiotic metabolism (phase I, II, III), oxidative stress, inflammation and growth factors. Primer sets used are shown in Table 3.2.1.

Statistical analysis

Microsoft Excel® 2014 and JMP® Pro 13 (SAS Institute Inc., Cary, NC, USA) were used for data analysis. Dunnett's test was performed to evaluate statistical significance between exposure groups, with a *p*-value of <0.05 considered significant.

Table 3.2.1: Primer sets used for qRT-PCR analysis in this study.

Symbol	Description	Function	Sequence		Accession number	Product length (bp)
			Forward	Reverse		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	(Housekeeping gene)	ACCCAGAAGACTGTGGATGG	CAGTGAGCTTCCCGTTCAG	NM_001289746.1	139
ACHE	Acetylcholinesterase	Phase I metabolism	CATCAACGCGGGAGACTT	GAGACTCGTTGTCTTTGCTGAA	NM_001302621.1	113
CYP1A1	Cytochrome P450 family 1 subfamily A member 1	Phase I metabolism	CTATCTGGGCTGTGGGCAA	CTGGCTCAAGCACAACTTGG	NM_001319217.1	138
CYP3A5	Cytochrome P450 family 3 subfamily A member 5	Phase I metabolism	TGACCCAAAGTACTGGACAG	TGAAGAAGTCCTTGCGTGTC	NM_001291830.1	240
NQO1	NAD(P)H quinone dehydrogenase 1	Phase II metabolism	GGATTGGACCGAGCTGGAA	AATTGCAGTGAAGATGAAGGCAAC	NM_001286137.1	140
SULT1A1	Sulfotransferase family 1A member 1	Phase II metabolism	AAAGCCCCAGGGATTCCCTCA	GGAAACTGCCACATCCTTTGCGT	NM_177530.2	162
ABCC2	ATP binding cassette subfamily C member 2	Phase III metabolism	CTTCGGAAATCCAAGATCCTGG	TAGAATTTTGTGCTGTTCACATT	NM_000392.4	284
CXCL8	C-X-C motif chemokine ligand 8	Inflammation, oxidative stress	ACTTTCAGAGACAGCAGAGCACACA	CCTTCACACAGAGCTGCAGAAATC	NM_001354840.1	151
HMOX-1	Heme oxygenase 1	Oxidative stress	ATGGCCTCCCTGTACCACATC	TGTTGCGCTCAATCTCCTCCT	NM_002133.2	55
NFE2L2	Nuclear factor, erythroid 2 like 2	Oxidative stress	CTTGGCCTCAGTGATTCTGAAGTG	CCTGAGATGGTGACAAGGGTTCTA	NM_001313904.1	124
TNF	Tumor necrosis factor	Inflammation	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA	ENST00000376122.3	123
FGF2	Fibroblast growth factor 2	Growth factor, fibroblast cells	GGCTTCTTCCTGCGCATCCA	GCTCTTAGCAGACATTGGAAGA	NM_002006.4	354
VEGFA	Vascular endothelial growth factor A	Growth factor, especially vascular endothelial cells	ACATTTACACGTCTGCGGATCT	AGGGAAAGGGGCAAAAACG	NM_001025367.2	104
AHR	Aryl hydrocarbon receptor	Regulatory element	ATCACCTACGCCAGTCGCAAG	AGGCTAGCCAAACGGTCCAAC	NM_001621.4	137
ESR1	Estrogen receptor 1	Regulatory element	ATTGGTCTCGTCTGGCGCTCC	CCCTGCAGATTCATCATGCGG	NM_001328100	161
NR1I2	Nuclear receptor subfamily 1 group I member 2	Regulatory element	CATGAGGGGGGTAGCAAAGC	TGCAGGGGATCTCCCTCTTC	NM_022002.2	248

Key: adenine (A), cytosine (C), guanine (G), thymine (T)

Results

Cellular viability

After 24 h exposure to either the carrier compound, DMSO, or the carrier with *p,p'*-DDT, cellular viability was determined by CCK-8 assay. As shown in Figure 3.2.1, there were no statistically significant effects observed on cell viability between wells containing the carrier (DMSO) and carrier with *p,p'*-DDT concentrations up to 50 µM. However, there was a significant reduction in cellular viability at 100 µM ($p < 0.0001$), and on microscopic evaluation some non-

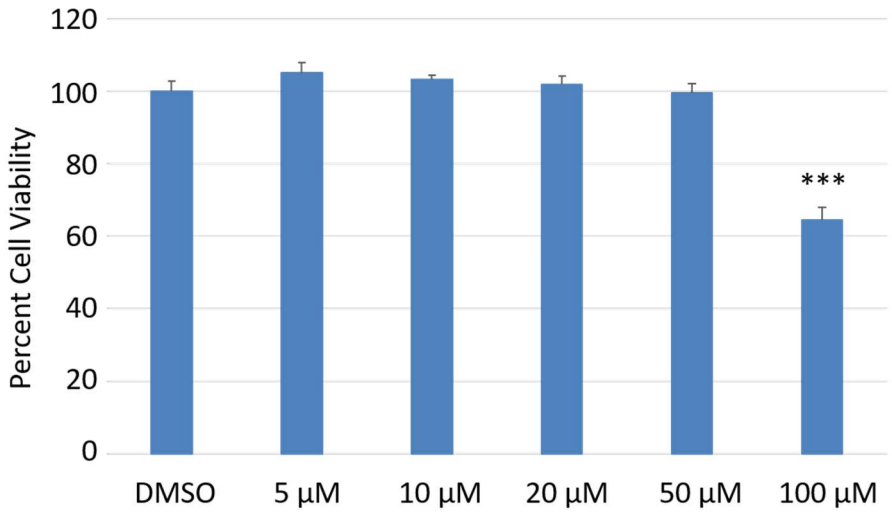


Figure 3.2.1: Comparison of *p,p'*-DDT on MCF-7 cell viability, as evaluated by the CCK-8 assay after 24 h exposure. DMSO concentration in all wells was 0.1%. Data are mean ± SE of absorbance compared to DMSO. Range of *p,p'*-DDT concentrations was 5–100 µM. Column carrying *** mark is significantly different at $p < 0.0001$ compared to DMSO wells (Dunnett's test).

adherent cells were seen in wells with this concentration. Comparisons of gene expression were conducted using solvent carrier samples as controls.

Gene expression

Results of gene expression analysis are shown in Figures 3.2.2 – 3.2.5. First, the enzymes involved in xenobiotic metabolism were analysed (Figure 3.2.2). No significant differences were seen between negative carrier control and *p,p'*-DDT treatments for the phase I enzyme *CYP3A5*, nor the phase II enzyme *SULT1A1*. For the other phase I enzymes, *CYP1A1* gene expression was down-regulated 5-fold at 5 μM *p,p'*-DDT ($p = 0.04$), and *ACHE* up-regulated 3-fold at 50 μM ($p = 0.03$) and 16-fold at 100 μM ($p < 0.0001$). The other phase II enzyme assessed, *NQO1*, was up-regulated 2–fold at 10 μM and 20 μM ($p < 0.0001$ for both). The phase III metabolising enzyme, *ABCC2*, was up-regulated 14-fold at 100 μM ($p = 0.01$).

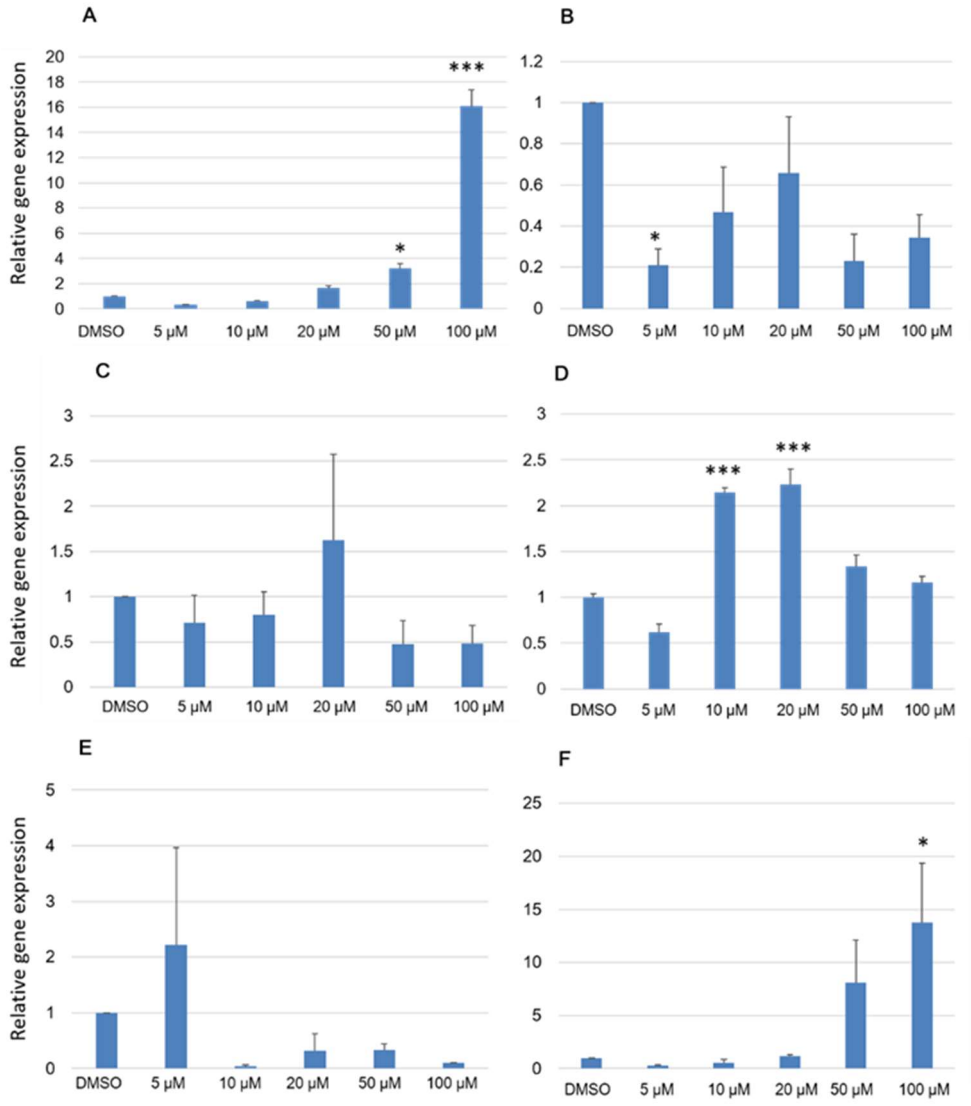


Figure 3.2.2: Effect of *p,p'*-DDT on enzymes involved in xenobiotic metabolism (XMEs) in MCF-7 cells after 24 h exposure. A) Acetylcholinesterase (*ACHE*), B) cytochrome P450 family 1 subfamily A member 1 (*CYP1A1*), C) cytochrome P450 family 3 subfamily A member 5 (*CYP3A5*), D) NAD(P)H quinone dehydrogenase 1 (*NQO1*), E) sulfotransferase family 1A member 1 (*SULT1A1*), and F) ATP binding cassette subfamily C member 2 (*ABCC2*). Values are expressed as mean ± SE of mRNA expression relative to the control, DMSO (ΔΔCt method, $n \geq 3$). Columns carrying a * mark are significantly different at $p < 0.05$, and *** at $p < 0.001$ (Dunnett's test).

Oxidative stress and inflammatory markers were then analysed (Figure 3.2.3).

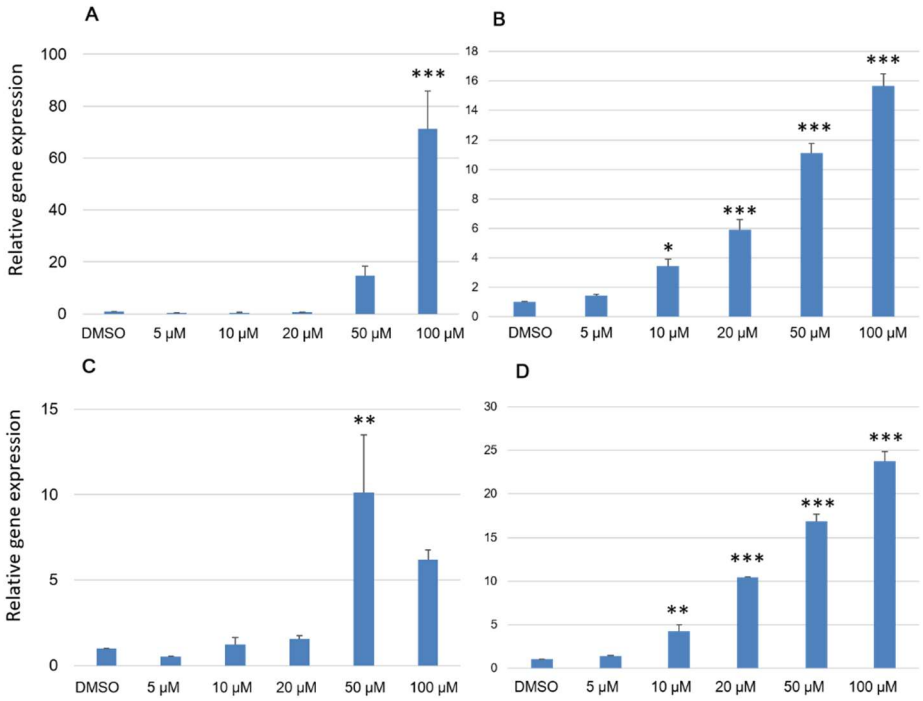


Figure 3.2.3: Effect of *p,p'*-DDT on oxidative stress and inflammatory markers in MCF-7 cells after 24 h exposure. A) C-X-C motif chemokine ligand 8 (*CXCL8*), B) heme oxygenase 1 (*HMOX-1*), C) nuclear factor, erythroid 2 like 2 (*NFE2L2*), and D) tumor necrosis factor (*TNF*). Values are expressed as mean ± SE of mRNA expression relative to the control, DMSO (ΔΔCt method, $n \geq 3$). Columns carrying a * mark are significantly different at $p < 0.05$, ** at $0.001 < p < 0.01$, and *** at $p < 0.001$ (Dunnett's test).

CXCL8 expression was up-regulated 71-fold at 100 µM ($p < 0.0001$), and *NFE2L2* up-regulated 10-fold at 50 µM ($p = 0.001$). Both *HMOX-1* and *TNF* showed dose-dependent up-regulation from 10 µM to 100 µM treatments. Gene expression for *HMOX-1* ranged from 3-fold up-regulation at 10 µM ($p = 0.02$) to 16-fold at 100

μM (with p < 0.0001 from 20 μM upwards). Up-regulation for *TNF* was from 4-fold at 10 μM (p = 0.008) to 24-fold at 100 μM (with p < 0.0001 from 20 μM upwards).

Next, cell growth genes were analysed (Figure 3.2.4).

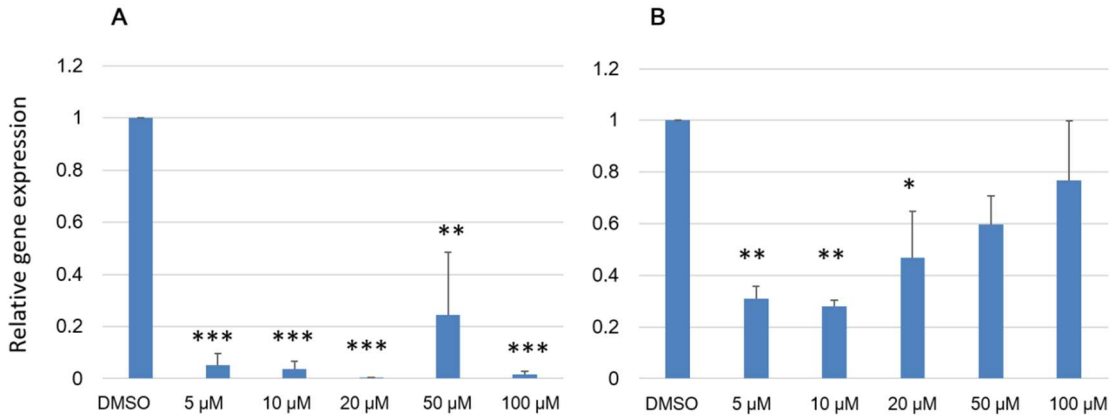


Figure 3.2.4: Effect of *p,p'*-DDT on cell growth in MCF-7 cells after 24 h exposure. A) Fibroblast growth factor 2 (*FGF2*), and B) vascular endothelial growth factor A (*VEGFA*). Values are expressed as mean ± SE of mRNA expression relative to the control, DMSO (ΔΔCt method, $n \geq 3$). Columns carrying a * mark are significantly different at $p < 0.05$, ** at $0.001 < p < 0.01$, and *** at $p < 0.001$ (Dunnett's test).

FGF2 was down-regulated at all exposure concentrations, to a low of 398-fold compared to control at 20 μM ($p = 0.0003$ at 50 μM, $p < 0.0001$ for all other concentrations). *VEGFA* was down-regulated between 5 μM ($p = 0.006$) and 20 μM ($p = 0.04$), with a low of 3-fold at 10 μM ($p = 0.005$).

Finally, regulatory elements were analysed (Figure 3.2.5).

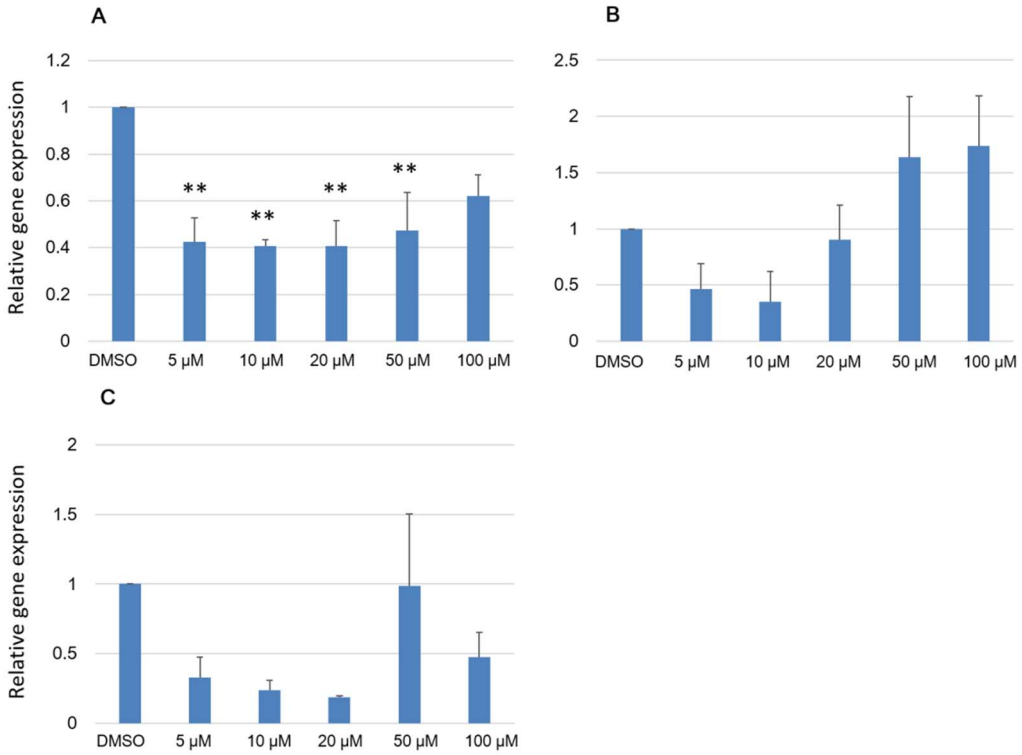


Figure 3.2.5: Effect of *p,p'*-DDT on regulatory elements in MCF-7 cells after 24 h exposure. A) aryl hydrocarbon receptor (*AHR*), B) estrogen receptor 1 (*ESR1*), and C) nuclear receptor subfamily 1 group I member 2 (*NR1I2*). Values are expressed as mean ± SE of mRNA expression relative to the control, DMSO (ΔΔCt method, $n \geq 3$). Columns carrying a ** mark are significantly different at $0.001 < p < 0.01$ (Dunnett's test).

No significant differences were seen between negative carrier control and *p,p'*-DDT treatments for either *ESR1* or *NR1I2*. *AHR* expression was down-regulated between 5 µM ($p = 0.003$) and 50 µM ($p = 0.006$), with a low of 2–fold at 10 µM ($p = 0.002$).

Discussion

Cellular viability

Concentrations up to 50 μM *p,p'*-DDT did not affect cell viability. However, the difference at 100 μM shows significant adverse cellular changes. Data for the highest exposure were considered with this in mind. Exposure of the neuronal PC12 cell line to *p,p'*-DDE induced apoptosis via TNF signalling at concentrations ≥ 20 μM (Wang et al., 2014). Wang et al's study also demonstrated dose-dependent neuronal apoptosis in zebrafish embryos exposed to *p,p'*-DDE. Cell cytotoxicity appears to be dependent on the cell line, exposure concentration, and congener (Nuñez et al., 2002). Reduction in cellular viability could be due to oxidative stress identified in some of the genes assessed in this study, for example *TNF* or *CXCL8*, which may lead to cell death.

Gene expression

Although previous studies suggested that significant changes may be expected in expression of the genes selected, this was not so in all cases.

Enzymes and transporters involved in xenobiotic metabolism

Environmental studies frequently use inhibition of ACHE activity as a biomarker for xenobiotic, including DDTs, contamination (Jung et al., 2012; Vázquez-

Boucard et al., 2014). Activity of ACHE in the brain appears to be an important biomarker but even serum levels are less reliable for toxicity assessment (Walker et al., 2012). Although a non-significant inhibition was observed at low exposure concentrations, this *in vitro* study has identified a substantial promotion of expression in MCF-7 cells at higher *p,p'*-DDT concentrations. Thus, ACHE may play a more important role in phase I metabolism at high levels of contamination.

No significance was detected in *NR1I2* expression. A systematic study conducted by Chaturvedi et al. concluded that different mouse cell types responded discordantly to xenobiotic exposure, with the DDT treatment resulting in up-regulation of the NR1I2 receptor in liver but unchanged in testis (Chaturvedi et al., 2010). Species-specific regulation of the *NR1I2* gene has also been demonstrated in mouse and rat liver experiments after *o,p'*-DDT exposure (Kiyosawa et al., 2008a). The nuclear receptor NR1I2 pathway (originating at the CAR/PXR ligand on the nucleus) is involved in *CYP3A5* expression. It is therefore unsurprising that this gene was not significantly affected by treatment.

Expression of the phase II enzyme *SULT1A1* and phase III enzyme *ABCC2* are also under control via the CAR/PXR ligand, but via the nuclear receptor subfamily 1 group I member 3 (NR1I3) pathway. Phase I enzyme *CYP3A5* expression is also influenced via this pathway. Of these three enzymes, only *ABCC2* expression showed a significant dose-dependent up-regulation at the highest treatment. This gene is also induced by NFE2L2 and NR1I2, and it may be useful

to assess comparative expression of these with NR1I3 to further clarify the mechanism involved. The ABCC2 protein plays a major role in elimination of endo- and xenobiotics, and regulation by *p,p'*-DDT is likely to impact detoxification in the body (Arana and Tocchetti, 2016). For the MCF-7 cell line, elimination of *p,p'*-DDT at phase III may be more important than at earlier stages. Further examination of other phase I and phase II enzymes should be conducted to elucidate this.

Expression of the phase I enzyme *CYP1A1* was significantly downregulated only at the 5 μM concentration. In the MCF-7 cells, *AHR* expression was also down-regulated after exposure to *p,p'*-DDT. AHR is known to induce expression of *CYP1A1*. This finding concurs with a study using placental cells, which found suppression of both AHR protein and CYP1A1 activity after exposure to *p,p'*-DDT, *o,p'*-DDT and *o,p'*-DDE (Wójtowicz et al., 2011). Another regulator of *CYP1A1* expression is ESR1; expression of *ESR1* was not significantly affected by *p,p'*-DDT in this study. Although some pesticides including DDTs have previously been linked to estrogenic effects, and CYP1A1 has been implicated in some cell line exposures, a study exposing MCF-7 cells with *p,p'*-DDE and another exposing peripheral blood mononuclear cells with DDE did not result in induction of the *CYP1A1* gene via this estrogenic mechanism (Gaspar-Ramírez et al., 2015; Liu et al., 2014; McDougal et al., 1997). Also, expression of *TNF* was significantly up-regulated in a dose-dependent manner. This protein is known to inhibit expression of *CYP1A1*. NFE2L2, which was up-regulated at 50 μM, also

inhibits *CYP1A1* expression. The balance between AHR, ESR1, NFE2L2 and TNF effects is likely to be important for *CYP1A1* expression.

NQO1 is a phase II enzyme, known to detoxify xenobiotics and provide cytoprotection to exposed tissues. Exposure to the carcinogen benzo(a)pyrene upregulated expression of NQO1 in both MCF-7 and HepG2 human cell lines (Hockley et al., 2007). Expression of the *NQO1* phase II enzyme was significantly up-regulated in MCF-7 at 10 μM and 20 μM *p,p'*-DDT concentrations. Reactive oxygen species activate NFE2L2 (up-regulated in this study) via the MAPK pathway, in turn inducing *NQO1*. AHR may induce *NFE2L2*, but down-regulation of *AHR* seen in this study suggests this pathway is not involved during *p,p'*-DDT exposure. TNF is known to induce expression of *NQO1*, and expression of *TNF* was significantly up-regulated in the study. The balance between NFE2L2, TNF and AHR effects is likely to be important for *NQO1* expression.

Oxidative stress and inflammatory markers

A highly significant ($p < 0.0001$) increase in gene expression of *CXCL8* was seen at the 100 μM exposure concentration. This inflammatory cytokine and indicator of oxidative stress may be related to the reduced viability seen at this concentration, and this mechanism should be further investigated. A study with exposure of THP-1 cells (a human monocytic cell line) to *p,p'*-DDT or *p,p'*-DDE resulted in a significant down-regulation of *CXCL8* expression, suggesting cell-specific effects on this gene (Buoso et al., 2017). CXCL8 is thought to be involved

in pathogenesis of bronchiolitis, and prenatal DDE exposure has been linked to children's respiratory health. However, although regression modelling suggested the chemokine interleukin 10 (*IL10*) plays a role in such respiratory pathology, the modelling did not confirm a link with *CXCL8* (Gascon et al., 2014).

The anti-oxidant enzyme HMOX-1 was up-regulated in a dose-dependent manner when exposed to *p,p'*-DDT. Inducers of this gene include heavy metals, endotoxin, and inflammatory cytokines (Choi and Alam, 1996). NFE2L2 has been shown to up-regulate expression of this gene (Salazar et al., 2006). In this study, a significant increase was also noted in *NFE2L2* at 50 μM concentration. Conversely, a study using HepG2 cells showed ROS-mediated down-regulation of *NFE2L2* following exposure to *p,p'*-DDE (Jin et al., 2014). It would be of interest to repeat exposure of MCF-7 cells with *p,p'*-DDE at similar exposures to compare effects. This indicator of oxidative stress is an important intermediary in several xenobiotic metabolism pathways and therefore a useful biomarker.

TNF is an important pro-inflammatory cytokine, regulating immune response to pathogens. Massawe et al. demonstrated an increase in TNF secretion after DDT exposure at 2.5 μM to various human immune cells, thought to occur via the MAPK pathway (Massawe et al., 2017). Conversely, Burow et al. proposed that suppression of TNF-induced apoptosis by *o,p'*-DDT occurred via an estrogen receptor pathway (Burow et al., 1999). However, a study on macrophages showed that although DDT alone induced *TNF*, suppression of *TNF* was seen

with DDT in the presence of lipopolysaccharide (found on the outer membrane of Gram negative bacteria and a strong immune stimulant) (Dutta et al., 2008). TNFα is a pro-inflammatory cytokine, and altered production will result in an imbalance in the immune system. An association was shown clinically between maternal *p,p'*-DDE and *o,p'*-DDD, *TNF* expression, and preterm birth of infants (Tyagi et al., 2016). A correlation has also been demonstrated between chlorinated pesticides, including DDT, in mothers' milk and depressed TNF secretion in infants (Schaalan et al., 2012). These data suggest immunotoxicity may occur in infants relating to organochlorine exposure during development.

Cell growth

Interestingly, expression of both cell growth factors—*FGF2* and *VEGFA*—were significantly down-regulated with exposure to *p,p'*-DDT. With *VEGFA*, the suppression is most obvious at lower exposure doses. These lower doses are closer to the environmental exposures seen with breast milk contamination. An 18 h exposure experiment with MCF-7 cells showed up-regulation of *VEGFA* expression after exposure 10 μM *o,p'*-DDT, suggesting a difference in effects between DDT congeners (Bratton et al., 2012). AHR induces *FGF2* and *VEGFA* expression, and thus the down-regulation of these genes seen in this study may be explained by concomitant *AHR* down-regulation (Lahoti et al., 2013). This is the first report of *FGF2* expression effects in association with DDTs exposure, and may indicate another mechanism by which *p,p'*-DDT exerts effects on cells, particularly adipose tissue.

Regulatory elements

There was a significant down-regulation of *AHR* gene expression at exposure doses between 5–50 μM. This differs from results in a study using MCF-7 cells with 1 μM *p,p'*-DDT exposure, which showed enhanced proliferation of cells associated with up-regulation of *AHR* expression after 12 h (Ociepa-Zawal et al., 2007). Peripheral blood mononuclear cells with 28 nM DDE exposure also showed up-regulation of *AHR* expression, which was abolished by TNF (Gaspar-Ramírez et al., 2015). It can be concluded that differing the exposure chemical, dose and period have very different effects on cells.

Although there appears to be a trend for increasing expression of *ESR1* with increasing concentration of *p,p'*-DDT, no statistical significance was detected between exposure groups. Previous work on mice brains has linked prenatal *p,p'*-DDT exposure to a depressive-like effect associated with a decrease in estrogen receptors including ESR1 (Kajta et al., 2017). An *in vivo* exposure study using *o,p'*-DDT in immature rats did not elicit estrogen receptor-mediated responses (Kiyosawa et al., 2008b).

At the concentrations used, no statistically significant effects were observed in *NR1I2* expression after exposure of MCF-7 cells with *p,p'*-DDT. Exposure of other cell lines to other DDT congeners is generally reported to result in induction of *NR1I2*: for example in HepG2 cells exposed to 10 μM *o,p'*-DDT, liver samples from rats which received technical grade DDT, and liver from salmon receiving

DDE (Kiyosawa et al., 2008b; Medina-Díaz et al., 2007; Mortensen and Arukwe, 2006). However, Kiyosawa et al. reported species differences in PXR and CAR activation in mice and rats (Kiyosawa et al., 2008a).

Many experiments have been conducted using DDTs to assess the molecular effects. Differences in cell line, tissues, species, DDT congener used and exposure time vary greatly between studies. Genes were selected in this study to give an overview of several metabolic processes that may be affected by xenobiotic exposure. Also, expression in the target cell line was considered (for example CYP1A1 is highly expressed in breast cells while CYP1A2 is not). This study has demonstrated a number of modulatory effects by *p,p'*-DDT on the transcriptome, and future studies can follow these to further elucidate pathways involved. It would be useful to investigate these areas of metabolism in more depth, for example selecting phase II enzymes from the glutathione S-transferase (GST) or UDP glucuronosyltransferase (UGT) families, oxidative stress markers such as superoxide dismutase (SOD), or inflammatory mediators such as Type 1 interferon or interleukins. We still have much to learn about the toxic effects of DDTs, especially at levels and mixtures present in the environment.

This acute exposure study used levels of *p,p'*-DDT that are higher (10–100 μM) than those previously reported in breast milk in Tunisian women (estimated to be 0.0395 μM using an average milk fat concentration of 3.2%) (Ballard and Morrow, 2013; Ennaceur and Driss, 2013). However, high-level exposure acutely may

mimic the chronic exposure experienced by people living in countries using DDT regularly. It would therefore be useful to assess genes identified in the study with expression in these populations.

Conclusions

This experimental study has identified several genes to be significantly up-regulated (*ACHE*, *NQO1*, *ABCC2*, *CXCL8*, *HMOX-1*, *NFE2L2,* and *TNF*) and down-regulated (*CYP1A1*, *FGF2*, *VEGFA*, and *AHR*) after acute exposure of MCF-7 cells to the *p,p'*-DDT congener. Effects are dose-dependent in some genes. These genes are involved in an array of metabolic processes, including inflammation, oxidative stress, and growth of fibroblast and vascular endothelial cells. These may be useful biomarkers for exposure to DDT contamination, and may also help identify the mechanisms of toxicity of DDT and its metabolites in breast cells and in nursing infants.

- In chapter 2, we confirmed environmental exposure of DDTs in both chicken and fish samples, which may pose a risk to animal health as well as human health (through consumption). However, clinical toxicity was not noted in the animals sampled.

- In chapter 3, molecular analysis was performed on chicken liver samples from the field, and showed significant alterations in expression of several genes associated with immune function, notably an array of β–defensin genes, and metabolic processes, in particular those relating to lipid and hormone metabolism, growth and reproduction.

- Using molecular techniques, we also detected alteration of several genes involved in metabolic processes following acute exposure of the MCF-7 human cell line.

Chapter 4

Conclusions

Conclusions

The aims of this doctoral thesis were to assess human health risk from DDTs through consumption of contaminated foods, particularly chicken and fish products, and to investigate metabolic effects of such contamination in both chickens and humans. A combination of a literature review, field sampling (from regions in South Africa and Mozambique where DDT is sprayed to control malaria), and *in vitro* laboratory exposure of a human cell line (MCF-7) was utilised to achieve these goals.

Organochlorine pesticide (OCP) use in agriculture and disease control over a prolonged time period has led to contamination of many food products. Ongoing use of DDT in indoor residual spraying programs over the past decade in KwaZulu-Natal has been an effective method of controlling malaria vectors and hence reducing disease cases and fatalities. However, this chemical and its metabolites are known to have adverse effects in many species, and the full extent of environmental contamination is not yet appreciated. Thus, this research interpreted reports of food contamination by OCPs, assessed the extent of DDTs contamination in exposed biota in the field situation, and probed the effects of such contamination in organisms at the molecular level.

- A literature review revealed extensive contamination of food products in many countries across the African continent by DDT and other organochlorine pesticides (OCPs). Vegetables, meat and dairy products (including processed foods) contained these chemicals. Contamination levels were also reported in human serum and breast milk. Clinical reports of toxic effects have been associated with such contamination.

- Field sampling during this work has demonstrated contamination of free-ranging chickens in KwaZulu-Natal homesteads where DDT is sprayed annually to control malaria, and also in fish living in the Indian Ocean which rivers reach after passage through similar DDT-spraying regions in South Africa, Mozambique and Swaziland. Such contamination levels are sufficiently high as to be a potential health risk to people from consumption, with chicken eggs a much greater risk than chicken meat, which in turn carries a greater risk than fish meat. This information is extremely useful for policy-makers as they plan their malaria control programs, and as they advise residents on how to minimise health risks from contamination with DDTs.

- Although no clinical toxic effects were detected, molecular analysis showed genetic changes in chickens associated with higher levels of contamination with DDTs. This is the first report of significant genetic changes associated with DDTs exposure in chickens. Notably, a number of avian β-defensin genes (AvBD1, AvBD2, AvBD6, and AvBD7) were

found to be significantly down-regulated in birds with higher DDTs, in association with higher p,p'-DDE and p,p'-DDD levels. Consequences of damage to these innate immune system genes by DDTs is a previously unidentified health risk to chickens, and implicates xenobiotics as a factor increasing susceptibility to infectious disease. Up-regulation of various genes involved in metabolism (ELOVL2, SQLE and CYP17A1) also implicate such contamination in metabolic effects including altered lipid and hormone metabolism, growth and reproduction. These data presented may be useful in amelioration of such matters, for example in counteracting specific genetic changes in livestock species by selectively breeding animals with polymorphisms that are less susceptible to the effects of DDTs and/or using dietary modifications to offset the effects.

A panel of potential biomarkers was assessed in culture of the MCF-7 human breast cancer cell line after acute exposure to p,p'-DDT. Regarding metabolism of the xenobiotic, the phase I enzyme ACHE, phase II NQO1 and phase III ABCC2 were significantly up-regulated. Phase I CYP1A1 was down-regulated at the lowest exposure. Oxidative stress and inflammatory markers CXCL8, HMOX-1, NFE2L2 and TNF all showed significant up-regulation. Interestingly, the NFE2L2 gene is also involved in cholesterol trafficking. Also supporting the reduction in expression of growth-related genes identified in the chicken analysis, FGF2 and VEGF genes were significantly down-regulated in MCF-7 culture. The AHR receptor involved in drug metabolism pathways showed down-regulation.

As with chickens, the implications for humans are potential effects on the immune system and metabolism, which may in turn potentiate other diseases. The possibility of nutritional manipulation of genetic expression could also be useful in people, and may provide a relatively simple solution to a long term environmental problem.

Environmental persistence of DDT and its metabolites will continue to expose biota for many decades. Further work remains to be conducted to monitor exposure levels over time and to ascertain subclinical effects in more detail. Other species at risk should be included in this assessment, such as other livestock species and wildlife. As species-specific effects are likely, different taxonomic groups should be included in the assessment. Meanwhile, it is important to discuss potential health risks to people from consumption of contaminated food products, and how to reduce those risks (for example by altering cooking methods or purchasing commercially-reared chicken products). Other interventions may be developed to counteract adverse effects from contamination, such as targeting pathways involved in these genetic effects. Trials with nutritional manipulations should be conducted to ascertain their suitability and practicality for general application. In people, remediation efforts should be focussed on the most at risk groups – young children and women who spend most time in the DDT-exposed homestead.

DDT has been shown to be a cost-effective insecticide for use in malaria control programs. However, the hidden costs to human, animal and environmental health

are difficult to quantify. Alternative control methods are under investigation, including use of other insecticides, greater use of insecticide-treated nets, improved access to health care in malaria-endemic areas, and education about mosquito control and avoidance. Due to continued exposure from legacy use, remediation measures may be required in some areas with high levels of environmental contamination. An increased identification of adverse effects, and understanding of mechanisms by which DDTs result in these, should enable focused research into alleviation of health problems in future generations of people, their livestock, and wildlife.

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May the road rise to meet you,
may the wind be ever at your back.
May the sun shine warm upon your face,
and the rains fall soft upon your fields.
And until we meet again,
may God hold you in the palm of his hand.

(Anon)

Between my finger and my thumb
The squat pen rests.
I'll dig with it.

(Seamus Heaney)