The effectiveness of the Australian pesticide regulatory system: A case study on chlorpyrifos exposure among an urban South Australian population

by

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DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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ABSTRACT

Australia has a well-developed agricultural industry with 394 million hectares of agricultural land out of 769 million hectares total land area. Agricultural pesticides are used on 73% of the total value of crops produced. In most countries, Australia included, exposure to pesticides is controlled by having regulations to restrict this chemical usage for the purpose of protecting the people and environment. Presently, there is no exposure monitoring programs to evaluate the effectiveness of pesticide regulation in Australia in controlling the population exposure to pesticides.

To understand the basis of Australian pesticide regulation, the differences between the regulatory systems in Australia (AU) and the European Union (EU) are explored in **Chapter 2**. It was discovered that the assessment to authorize a pesticide in the EU is based on the hazard of the pesticides. If a pesticide is not classified as hazardous, the pesticides will proceed to the next level of assessment. On the other hand, the assessment to authorize a pesticide in Australia is based on risks (the likelihood of being exposed to the pesticides and the potential of effects of the exposure to the pesticide). There is not any registration review period set for an active substance authorised in Australia. A registered active substance can be authorized for used until it is nominated for reconsideration. In contrast, active constituents are authorized for use in the EU for 10 years only. The Australian reconsideration process is conducted on an ad-hoc basis. This is concerning because as more research is conducted on various chemicals, a pesticide registered a number of years ago maybe uncovered to be more of a hazard than previously thought. Unless regular scientific review process is built into a regulatory system, there is a risk of hazardous pesticides continuing to be used. Another matter that is of concern is there are no regular systematic comprehensive chemical residue surveys for food commodities conducted in Australia. Therefore, we cannot make any formal conclusion on what pesticide residues are consumed

by the Australian public. On the other hand, the EU has a systematic monitoring program called the National Control Programmes which are reported every year.

To understand the effectiveness of pesticide regulation in Australia, this thesis narrowed down its focus on the restriction of chlorpyrifos (CPF) use among the general public of Australia introduced in 2000- 2001. The restrictions were introduced as a result of CPF review that was undertaken by APVMA as part of the reconsideration process. The reconsideration process was triggered by extensive reviews and regulatory changes to chlorpyrifos registration in other jurisdictions (US and EU). **Chapter 3** describes the literature review conducted to explore the availability of exposure data among the Australian population. The literature review revealed that the monitoring of CPF exposure among the Australian public is not done extensively. Pesticide contamination in food is not monitored frequently and systematically in Australia too. Therefore, we do not know the extent of CPF or any pesticides exposure among the Australian public. For this reason, there is not enough information to evaluate the effectiveness of Australian pesticide regulatory system to control pesticide exposure among the public. Particularly, the effectiveness of CPF use restriction introduced among the general public of Australia in 2000-2001 has not been reported because of the limited exposure monitoring done.

This thesis intended to address this gap by investigating the extent of chlorpyrifos exposures of an urban South Australia population after the interim regulatory measures introduced in 2000-2001. The overall aim of this thesis is to investigate the CPF exposure among an urban population in South Australia after the implementation of the said interim regulatory measures. Biomonitoring approach was chosen to be the means to investigate the exposure of CPF among the said population. Urinary 3,5,6-trichloro-2-pyridinol (TCPy) was selected as the biomarker of exposure for the study population, which was comprised of a random sample of adults and children. An analytical method

was developed to analysed urinary TCPy as described in **Chapter 4**. Urinary TCPy of the collected sample was analysed with GCMS with modified QuEChERS extraction.

Analyses of urinary TCPy for the population of this study were compared in **Chapter 5** with a study conducted in 2003-2006 when the implementation of restrictions of CPF usage was at the initial stage. The comparison revealed that there is a 76% decrease in the frequency of detection of urinary TCPy among this population. Moreover, the P95 level of this population is 0 ug/g (non-detected) whereas it was 12.5 ug/g in the 2003-2006 study. The range of concentration of TCPy in this study is 0- 69.53 µg/g while it was < LOD- 217.9 µg/g in the 2003-2006 study. We found that the CPF exposure among the urban South Australia population in this research has decreased seemingly because of the restriction of high concentration of CPF among the public of Australia. The findings of this study are unique and valuable in investigating the effectiveness of some parts as well as the whole Australian pesticide regulatory system.

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LIST OF ABBREVIATONS

3-PBA	3-phenoxybenzoic acid
AChE	acetylcholinesterase
ACCS	Advisory Committee on Chemical Scheduling
ABS	Australian Bureau of Statistics
ADHD	attention deficit hyperactive disorder
ADI	acceptable daily limit
ADME	absorption, distribution, metabolism, and excretion
agvet	agricultural and veterinary (chemical/code)
AHS	Agricultural Health Study
ANOVA	analysis of variance
APVMA	Australian Pesticides and Veterinary Medicine Authority
AS	active substance
ASD	autism spectrum disorder
ASTDR	Agency for Toxic Substance and Disease Registry
ATDS	Australian Total Diet Study
AUC	area under the curve
BPR	Biocidal Products Regulation
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
BuChE	butyryl cholinesterase
CCCEH	Columbia Centre for Children's Environmental Health
CDC	Centres for Disease Control and Prevention
CHAMACOS	Centre for the Health Assessment of Mothers and Children of Salinas
CPF	chlorpyrifos
Cth	commonwealth
DALY	disability-adjusted life-years
DAP	dialkyl phosphate
DCM	dichloromethane
DE	diethyl
DEP	diethylphosphate
DETP	diethylthiophosphate
DDT	Dichlorodiphenyltrichloroethane (a pesticide)
DPR	Department of Pesticide Regulation
DSCI	Department for Communities and Social Inclusion
EC	European Commission
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FFDCA	Federal Food, Drug, and Cosmetic Act

FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FOD	frequency of detection
FQPA	Food Quality Protection Act
FSANZ	Food Standards Australia New Zealand
FVO	Food and Veterinary Officer
GBR	Great Barrier Reef
GCMS	Gas chromatography mass spectrometry
GLP	Good Laboratory Practice
HOME	Health Outcomes and Measures of the Environment Study
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer
ISTD	internal standard
K_{ow}	partition coefficient
LCMS	Liquid chromatography mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
m/z	mass to charge ratio
MACCP	Multi-Annual Co-coordinated Control Programme
$MgSO_4$	magnesium sulphate
MRL	maximum residue limit
MS	Mass spectrometry
MTBE	methyl tertiary-butyl ether
MTBSTFA	N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide
NaCl	sodium chloride
NHANES	National Health and Nutrition Examination Survey
NOAEL	no-observed-adverse-effect-limit
NRA	National Registration Authority
NRDC	National Resources Defense Council
NRS	National Residue Survey
NTE	neuropathy target esterase
OECD	Organization for Economic Co-operation and Development
OP	organophosphorus
OPIDP	organophosphate-induced delayed polyneuropathy
PANNA	Pesticides Action Network North America
PBT	persistent, bioaccumulative and toxic
PON	paraoxanose
POP	persistent organic pollutant
PPE	personal protective equipment
PPP	plant protection product
PUBCRIS	Public Chemical Registration Information System Search
PYR	pyrethroid
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, Safe

RA	risk assessment
RASFF	Rapid Alert System for Food and Feed
RAR	renewal application report
RMS	rapporteur member state
RSD	relative standard deviation
SA	South Australia
SPE	solid phase extraction
SPS	Sanitary and Phytosanitary (measures)
TBDMS	tert-butyldimethylsylil
TCPy	3,5,6-trichloro-2-pyridinol
US EPA	United States Environment Protection Agency
WHO	World Health Organization
WTO	World Trade Organization

CHAPTER 1: INTRODUCTION

1.1 Pesticides

Pesticides are defined as “any substance or mixture of substances deliberately added to the environment and intended for preventing, destroying, repelling or mitigating pests” (Costa, 2010). Pesticides were first developed to protect the supply of human food against vertebrate, invertebrate and microorganisms, with the earliest reports of pesticide use appearing before 1000 BC (Costa, 2013). Pesticides are grouped into sub-classes with different names, mainly according to their target organism. This includes insecticides, herbicide, miticides, rodenticides, nematocides, fungicides, fumigants, wood preservatives and plant growth regulators. “Plant protection products” (PPP) is another term used in describing pesticides in the European Union pesticide regulations. PPP are essentially “pesticides that protect crops or desirable or useful plants” (European Commission (EC), 2018).Today, the primary use of pesticide or PPP is to meet the food supply needs of our increasing world population. In 2012, it was estimated that the world pesticide expenditure (data from manufacture and formulator) was nearly US$56 billion with herbicides and plant growth regulators taking up 44% of the total expenditure (Atwood & Paisley-Jones, 2017).

Australia has a well-developed agricultural industry with 394 million hectares agricultural land out of 769 million hectares total land area (for the financial year 2016-17) (ABS, 2018). The application of pesticides in the Australian agricultural industry is said to “have a “major” role to secure certainty of crop and food production in Australia” (Neales, 2013). Presently, there is no pesticide use reporting program in Australia that is available publicly. The exact amount of pesticide use in Australia is not calculated. However, in 2015-16, it was reported that A$20.6

billion worth of crops yielded from the usage of agricultural pesticides, which is 73% of the total value of crops produced in that year (Deloitte, 2018).

1.1.1 Benefits and risks of pesticide usage

The use of pesticide is imperative in food supply because managing pests and undesirable organism in agricultural scenes increases yields and quality in food production (Cooper & Dobson, 2007). The impact of pests in destroying food crops can be enormous. In the 19th century, an attack of unfamiliar fungus on potatoes in Ireland resulted in famine and one million people died of starvation (Vanhaute, Papring & Ó Gráda, 2006). Furthermore, application of pesticides may increase efficiency in producing crops which in turn leads to the reduction in the price of food to the consumer (Cooper & Dobson, 2007) .

Pesticides have been used for vector control for disease protection too (WHO, 2009). For example, the widespread use of an organochlorine insecticide DDT (now banned), in the WHO Global Malaria Eradication Program resulted in removing malaria from Europe, North America, the Caribbean, parts of Asia, and South-Central America in 1955 (Tanner & de Savigny, 2008). Finally, pesticides are used to manage organisms that harm human activities or structures. For instance, herbicides may be applied to control plants growth that causes obstructions in the driveway, while insecticides are used to control termites. Pesticides are also applied to the museums' objects for conservation (National Museum of the American Indian, 2020).

With all these undeniable benefits, the usage of pesticides in protecting crops and for vector control also comes with its risks or unintended effects (Table 1). A lack of awareness of

pesticide users in its application may have caused adverse effects such as pesticide resistance among pests, pest resurgence, the decline of beneficial organisms, alteration of soil microbial diversity and microbial biomass and acute and chronic human diseases (Begum, Alam & Uddin, 2017).

Table 1 The risks and benefits of pesticides

Benefits (Saeedi Saravi & Shokrzadeh, 2011; Cooper & Dobson, 2007)	**Risks (Begum, Alam & Uddin, 2017)**
Increase food production **Decrease food price** **For human health (vector control)** **Controlling organism that harm human activities and structures**	**<u>Effects to pests</u>** Pesticide resistance Pest resurgence Secondary pest outbreaks **<u>Effects on beneficial organism</u>** Predators Pollinators Earthworms **<u>Effects on human health</u>** Acute disease Chronic disease **<u>Effects on soil environment</u>** Effects on different soil enzyme Toxic residue in food, water and air

1.1.2 Pesticide exposure

Humans can get exposed to pesticide through occupational route (exposure at workplace; example: Fenske & Elkner, 1990; Sánchez-Peña *et al.*, 2004; Recio *et al.*, 2001; Recio-Vega *et al.*, 2008) , indoor exposure (application at home; example: Whyatt *et al.*, 2007, 2004, 2009) , dietary exposure (from foods and drinks; example: Chen *et al.*, 2011; Bakirci *et al.*, 2014; Berrada *et al.*, 2010; Tadeo *et al.*, 2004) and the environment (pesticides are found in water, soil and air; example: Kuranchie-Mensah *et al.*, 2012; Yao *et al.*, 2006; Jiang *et al.*, 2009). Pesticide exposure has been linked to several acute and chronic health effects along with multiple health conditions (*reviewed in* Koureas *et al.*, 2012; Reiss *et al.*, 2015). There are several health effects associated with chlorpyrifos exposure, which will be discussed in **Chapter 3** of this thesis. For this reason, the introduction of new pesticides to the public are regulated by the government, which will be discussed in **Chapter 2** of this thesis.

1.2 The Importance of Pesticides Regulations

1.2.1 The Impact of Pesticides Regulations Globally

Pesticide laws and regulations have been one of the tools to control pesticide exposure among occupational groups and the general population. In some cases, introducing restrictions on pesticide use has been proven to be effective. As an example, US EPA has banned residential use of an organophosphorus(OP) insecticide chlorpyrifos (CPF) in the year 2000 when the Food Quality Protection (FQPA) Act signed into law (US EPA, 2018). Restrictions of chlorpyrifos (CPF) use has resulted in the reduction of its use, reduction of exposure and

reduction of CPF level in indoor and personal air level. The agricultural market sector usage of chlorpyrifos in the US was reduced from 8-11 million pounds in the year 2001 to 7-9 million pounds in the year 2007 because of the residential restriction applied in the year of 2000 (Grube *et al.*, 2011). In New York, indoor chlorpyrifos levels decreased 5-fold from homes monitored in 2004 (3 or 4 years of CPF residential ban) than in 2001 (Whyatt *et al.*, 2007). Furthermore, banning CPF for residential use in the US showed reduced exposure among the population. CPF residential restriction in the US has reduced internal dose of CPF during pregnancy in women in New York (Whyatt *et al.*, 2009). This was assessed by the biomarker of exposure measurement in urine, meconium and maternal and cord blood (Whyatt *et al.*, 2009). In the US too, the median level of dialkylphosphorus (DAP) metabolites has decreased by more than half which may imply the decline of human exposure to organophosphorus insecticides since the implementation of the Food Quality Protection Act (FQPA) (Clune, Ryan & Barr, 2012). In another example in South Korea, herbicide paraquat re-registration cancellation in 2011 has reduced total DALYs (disability-adjusted life-years) due to acute poisoning and intentional poisoning (Ko *et al.*, 2018). DALY is a measure of the burden of disease where one DALY represents "the loss of one year of life lived in full health" (WHO, 2018). While in Israel, regulations restricting agricultural use of organophosphorusm(OP) insecticides have been demonstrated to have reduced urinary DAP metabolites between the year 2012 and 2016 among urban pregnant women and their infants (Ein-Mor *et al.*, 2018).

1.2.2 Pesticide Regulations in Australia

In Australia, pesticide use is governed federally by the Australian Pesticides and Veterinary Medicine Authority (APVMA) (*Agricultural and Veterinary Chemicals (Administration) Act* 1992 (Cth)) according to the *Agricultural and Veterinary Chemicals Code Act 1994* (Cth) (the

Agvet Code) "up to—and including the point of retail sale*"* (APVMA, 2017e). The states and the territories regulate the control of pesticides use beyond the point of sale. Fundamentally, the Agvet Code covers the evaluation, approval and control of supply for active constituents and control of supply and manufacture of agricultural chemical products and veterinary chemicals products (pesticides included). Further details of Australian pesticide regulations are discussed in **Chapter 2** of this thesis.

Despite all requirements spelled out in the Agvet Code, there is not enough information available to directly evaluate the effectiveness of pesticides regulations in controlling pesticide exposure among the population in Australia. There are no government programs that systematically conduct exposure measurement data (e.g. biological monitoring or environmental monitoring). So there is currently no objective data to answer the question: does the pesticide regulatory framework in Australia effectively protect the population from pesticide exposure?

The effectiveness of Australian pesticide regulation was also questioned when it comes to the protection of the Great Barrier Reef (GBR). King, Alexander & Brodie (2013) argued that the pesticide regulatory system in Australia does not seem to "working adequately" because of extensive pesticide contamination found in all rivers discharging to the GBR. This might have been causing stress to the ecosystem. In another issue, Larsen (2018) made an analysis of the potential conflict of interest on how APVMA makes regulatory decisions and suggested that the approval process is neither independent nor free from political pressure. Finally, to date, some pesticides that were once approved in the European Union that are still in use in Australia (Table 2). This raises the question on how the re-evaluation of an active substance took place by the APVMA.

Table 2 Status of pesticide approval in Australia and in the EU

Pesticides	Type of pesticides[1]	Status in Australia (APVMA, 2018e)[1]	Status in European Union (European Commission, 2016)[2]
Azamethiphos	Organophosphorus insecticide	Approved	Not approved
Azinphos-methyl	Organophosphorus insecticide	Approved	Not approved
Chlorfenvinphos	Organophosphorus parasiticide	Approved	Not approved
Diazinon	Organophosphorus parasiticides and insecticide	Approved	Not approved
Dichlorvos	Organophosphorus insecticide	Approved	Not approved
Fenitrothion	Organophosphorus insecticide	Approved	Not approved
Omethoate	Organophosphorus miticide	Approved	Not approved
Permethrin	Pyrethroid insecticide, parasiticide, miticide, and mixed function pesticide	Approved	Not approved
Terbufos	Organophosphorus mixed function pesticide	Approved	Not approved

1- https://portal.apvma.gov.au/pubcris (accessed on 11th February 2020); 2-http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.selection&language=EN(accessed on 11th February 2020

In estimating pesticide exposure among the population through dietary exposure, pesticide residue may be monitored in food and beverages. Monitoring of pesticide residue in food and beverages in Australia has been done on some occasions by Food Standards Australia and New Zealand in the Total Diet Survey (FSANZ, 2012). However, pesticides are not monitored routinely and were not included in the latest survey (the year 2014 (FSANZ 2014)). The most recent surveillance of pesticides in food was done in 2011 as part of the 23rd Total Diet Survey. There was a range of agvet chemicals tested in this survey including chlorinated organic pesticides, organophosphorus pesticides, carbamate pesticides, synthetic pyrethroid pesticides, herbicides and fungicides. All chemicals were tested in 92 foods and beverages. According to the result of 23rd Total Diet Survey, there were detections of agvet residues that are not approved for use in any foods according to the *Australia New Zealand Food Standard Code- Standard 1.4.2- Agvet chemicals* 2016 (Cth). For example, allethrin, an insecticide was detected in beef sausage and mushroom. There were also some agvet chemicals residue that were not approved for use detected in some foods and beverages tested (FSANZ, 2011). Routine systematic monitoring of pesticide residues in food is important because the results will help to detect and appropriately manage the potential for pesticide misuse that directly impacted the consumers' health.

As for pesticide exposure monitoring among the public, there are reports of biomonitoring studies conducted in South Australia (Babina *et al.*, 2012) and Queensland (Heffernan *et al.*, 2016; Li *et al.*, 2019) for some pesticides used in Australia. A cross-sectional study by Babina et al. (2012) showed that there was a widespread of exposure of neurotoxic pesticides (organophosphorus and pyrethroids) among the children of South Australia. The children in urban population recruited in Babina's research had levels of urinary metabolites of organophosphorus (OP) pesticides (diethyl phosphate, diethyl thiophosphate and dimethyl

dithiophosphate) 1- 4 times higher than the 0-6 years urban children population in Germany (Heudorf, Angerer & Drexler, 2004). Heffernan and colleagues (2016) reported age and sex-stratified urinary metabolite concentrations of some commonly available pesticides in Australia among the Queensland population. Similar to the findings of Babina *et al.* (2012), the levels of some metabolites were found to be higher than in the US and in Canada. The levels of pyrethroid metabolites (3-PBA and trans-DCCA) in Canadian children were six and eight times respectively higher in the Queensland population study by Heffernan and colleagues (2016) than in the US and Canada. The levels of chlorpyrifos metabolite (TCPy) was three times higher in the Queensland population than in the US population (Heffernan *et al.*, 2016). These few studies cannot make any conclusion of how the dynamics of exposure in the general population changes in response to various changes in policies and regulations in the Agvet Code. Thus, the effectiveness of the regulations to protect the public from pesticide exposure cannot be fully concluded from these small scale biomonitoring studies.

It is important to know whether the existing regulations are effective in protecting people from pesticide exposure. However, the lack of relevant monitoring data and accountability prevent an objective evaluation of the Australian pesticide regulation.

1.3 Measuring effectiveness of pesticides regulations in controlling pesticide exposure with biomonitoring

The effectiveness of pesticide regulations in controlling pesticide exposure among the general population may be evaluated using human exposure assessment (Clune, Ryan & Barr, 2012; Ein-Mor *et al.*, 2018; Ganzleben *et al.*, 2017; Whyatt *et al.*, 2007, 2009) pesticide residue

monitoring in food (FSANZ, 2011) and in the environment (Haynes, Müller & Carter, 2000; King, Alexander & Brodie, 2013).

Biological monitoring can be used to monitor the exposure via the measurement of pesticides, their metabolites and/or measures of biological responses in biological samples collected from the potentially exposed population. Biomonitoring study allows the determination and quantification of chemical substances in the human population. It is done for a variety of purposes, one of them is to investigate the trends and changes in chemicals exposure. Biomonitoring results display aggregate exposure regardless of its source and route of uptake (Needham, Calafat & Barr, 2007). When biological monitoring is conducted routinely, the trends in the biomonitoring data can inform the evaluation of whether or not the regulations in place are effective in controlling pesticide exposure.

The application of biomonitoring to investigate trends in exposure is exemplified in biospecimen program as part of National Health and Nutrition Examination Survey (NHANES) run in the US for the past 40 years (CDC, 2017). NHANES conducts biomonitoring of US population for hundreds of chemicals, including pesticides. Of note, the implementation of the Food Quality Protection Act (FQPA) 1996 was evaluated through biomonitoring as part of NHANES. FQPA 1996 enforces that before registration on food and feed, a pesticide must have "reasonable certainty of no harm" (US EPA, 2017d). Clune, Ryan & Barr (2012) suggested that the implementation of FQPA 1996 may have resulted in the decline of urinary DAPs (general metabolites of OP pesticides) concentration in the US population (the year 1988-1994 versus 1999-2004). This conclusion was derived with the assistance of biomonitoring studies conducted in those years. In another example, in Israel, regulations restricting agricultural use of OPs have been demonstrated to have reduced urinary

DAP metabolites between year 2012 and 2016 among urban pregnant women and their infants (Ein-Mor *et al.*, 2018).

As described in Section 1.2.2, biomonitoring studies of pesticide exposure among the Australian population are few and far between. Australian government does not conduct routine systematic biomonitoring data collection. Fundamentally, there are no biomonitoring studies published with the purpose to evaluate the effectiveness of pesticides regulations and policy in Australia.

1.4 Knowledge gaps and rationale for this study

Pesticide regulations can be effective in controlling the exposure to pesticide among the population. However, in Australia, there is not enough information available in the literature to evaluate whether the regulations in place are effective in protecting the population from pesticide exposure. The issues with regards to the evaluation of the effectiveness of the Australian pesticide regulatory framework raised in this chapter include:

1) The biomonitoring studies investigating pesticide exposure among the population are not conducted systematically in Australia. Therefore, there is nothing can be said on the trends in pesticide exposure among the population. The trends of pesticide exposure will provide information on the effectiveness of the pesticide regulatory system in Australia in controlling pesticide exposure among the population.

2) APVMA is suspected to have or poorly manage the, potential conflicts of interest when making regulatory decisions because the pesticide approval process is neither independent nor free from political pressure.
3) Pesticide residue monitoring in food is not conducted in a systematic manner by FSANZ in the Total Diet Study.

Following all these issues, there is a need to do a review of Australia's pesticide regulatory system from the perspective of how it protects the population from being exposed to pesticides. **Chapter 2** of this thesis will compare the Australian regulatory system with the European Union. The EU regulatory framework was chosen for this comparison for the following reasons:

1) The EU pesticides regulations framework as prescribed by the Regulation 1107/2009 is clear and transparent with the emphasis on the industry being responsible for objectively demonstrating that the pesticides product that placed on the market do not have any harmful effect on human or animal health or any unacceptable effects on the environment.
2) There is sufficient information provided by the EU agencies on their role in the overall framework allowing for informed comparison.
3) The decision making process in the EU pesticide regulatory system involves extensive peer-review system (**Chapter 2**).

Following the issue brought up in (1) with regards to the absence of biomonitoring studies conducted periodically, this research will conduct a biomonitoring study of chlorpyrifos exposure among an urban population of South Australia (**Chapters 5**). The result of this biomonitoring study will be compared with a biomonitoring study conducted in an urban

population in South Australia back in the year 2003-2006 (Babina, 2007; Babina *et al.*, 2012). This research does not aim to measure the overall population exposure levels, rather, it will provide a general idea if the regulatory interim measure set for CPF control has an impact on the actual exposure measure.

Chlorpyrifos (CPF) is an insecticide that is undergoing a reconsideration process in Australia since the year 1996. The general reconsideration process is described in **Chapter 2**, **Section 2.3.2**. As part of this process, in the year 2000, a preliminary report was published by the National Registration Authority (NRA – the APVMA back then). The report highlighted that the home and garden usage "could pose unacceptable health and safety risk and that there were of environmental concern" (APVMA, 2019). For that reason, there were several regulatory measures introduced and implemented in response to the reconsideration process of CPF since approximately 18 years ago (Table 21). One of the most significant restrictions was that the CPF product usage of home garden and domestic control was approved for concentration not more than 50g/L. With these measures implemented in controlling this pesticide use, it is not clear whether the regulatory approach taken by the APVMA has been effective in reducing the exposure to CPF in the general Australian population over the past 18 years.

The research questions of this thesis are:

1. What is the overall outlook of the Australian pesticide regulatory framework in relation to protecting the population from pesticide exposure?
2. What are the levels of CPF exposure among the general population of Australia?
3. Has the interim regulatory measure introduced during the process of reconsideration successfully reduced the exposure of the general population in Australia?

1.4.1 Aims and objectives

The overall aim of this thesis is to investigate the CPF exposure among the urban population in South Australia after the implementation of interim regulatory measures described in Table 21. Based on this, the specific objectives of this thesis are:

1. To do a review of Australian pesticides regulatory framework and compare and contrast the Australian system with the European Union system.
2. To do a review of chlorpyrifos and its status in Australia including exploring the availability of the data on the extent of CPF exposure in the Australian population.
3. To select a biomarker for CPF exposure assessment.
4. To develop an analytical method to measure the selected biomarker (TCPy) in urine samples.
5. To do analysis of urine samples for CPF exposure among the selected populations.
6. To compare the exposure of CPF in this research with the analysis done during the earliest stage of the implementation of restriction of CPF use among the public.
7. To discuss the effectiveness of the Australian regulatory approach in controlling exposure to CPF.

1.5 Structure of the thesis

- **Chapter 2** describes the overview of Australian pesticide regulatory system that is directly connected to public health protection and its comparison with EU.

- **Chapter 3** describes the literature review done on CPF to establish the importance of doing exposure assessment of CPF for protection of the public.
- **Chapter 4** gives details on the method of development to analyse TCPy, a CPF metabolite in urine.
- **Chapter 5** describes the result of the analysis of the sample and will be the case study on the levels of TCPy measured in the urine of urban Adelaide population.
- **Chapter 6** describes conclusions and some suggestions for future studies.

CHAPTER 2: REGULATIONS FOR THE CONTROL OF EXPOSURE TO PESTICIDES AMONG THE GENERAL POPULATION - A COMPARATIVE ANALYSIS BETWEEN THE AUSTRALIAN AND EUROPEAN REGULATORY FRAMEWORKS

Pesticide regulations play an important role in controlling the population's exposure to pesticide **(Chapter 1).** For example, the ban of Chlorpyrifos (CPF) in the US in the year 2000 has been shown to reduce i) its use, ii) the internal dose of CPF metabolite of the population and iii) the level of CPF indoor air and personal level **(Chapter 1)**. However, in Australia, nothing can be said regarding the effectiveness of pesticide regulations in controlling the exposure of the public to pesticides. For that reason, one of the objectives **(Objective 1)** of this research was review the Australian pesticides regulatory system and compare it with other international systems. This chapter will explain why the European Union regulatory system was chosen to compare with the Australian's pesticide regulatory system in the aspect of protecting the population from pesticide exposure. It will also describe and discuss both regulatory systems and provide suggestions to improve Australian's pesticide regulatory system.

2.1 Introduction

As discussed in **Chapter 1**, pesticide use is important for food supply and disease control, but pesticides may not always be selective to its target organism. Humans, animals and the environment have suffered negative impacts of pesticide contamination and exposure (Ahmed & Naqvi, 2011; Pingali, 1995; Mathew *et al.*, 2015; Gill, Ramos-Rodriguez & Raine, 2012) . As much as pesticides are essential to protect crops for food supply, there should be controls in place so that the benefits and risks are balanced out.

One of the ways to reduce risk to the population and the environment is to reduce pesticide exposure by controlling availability and use (Whyatt *et al.*, 2007, 2009; Clune, Ryan & Barr, 2012; Ko *et al.*, 2018; Ein-Mor *et al.*, 2018). This is achieved through the government regulations of i) pesticide approval for sale, ii) labelling, iii) application rates and procedures, iv) post application harvesting delays, v) storage, and vi) waste disposal. There is no worldwide harmonized legislation to control pesticide use and regulations can be different from one country to another in many aspects. For example, a pesticide may be banned in a country but it is still used in another country (Bozzini, 2017b). In addition, the maximum residue levels of pesticide on food sold in a country may be lower or higher in another country (Handford, Elliott & Campbell, 2015). Pesticides can be regulated from the beginning, where the decision is made whether to put it on the market up until the end - how it is used, stored and disposed of (from-cradle-to-grave approach) (Natarajan, Tsvetkova & Webber, 2007). Decisions regarding the assessment of a pesticide by regulatory authority bodies in countries can differ markedly. For example, Australian Pesticides and Veterinary Medicines Authority (APVMA) reported in Chlorpyrifos Toxicology Supplementary Report that "there is no evidence to indicate potential

neurodevelopment effects reported in some studies to occur at or below doses that inhibit acetylcholinesterase (AChE) activity" (APVMA, 2017c) while US EPA concluded that "there are neurodevelopmental effects occurring at chlorpyrifos exposure levels below that required for AChE inhibition." (US EPA, 2015b).

There is no "gold standard" of pesticide policy and regulations. To put Australia pesticide regulations into perspective, comparisons can be made between Australia and other nation's pesticides regulations. US and EU were once called as the "green giants" because both were the leaders in enacting legislation to control pollution and in promoting agreements in international level to diminish human development impact to on the environment (Vig *et al.*, 2004). Moreover, the US and EU are both among the largest global importer and exporter in 2018 (Eurostat, 2019) and among the largest agricultural producers in the world (Donley, 2019). Hence, the US and EU standards are highly likely to be relevant for the rest of the world (Bozzini, 2017b).

In the EU, pesticide approval, restriction and cancellations are in accordance with Regulation 1107/2009. The basis of Regulation of 1107/2009 is to "ensure that industry demonstrates that substances or products produced or placed on the market do not have any harmful effect on the human or animal health or any unacceptable effects on the environment" (Regulation 1107/2009). This means that the industry must demonstrate that the substances or products can be applied/used without giving harm to humans, animals and the surrounding environment. In addition, the EU also put a clear ban in approving and use of pesticides that are categorized as carcinogenic, mutagenic, reproductive toxic, and endocrine disruptor to humans unless the effects are considered negligible. These may be the reason that the EU is said to have the most stringent pesticide regulations globally (Bozini, 2017a). The success of the stringency of the

EU pesticide regulations is reflected in *2016 EU Report on Pesticide Residue in Food*, with 96.7% of the 84,657 samples analysed having levels within the permissible limit (EFSA 2018). Moreover, as mentioned in **Chapter 1** in Table 2, there are multiple pesticides banned in the EU that are allowed for use in Australia. The EU regulatory system also appears to make great efforts in protecting human and the environment. For example, there is work going on in developing methodology to consider cumulative effects of pesticides on human (European Commission, 2019a). In addition, action towards sustainable use of pesticides was implemented for the first time in November 2012 (European Commission, 2017). Sustainable use of pesticide in the EU is achieved by "reducing the risks and impacts of pesticide use on human health and the environment and promoting the use of IPM and of alternative approaches or techniques, such as non-chemical alternative to pesticides" (Directive 2009/128/EC).

In contrast with the EU, application to get pesticides approved in the US only requires the applicants to show that using the pesticide according to specifications "will not generally cause unreasonable adverse effects on the environment" (Federal Insecticide, Fungicide, and Rodenticide (FIFIRA) Act 1996). "Unreasonable adverse effects on the environment" being partially defined as "any reasonable risk to man or the environment, taking into account the economic, social, and environmental costs and benefits of the use of any pesticide" (US EPA, 2019). The US EPA's activities are subject to heavy political influence as the leadership is appointed by the Federal Government. The current US EPA administration has recently reversed the plan to ban chlorpyrifos despite backlash from the scientific community (Trasande, 2017). This example demonstrates that the US system allows for the regulatory decisions to be made to suit current politics rather than to be solely based on scientific evidence (US EPA, 2017c; Hiar, 2018). With all these points highlighted for both the US and EU, the latter was chosen to compare with the AU pesticide regulations.

The elements of the regulation process that are covered in this chapter are related directly to the protection of the public (the end-user) from undue pesticide exposure through legislative instruments. Frameworks for registration, renewal of registration and permissible pesticide residue levels in food in both Australia and the EU regulations will be mainly discussed. Occupational health and safety will not be covered in this review.

2.2 Method

To assess the differences between the EU and Australian pesticide regulation, the publicly available information on the official websites of each regulatory authority as well as published research articles in the English Language were reviewed. The pesticide regulatory information was assessed from the official website of APVMA (APVMA, 2020). The specific information on monitoring of pesticide residue in food is obtained from FSANZ website (FSANZ, 2019) and the Department of Agriculture, Water and the Environment (Department of Agriculture Water and the Environment, 2020). The EU pesticide regulation was assessed from the European Commission (EC) (European Commission, 2020), European Food Safety Authority (EFSA) (EFSA, 2020a), and European Chemicals Agency (ECHA) official websites (ECHA, 2020) (Table 3).

It is worth mentioning that Australian regulations and guidelines use the term “active constituent” while the EU uses “active substance” for the active component that primarily against pests/plant disease. The term “pesticides” is also not used in both the EU regulation and Australian. Australia use the term agvet chemical while European categorize pesticides into plant protection products (PPP) and biocidal products (BP).

Table 3 Sources of information for Australian and EU pesticide regulatory system

Information obtained	Source of information	Date Accessed
Australia Pesticide Regulatory System	All information on APVMA and Australia pesticide regulatory system are obtained from https://apvma.gov.au/ https://www.legislation.gov.au/ https://www.foodstandards.gov.au/Pages/default.aspx https://www.agriculture.gov.au/agfarm-food/food/nrs The list of the webpages visited are also provided in the reference list.	The information was accessed in February 2018 until March 2020. The exact date of the webpages visited is provided in the references list.
EU Pesticide Regulatory System	Information on EU Pesticide Regulatory Framework are obtained from EU law- https://eur-lex.europa.eu/homepage.html?locale=en EFSA- http://www.efsa.europa.eu/ ECHA- https://echa.europa.eu/ EC- https://ec.europa.eu/food/plant/pesticides_en	The information was accessed in February 2018 until March 2020. The exact date of the webpages visited is provided in the references list.

2.3 Australia Pesticide Regulatory Framework

Section 6 of the *Agricultural and Veterinary Chemicals (Administration) Act 1992* (Cth) states that the regulation of agricultural chemicals and veterinary medicines (known also as agvet chemicals) in Australia is administered by the APVMA under the regulatory framework referred to as the National Registration Scheme. The regulation shares the responsibility between the Commonwealth and the states and territories under the portfolio of Minister for

Agriculture (APVMA, 2017b). APVMA has the responsibility to oversee applications, regulations, permits, licences, chemical reviews, taking on enforcement and conformance activities, and import and export of agvet chemical products according to the agvet code (APVMA, 2017b). Once the product is sold, the participating states and territories should oversee the control of the chemical use (APVMA, 2015a). Australian pesticide regulations comprise of agvet code administration, APVMA establishment as a regulatory body to regulate agvet chemicals, APVMA roles, agvet code regulations, prosecution, collection of levy, and prescription of functions in relation to Director of Public Prosecution of the Commonwealth (Table 4). There are also legislative instruments to support agvet code act (APVMA, 2018b).

For states and territories, there may be one or more agencies responsible for overseeing the after retail use of pesticides (APVMA, 2018c). Some of the activities regulated by states and territories are enforcing condition of use (according to the label approved), monitoring residue, licensing applications, and record-keeping related activities. To differentiate federal and states and territories roles, APVMA assesses, registers and develops condition for use and does enforcement and compliance activities under the legislation in Table 4 below. On the other hand, agencies of states and territories control agvet product use through acts, regulations, codes of practice, guideline and standard operating procedures (APVMA, 2018c).

Table 4 Legislation of Australian Pesticide Regulations (APVMA 2018a)

Legislation	Purpose
Agricultural and Veterinary Chemicals (Administration) Act 1992	To establish that APVMA the responsible authority to regulate agvet chemicals up until the point of sale.
Agricultural and Veterinary Chemicals Act 1994	To allow agvet code to take effect.
Agricultural and Veterinary Chemicals Code Act 1994	Provide detail of APVMA roles that enabled APVMA to evaluate, approve, authorize, renew active constituent and chemical products. Furthermore, this act allowing APVMA to issue permit and license to manufacture chemical products.
Agricultural and Veterinary Chemical Products (Collection of Levy) Act 1994	To enable the collection of levy agvet chemicals product.
Agricultural and Veterinary Chemicals (Administration) Regulations 1995	Provision of controlled chemicals in Schedule 1, information for APVMA to include in the annual report, the prohibition to import certain active constituent and chemical products, and others.
Agricultural and Veterinary Chemicals Regulations 1999	Prescribed function of Director of Public Prosecutions of the Commonwealth.
Agricultural and Veterinary Chemicals Code Regulations 1995	Prescribed matters related to the Agricultural and Veterinary Chemicals Code Act 1994.
Agricultural and Veterinary Chemical Products (Collection of Levy) Regulations 1995	Prescribed the rate of levy.

2.3.1 Registration and assessment of pesticide in Australia

Fundamentally, the basis of Australian pesticide regulation is that no agvet product can be sold, supplied or used in Australia before being registered by the APVMA unless exempted (APVMA, 2018h). The active constituents of the new product must be first approved or exempted from registration. A registered product means that it can be sold, supplied and used

safely according to the directions on the label. This means that if a new product has a new active constituent which has not been approved before, the applicant must place two applications: 1) approval of a new active constituent; 2) registration of the new product. The process of approval of a new active constituent is similar to the process to register a new product but with two additional criteria to be met other than safety criteria. These two extra criteria are trade and efficiency.

The applicant first must lodge the application for both the approval of an active constituent and the registration of a new product online. Within one month, APVMA must complete the preliminary assessment. If the preliminary assessment passed, the applicant will be notified by APVMA that the application will be evaluated under Section 14 of the *Agricultural and Veterinary Chemicals Code Act* 1994 (Cth). APVMA then must publish a notice in the Gazette (or anywhere appropriate such as APVMA website) engaging anyone (the public) to provide written comments whether to approve the active constituent or whether to register the product with sound justification within at least 28 days (Section 12 and 13 of *Agricultural and Veterinary Chemicals Code Act* 1994 (Cth)). Public comments are assessed by APVMA but there is no evidence shown that the public comments have any influence in the decision-making process.

The data required for the applicant to submit during registration and authorization of active constituent are listed on the APVMA website (APVMA, 2017a). Some of the data that are relevant to register a new active constituent or a new product are chemistry, toxicology, residues, occupational health and safety, environment, overseas trade, pesticide efficacy and crop safety general guideline and special data of products of gene technology and nanotechnology. APVMA does the assessment of a new active constituent by reviewing dossier

submitted by the applicants. According to the guideline on toxicology data, applicants may present arguments from data published in the peer-reviewed scientific journals toxicology data (APVMA, 2018g). Studies must be conducted according to the principle of Good Laboratory Practice (GLP) and other OECD guidelines. "Toxicology data and/or scientific argument" information is imperative so that APVMA could formulate recommendations including poison scheduling, ADI (acceptable daily intake), acute reference dose, first aid scheduling and other relevant health recommendations.

The next step in a chemical's journey to the consumer is Poisons Scheduling. The Poisons Scheduling is a process which it determines the accessibility of the chemicals to the users. It is conducted under the Scheduling Policy Framework for Medicines and Chemicals (Department of Health, 2018a) and intended to determine the classification of medicines and chemicals including agvet chemicals according to the chemical's potential to cause harm to humans health (*Poison Standard February 2019* (Cth)). For instance, poisons (or chemicals) in Schedule 3 and Schedule 4 listed in the Poisons Scheduling Standard are only be sold by pharmacists or medical, dental and veterinary practitioners. The Schedules (classifications) determine how a product should be 1) stored, 2) labelled, 3) disposed, 4) sold, supplied, possessed or used and 5) record keeping (Table 5).

Table 5 Australia scheduling medicine and poisons

Schedule 1	Not currently in use
Schedule 2	Pharmacy Medicine
Schedule 3	Pharmacist Only Medicine
Schedule 4	Prescription Only Medicine Or Prescription Animal Remedy
Schedule 5	Caution
Schedule 6	Poison
Schedule 7	Dangerous Poison
Schedule 8	Controlled Drug
Schedule 9	Prohibited Substance
Schedule 10	Substances of such danger to health as to warrant prohibition of sale, supply and use.

An Advisory Committee on Chemical Scheduling (ACCS) is a committee comprised of representatives from each state and territory as well as independent experts in toxicology (Department of Health, 2018b). The ACCS is responsible for making recommendations on what schedule a chemical or a product should be assigned to and at what concentrations. At times, there is an absence of toxicology data because of the lack of research for a particular chemical/agvet chemical. Thus, some agvet approved chemicals are not assigned to any of the schedules. At the moment, this gap seems to create a possibility that such chemicals can be marketed, including to the general public, unscheduled and therefore without appropriate warning labels and other restrictions. This is a significant process gap that creates a potential of undue chemical exposures in the community and it needs to be addressed in Australia.

2.3.2 Renewal of registration and chemical review (reconsideration process) in Australia

In 2014, a bill to remove the requirement for re-approval and re-registration of active constituents and chemical products in *Agricultural and Veterinary Chemicals Code Act 1994* (Cth) was passed. This happened because the re-approval and re-registration of active constituents and chemical products provisions were said to be redundant because there is already chemical reconsideration process in place (Agricultural and Veterinary Chemicals Legislation Amendment (Removing Re-approval and Re-registration) Bill 2014 (Cth)). Originally, active constituents and chemical products were required to have a periodic examination every 7 to 15 years for the purpose of re-registration (*Agricultural and Veterinary Chemicals Legislation Amendment Act 2013* (Cth)). With the passing of the bill, there is no period of approval of an active substance unless it is cancelled by APVMA (*Agricultural and Veterinary Chemicals Code Act 1994* (Cth) s. 47). The licence holder may apply for the renewal of product registration every financial year or for a period of 5 years. The process of the evaluation of a chemical product renewal application is not as clear because it is not openly shared with the public as there is no relevant information on the APVMA website (APVMA, 2018a). It is likely that the renewal process is a simple administrative procedure without any scientific appraisal. Renewals are allowed as long as the registration is not cancelled.

Pesticides may be re-considered/re-evaluated (or in chemical review) if there is new scientific information revealed that challenges previously understood risks to human, environment, animal, the safety of crops and trade (APVMA, 2017d). To give an example, the reasons of chlorpyrifos review by the APVMA (NRA then) were reported to be *"*a) its very high toxicity to birds; b)water pollution potential and US restriction imposed to fish, birds, and other wildlife; c)demonstrated potential adverse effects in users; and d) high potential chronic and

moderate potential acute toxicity risk*"* (APVMA, 2009). These facts were not known when chlorpyrifos was first registered for use in Australia.

To trigger APVMA's process or reconsideration, a chemical must be firstly **nominated for reconsideration** (APVMA, 2017d). The nomination may be triggered if another jurisdiction makes a decision to deregister a chemical or if "a compelling scientific case" challenged the basis of evidence supporting the safe and effective use of the already approved chemical. Some examples of information that may be considered for a chemical nomination for reconsideration cited by the APVMA includes a) regulatory decisions from counterpart authorities in other countries; b) adverse experiences report (which resulted despite the usage of the chemical according to the label); c) pesticide residue violations cases (confirmed cases); d) new reliable scientific information (such as from high quality, peer-reviewed literature or reports from major international jurisdiction and organisations such as WHO); d) product failure or reduced efficiency report; e) information submitted to the APVMA in compliance with the existing statutory obligation; f) information obtained by state and territory authorities related to administration of control-of-use function. APVMA will then assess the available information and decide if reconsideration is justified for that chemical and the chemical be accepted into the Chemical Review Program. Once nominated, the chemical will go through a prioritization process and then planning and scoping, and then the work plan. The document of the work plan will include significant dates and deadlines and the scope of the assessment. Next, a Notice of Reconsideration will be sent to each approval holder and product registrant and this marks the start of the chemical review process. This is when the actual assessment is initiated. While the whole process of reconsideration presented in Figure 1 appears linear, it is said to be "a complex iterative process*"* in practice (APVMA, 2017d). The APVMA may need to collaborate with experts nationally or internationally at this phase. The result of the assessment

will then be compiled and released as a draft regulatory measure for public consultation. After the period of public consultation is closed, the APVMA and other agencies involved will assess all comments, data submissions, and recommendations. Finally, the APVMA will make a regulatory decision whether to: a) maintain the status quo – the chemical remains as a registered product; b) change, remove or add the label or the condition of approval or registration or c) cancel or put the chemical on hold pending the approval or registration.

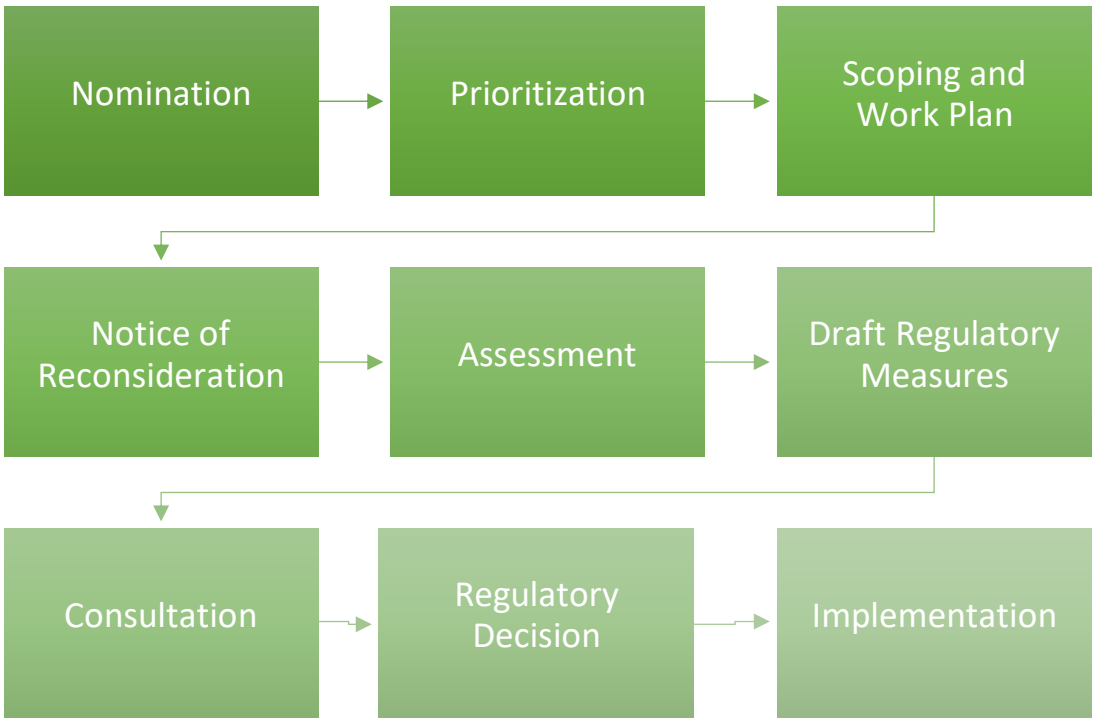

Figure 1 The reconsideration process of agvet chemical in Australia (APVMA, 2017d)

Overall, there is no systematic process or duration set for a pesticide to be reviewed, re-registered and re-approved as the one implemented in the EU. On the other hand, chemical re-evaluation and review (the reconsideration process) occurs on an ad-hoc basis because a pesticide can only be reviewed if it was "nominated" (APVMA 2017b). As said above, chemical reconsideration can take several years and, as a consequence, there is a potential that

highly hazardous pesticides will remain registered and thus the regulatory system would be failing to protect the consumer and the environment.

2.3.3 Pesticides Residue Regulation in Australia (MRL)

In principle, Standard 1.1.1 and Standard 1.4.2 of *Australia New Zealand Food Standards Code* (Cth) states that food for sale must not contain amounts of agvet chemical (and/or its metabolite and degradation products) in amounts that can harm the consumer. To control this, the maximum residue levels (MRL) of agvet chemicals in commodities are set by APVMA during approval of new active constituents and/or pesticide products (FSANZ, 2018a). MRL are defined by Food Standard Australia New Zealand (FSANZ) as the highest level/amount of an agvet chemical residue allowed in a food product sold in Australia (imported and produced locally) (FSANZ, 2018b). Decisions to set the MRLs of a product on agricultural produce are based on the data submitted by the applicants/manufacturers of products. The detail of information needed to determine the value of MRL of pesticides in food items is described in *Residues (Part 5A)* guideline published in APVMA website (APVMA, 2018f). Data required includes the pattern of use of the products which include the proposed use pattern of the product. The manufacturers/applicants are also required to nominate the MRL value for the active constituents and the withholding period. Evaluation of data to set the MRL value is said to be conducted through a peer-review process (APVMA, 2018f). The details of the peer review, e.g. who is engaged and how the peer review reports are evaluated are not available to the public. However, it is said that APVMA may consider information by other recognized bodies to evaluate the proposed MRL (APVMA, 2018f). Comments from the public are sought too, but only if the product contains a new active constituent. The proposed MRLs for other pesticides on food items are listed in *Agvet Chemicals Code Instrument 4 (MRL Standard)*

2012) (Cth) as well as in *Australia New Zealand Food Standards Code—Standard 1.4.2—Maximum Residue Limits 2012* (Cth).

It is established that the role of APVMA is to review data by applicants (pesticides manufacturer) and make the decision about the MRLs of the pesticides on food items. APVMA also works closely with Food Standard Australia New Zealand (FSANZ) on the assessment of agvet chemical residue in diet. For any variation of MRLs, FSANZ is the responsible body to consider the said requests (FSANZ, 2018a).

2.3.3.1 MRL Monitoring

MRL monitoring system of food at the point of retail sale and consumption in Australia is "complex" where it involves national, state, industry regulatory regimes that "governs and (sporadically) tests for pesticide residue" (Parker, 2015). FSANZ conducts the Australian Total Diet Survey (ATDS) of chemical residue in Australian food periodically. However, pesticides or agvet chemical are not always selected to be part of the survey. The last time pesticides were included in the ATDS was in the year 2011. No agvet chemicals were included in the latest ATDS (24th Australian Total Diet Surveying the year 2014-2016).

There is another industry-funded monitoring called National Residue Survey (NRS) (*National Residue Survey (Customs) Levy Act 1998* (Cth)). NRS is part of the Department of Agriculture Australia's strategy to keep chemical residues in agricultural produce at minimum (Department of Agriculture and Water Resources, 2019). This survey monitors residues of chemicals and environmental contaminants in some Australian commodities. This is done to confirm that Australia is a producer of clean food and subsequently to assist Australia's primary producers and agricultural industry to access domestic and international markets. This is different from

the ATDS because NRS is not conducted for the purpose of the population health, rather it is performed to facilitate trade. The information management of the survey data is under the responsibility of the NRS. From the National Residue Survey webpage, the residue testing datasets are made available to the public. Information of the survey that specifically relates to particular people and property is only released to government authorities or to approved individuals (*National Residue Survey Administration Act 1992* (Cth)).

The enforcement and monitoring of MRLs (*Australia New Zealand Food Standards Code* (Cth)) are to be done by the states and territories food regulatory agencies (FSANZ, 2018a). This means that the enforcement and monitoring are not uniform and may vary from one state to another (Parker, 2015). There is no confirmation because of no reports on the outcomes of such monitoring that are publicly available except for Western Australia (Department of Health Government of Western Australia, 2015).

Other than NGO and government regulators, there are also commercial tests available conducted by FreshTest and other service providers. FreshTest provides services to do chemical residue (MRL) and microbial testing at low cost for Wholesalers and their Growers in Australia (FreshTest, 2020). It is the largest and the most comprehensive horticultural residue testing program by the Australian Chamber of Fruit and Vegetables Industry (FreshTest, 2020). Coles and Woolworths, the two dominant supermarkets in Australia also require their suppliers of their fresh produce to comply with regulations including the Food Standard Code (Woolworth Supermarket, 2020; Coles, 2020). The monitoring and audits are paid by the producers themselves and this potentially creates conflicts of interest (Parker, 2015). This is because in this case, the producers may have full control of the monitoring tests

and results because they are the clients of the service provider/s (FreshTest, as an example). Therefore, any problems of the produce/food that were tested may not fully revealed.

To summarize, there are three major problems with the Australian pesticide residue monitoring system. Firstly, there are no regular systematic comprehensive chemical residue surveys for food commodities conducted in Australia. Pesticide residue testing in food in Australia is sporadic because only selected pesticides tested for selected food in the ATDS and NRS program. Moreover, while fresh produce sold in the dominant supermarkets have suppliers comply with the quality assurance program, it is not clear whether the test conducted and ordered by the supplier are free from conflict. Finally, for tests and monitoring conducted by the states and territories, most reports are not available publicly except for Western Australia. Therefore, it is not clear whether other states and territories are actively monitoring pesticide residue in foods. Along with the fact that there is no agvet chemical tested in the latest Total Diet Survey and most reports are not available publicly, we cannot make any formal conclusion on what pesticide residues are consumed by the Australian public.

2.4 European Union Regulatory Framework

Pesticides in EU legislation are classified into two groups: (1) plant protection product (PPP) and (2) biocidal products. By definition, PPPs are ”pesticides’ that protect crops or desirable or useful plants” (European Commission, 2018); while biocidal products are products that are used to control other organisms. Biocides cover a wide range of products such as disinfectant, parasiticides and bacterial killer. Pesticides are included in both categories (PPP and biocides) (European Chemicals Agency, 2018). Both groups of products are regulated under different

regulations with the same aim to protect humans, animals and the environment. Instead of EFSA, ECHA (European Chemicals Agency) is the authority responsible to make assessments of the biocides products. Biocidal products are regulated under Regulation (EC) 528/2012. This chapter will only review PPP because the biocides approval process is similar to PPP approval process.

PPPs in the EU market are regulated under Regulation (EC) 1107/2009 together with other regulations (Table 6). Regulation (EC) 1107/2009, however, is essential because it encompasses detailed information on active substances (AS) and PPP approval process in the EU. This regulation covers approval of AS and products, labelling, and monitoring that are applicable in all member state. The main authorities that work with this legislation are the European Food Safety Authority (EFSA), European Commissions (EC) and the member states. EFSA is an independent authority (EFSA, 2019c) that is responsible for current extensive and comprehensive peer review of the data available for all active substances.

Table 6 Some of European pesticide regulations

Regulations	Functions
Regulation EC 1107/2009	To get product authorized on the market
Regulation EU 540/2011	List of approved substances
Regulation EU 546/2011	Principle to evaluate and authorize PPP
Regulation 547/2011	Requirement of PPP labelling
Regulation EU 283/2013	Data requirement for active substance approval
Regulation EU 284/2013	Data requirement for PPP product authorization
Regulation 396/2005	Assessing residue in food

2.4.1 Stages to get pesticide authorized in the EU

The requirements, procedures, and timeframes for authorization of PPP are described in Regulation (EU) 1107/2009. The whole procedure to get the AS and its PPP into the market is not simple and it is illustrated in Figure 2.

First and foremost, the active substances will be undergoing a cut-off process where the chemicals that are in the list of banned chemicals in the EU will not be going through any assessment at all (Bozzini, 2017a). Active substances and (other constituents, i.e. safeners (chemical compounds that are added to make pesticide safer) and synergists) that are carcinogenic, mutagenic, toxic to reproduction, or endocrine disruptive to humans cannot be authorised in the EU (Regulation 1107/2009, Annex II). In addition, AS, safeners and synergists are not approved if they are a POP (persistent organic pollutant), a PBT (persistent, bioaccumulative and toxic), and/or harmful to bees. This is in line with the aim of EU pesticide legislation: the AS (and its other constituents) must not do harm to human health and the environment. The application for approval of AS that falls into the abovementioned hazard is rejected right away and it will not go through further evaluation.

If the AS is identified not in the banned categories, it will proceed in two phases before the PPP is released to the market (EFSA, 2018a). First, the manufacturer (applicant) must get the active substances (AS) in the product (formulation) approved by the EC. The manufacturer/company will send application (dossier) to the rapporteur member state (RMS) of choice with required data to support the application. Regulation EU 283/2013 (for PPP) spells out the details of data required for AS approval. Applicants must demonstrate in the dossier that the AS, once it is approved for use, does not put the safety of humans and the

environment in jeopardy. A set of mandatory safety studies and the literature review of research done in the last 10 years for adverse effects of the active substance are required to be done by the applicant (EFSA, 2017). There is also a set of mandatory safety studies required for applicants that are funded by the applicants and conducted by a certified laboratory that is subjected to regular audits (EFSA, 2017).

The RMS will do an initial risk assessment and then prepare a draft assessment report to be sent to EFSA. During this process, the RMS may request additional information from the applicant. This process can take 12 months or more if further information is requested by the RMS. EFSA will then do an independent scientific (Regulation (EC) 178/2002) review of the draft assessment report (DAR) in consultation with other RMSs. The conclusion of the peer review is then sent to the European Commission (EC). Finally, the decision of the AS authorization is done by the EC based on the EFSA's peer review. The recommendation made by EFSA is not legally binding (Pintado, 2014). An authorized AS is approved for 10 years.

The next phase is getting the product (PPP) authorized in the EU countries. This is the role of the EU member states. The basis of PPP authorization is that the applicant shall apply to each country where the product is intended to be used. There are three zones in the EU: North, Central and South (European Commission, 2019b). The applicant may select the RMS in the related zone that will examine and assess the application. The data requirements for the application to get PPP authorized are provided in Regulation (EC) No. 284/2013. Having two different authorities approving each AS and PPP is criticized (RMS and EC respectively) because this may have caused a delay in the discovery of the risks of the AS and its PPP. In this phase too, information on MRLs is required (Regulation EC 396/2005). Whenever

necessary, the RMS makes the assessment available to another member state in the same zone because cooperation from other member states in the zone is needed too for peer-review.

Finally, the other member states in the zone shall grant or refuse the application of the authorization of the product in countries where it is intended to be used. If authorization is granted, and if in the future, the applicant would like to place the product (with the same use and comparable agricultural practice) in another member state, an application can be made through "mutual recognition" as explained in Article 40 to Article 42 in Regulation (EU) 1107/2009.

From the description above of the phases involved in getting an AS and its PPP into the market, it is noteworthy that there is a clear cut separation in risk assessment and risk management process in getting pesticides authorized in the EU (Bozzini, 2017a). In other words, the EFSA only makes risk assessment of the AS only while the EC will make the approval of the EC and recommend steps to mitigate the risks (Bozzini, 2017a; Storck, Karpouzas & Martin-Laurent, 2017). In the same context, recommendations made by EFSA is not legally binding (Alemanno, 2014). The reason for the distinct role and players in risk assessment and risk management is to maintain independence and objectivity when doing scientific evaluation in the risk assessment process as well as to ensure accountability for the decision made by the decision-makers in the risk management phase (Bozzini, 2017a). Another feature to highlight is that the EFSA does not make any risk assessment during the PPP authorization (Figure 2 below). The EU member states make the decision and authorize the products to be used at the national level (Figure 2).

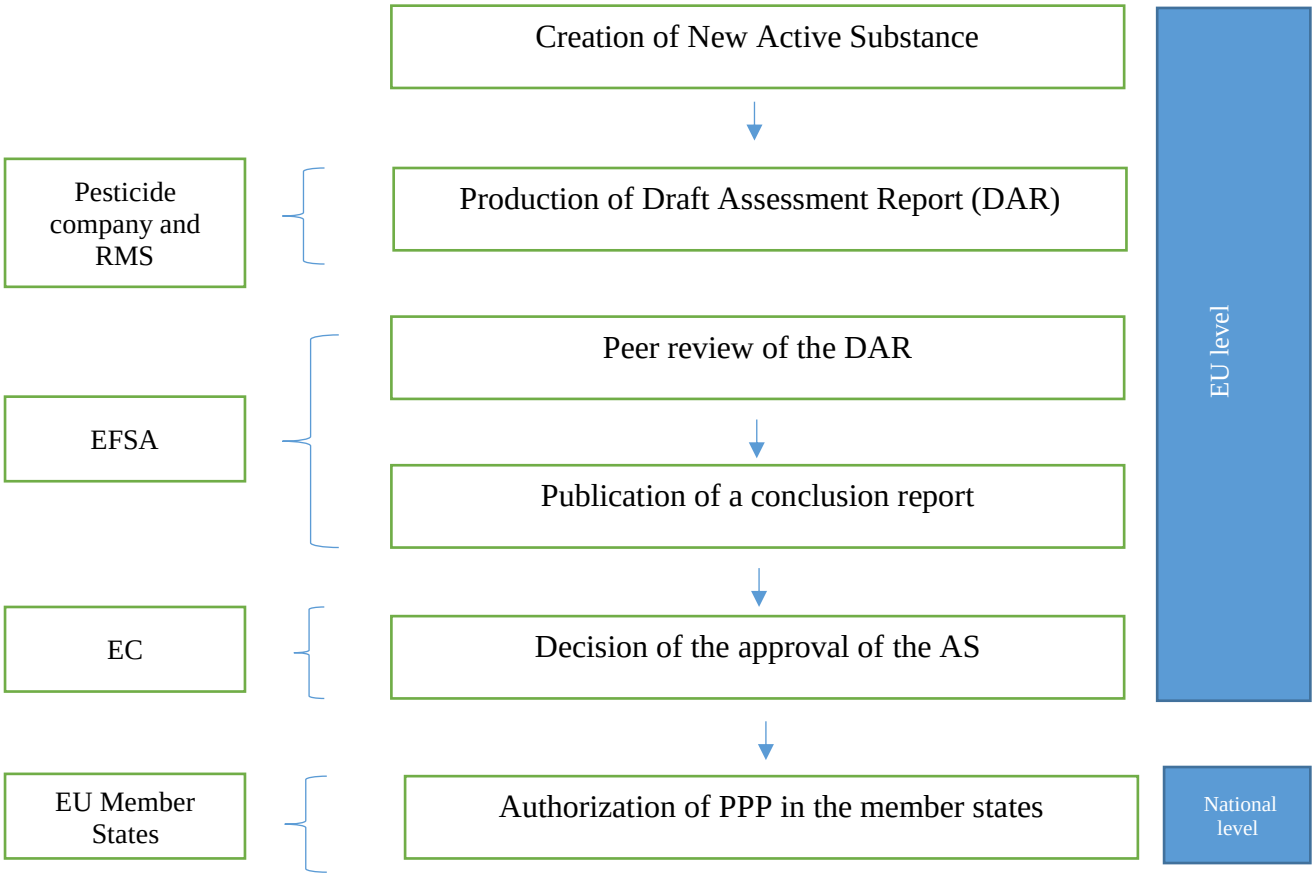

Figure 2 The process of approval of AS and authorization of PPP in the EU (Storck et. al. 2017). Reproduced with permission.

Although there are claims that the EU pesticide regulatory system is one of the most stringent in the world (Bozzini, 2017a), the process of approval of AS and PPP in the EU are not exempted from criticism by the NGOs and peer-reviewed publications. However, the criticism by NGOs is not going to be discussed here because it is already stated that this chapter will only review information from the official websites and peer reviewed journal articles (Section 2.1).

The first critic of note is having multiple authorities in making the decision to approve and authorize the AS and PPP may cause incoordination of AS and PPP approval (Storck, Karpouzas & Martin-Laurent, 2017). As mentioned before, the approval of AS is done by the

EC (EU level) while the authorization of PPP is done by the EU Member States (national level). This leads to responsibility issues as exemplified in the case of glyphosate (a herbicide). The EFSA stated that glyphosate (the AS) is unlikely to be carcinogenic and genotoxic (EFSA, 2015). However, the glyphosate as AS and PPP (along with other ingredients) is considered as potentially genotoxic and carcinogenic by the IARC (IARC, 2016). Since the application of glyphosate in the field is highly likely to be as PPP, it is only logical that the decisions are made based on glyphosate PPP, not as an active substance and the risk assessment of both AS and PPP are done by the EFSA, not by two authorities. Having separate risk assessments and separate authorities to approve AS and authorize PPP lead to "poor coordination and weigh-down authorization process" (Storck, Karpouzas & Martin-Laurent, 2017). Thus, having EFSA as the only authority to do risk assessment in the process to approve the AS as well as in the process to authorize PPP is suggested to solve this problem.

Secondly, the EU's procedure to get an AS approved is not free from conflict of interest (Storck, Karpouzas & Martin-Laurent, 2017). This is because safety testing and data are provided by the pesticide manufacturer to the RMS. It is later that the EFSA do the risk assessment based on the dossier by the pesticide manufacturer. Although the EFSA may do their own review from the peer-review publications, it is important to highlight that the testing done by the pesticide manufacturer may not be independent and free from conflict of interest.

Finally, the lack of transparency of the process of authorization is also criticized by various NGOs as also reviewed by Storck, Karpouzas & Martin-Laurent (2017). Although the conclusion of the risk assessment conducted to approve an AS is published, the details of the studies are not known. The impact of this lack of transparency in reporting the results and process of decision making is said to have hindered the follow- up research of the approved

AS and its products. As a result, it usually took years to have the risks to human and environment of the particular AS or PPP disclosed.

2.4.2 Renewal of Approval of Active Substance in the EU

As previously described in Section 2.4.1, the first approval of active substances (AS) is valid for not more than 10 years but the review of approval can be requested by the EC in the light of new scientific and technical knowledge (Article 21 of Regulations 1107/2009). Applicants may apply for renewal of registration, where the AS still conforms to approval criteria in Article 4 of Regulation 1107/2009. The new period shall not exceed 15 years except the AS that falls into article 4 (7) criteria. This AS (article 4(7)) can only renew for 5 years. The application shall be directed to any member state where the AS is registered along with the submission for notification to other member states, EFSA, and the EC. For the application of renewal, the applicant may submit new data to support the renewal that was not required during registration or last approval. The EFSA will make that information available to the public except for any confidential detail.

Unlike in Australia, the process of renewal in the EU is as rigorous as the process of approval of active substance. The application for renewal is sent to the RMS that provides initial Renewal Application Report (RAR). EFSA will conduct a peer review of RAR with other member states. The peer-review process includes expert and public consultation. EFSA will then produce a draft conclusion on the renewal of the active substance and the Commission will decide whether to provide renewal of approval of the active substance.

Under article 18 of Regulation (EU) 1107/2009, the Commission may establish work program which is a renewal programme by grouping similar active substances to set a high priority on the safety of human and animal health and the environment. To date, five renewal programmes have been initiated (European Comission, 2018). Overall, active substances and PPPs in the EU have fixed approval period of not more than 10 years and the EC may request to review the approval when they are any new risks emerge of the registered AS or PPPs. Having a fixed approval period is important because it will initiate a renewal of approval at certain time and not on ad-hoc basis for at least in 10 years since the first approval. This is because in science, not all facts are revealed or concluded in one instance. Having a non-ad hoc basis renewal program also ensures confidence of the consumer in the pesticides market.

2.4.3 Pesticides Residue Regulation in the EU

Pesticide residue levels in food in the EU were previously not uniform among the member states (European Commission, 2008). It is now harmonized under Regulation EC 396/2005. Council Directive 91/414/EEC emphasized that public health is a higher priority matter than the interest of crop protection. Therefore, there is a need to monitor food to ensure that the residue levels do not exceed the maximum residue limits (MRL). To get PPP registered in the EU, the applicant must incorporate the minimum amount of pesticides needed to protect the crops and the level of residue after pesticides being applied on crops in the dossier. EFSA will play the role to investigate that these residue levels are safe for consumption for all consumer groups (babies, elder and vegetarian included) and the outcome of this assessment is called “reasoned opinion” (Regulation (EC) NO 396/2005). The process is similar for both the approval of AS and PPP where the EFSA take charge of doing risk assessment which is the

proposed MRL value of pesticides on food and beverages while the European Commission will decide whether to approve the proposed MRL. In cases where the residue levels are considered high risk for certain food for any of consumer groups, MRL for the product is rejected and the product will not be registered for application on that crop. In the event where the substantive quantity of a product is lower than the maximum level allowed in that crop, the lower level is set as the MRL to ensure lower application of pesticides.

Generally, there are some of MRL value in the EU is lower than other countries and what the Codex Alimentarius set (see Table 7 for example). The Codex Alimentarius is a committee set up jointly by the FAO and WHO in 1963 that provides international food standards, guidelines and codes of practice (Codex Alimentarius FAO-WHO, 2020). The MRL standard set by Codex may be one of the standards referred to by the exporters (Handford, Elliott & Campbell, 2015) because it is included in the World Trade Organization agreement on the Application of Sanitary and Phytosanitary Measures (the SPS agreement) (International Trade Cantre, 2010). Even if the Codex MRL standards have been established, "countries routinely reject crops containing pesticide residue levels above their national MRL level" (Handford, Elliott & Campbell, 2015). Since the MRLs of pesticides in the EU are the same for domestic and imported foods in the EU, there are issues raised affecting the international trade. Import restrictions may be imposed on crops from countries that presented pesticide residue more than the EU MRL level. As an example, the low residue limit of thiabendazole imposed by the EU has caused a reduction in Peruvian mango exports. Thiabendazole is a common pesticide to control fungal infection in mango (World Trade Organization, 2017).

Table 7 MRL values for the EU, US and Codex. The bold MRL values are the ones that is lower than Codex and the US

Pesticide	**EU MRL** (European Commission, 2016)	**US MRL** (Code of Federal Regulations, 2020)	**Codex MRL** (CODEX ALIMENTARIUS FAO-WHO, 2018)
Buprofezin	**0.01**	0.05	3.00
Boscalid	2.00	10.0	2.00
Acibenzolar-s-methyl	0.30	-	0.30
Deltamethrin	0.20	1.00	0.20
Diphenylamine	**0.05**	30.00	10.0
Ethephon	0.80	5.00	0.80
Fenamiphos	**0.02**	-	0.05
Fenitrothion	**0.01**	-	0.50
Folpet	**0.3**	5.00	10.00
Fenpyroximate	0.3	-	0.20
Imidacloprid	0.5	0.50	0.50
Indoxacarb	0.5	3.00	0.50
Malathion	**0.02**	8.00	0.05
Methidathion	**0.03**	-	0.50
Methomyl	**0.01**	1.00	0.30

To control MRLs among member states in a uniform manner, there are three different instruments introduced by the EC for all member states authorities (European Commission, 2008). Firstly, for the actual monitoring, there is a coordinated program called EU Multi-Annual Cooordinated Control Programme (MACCP) for each member state to assess consumer exposure and to ensure compliance (Regulation (EU) 2017/660). Secondly, the staff responsible for the residue analysis are trained through the Community Reference Laboratories program. Finally, to assess the control activities, inspections in the member state are done by the Food and Veterinary Office (FVO) of the commission (European Commission, 2008). FVO does inspections to member states to ensure compliance with EU food safety and quality legislation. With all these controls in place, there is also a mechanism in place to assist member states to notify the Commission if the level of pesticide residue in a food or feed pose risks to consumers' health (Reynolds, 2014). This system called the Rapid Alert System for Food and

Feed (RASFF) (European Commission (EC), 2019) gives alert or merely notification to all other member states and subsequent necessary actions are taken accordingly for consumer protection.

The EU has a systematic monitoring program called the National Control Programmes (Article 30, Regulations 396/2005). The EU Member states are responsible to establish multiannual control programmes for pesticide residue. The programmes must be updated (and submitted to the Commission) every year with the aim to assess consumer exposure and compliance with the latest and updated legislation. The EFSA prepares and publishes the annual reports on the control activities conducted by the EU member states (EFSA, 2020b). The EFSA also make recommendations regarding the future monitoring programs in the annual report. The EU pesticide residue monitoring program is one of the most comprehensive food survey programs in the world as it analyses more than 75 000 food samples for over 600 different pesticides every year (EFSA, 2020b). The report of the monitoring program is required to be updated on the internet for the public (Regulations 396/2005). All the criteria of the monitoring program and the reporting is spelled out in the regulations. In the latest annual monitoring report (the year 2017), the EFSA reported that 95.9% of food samples analysed were found to be within the legal limit (EFSA, 2019b).

2.5 The comparison of Australia and the EU pesticide regulatory system

2.5.1 Assessment for active constituent/product approval

The process to get approval and authorization is more complex in the EU than in Australia. It is important to acknowledge that the structures of the two regulatory systems are different markedly. Australia is one country, a federation of several states and territories while the European Union is a political and economic union of 28 independent countries. Although EU pesticide regulation is highly harmonised, it has more regulatory authorities involved than in Australia. As an example, authorization of a pesticide product in Australia is only a one-off process to cover the whole nation, while in the EU, an authorized product may need another step if it was to be extended (after authorization) to another zone or even the whole the EU. Therefore, an approach to control pesticides authorization may work for Australia but it will more likely to not work in the EU and vice versa.

In the EU, pesticides are divided into plant protection product (PPP) and biocidal product (BP). With this structure, there are different separate authorities involve in approving PPP as well as BP. This is different than in Australia where only one authority (the APVMA) involved in approving the pesticide product. This may not necessarily be complex but this structure may be helpful to harmonize the pesticide regulation in the EU as there are 28 independent countries involved in the process of getting a pesticide approved. Having separate authorities may have responsibilities distributed to more than one party and this, in turn, will increase the efficiency of the whole process. However, as previously mentioned, having multiple authorities involved in this process is also argued to have caused incoordination between the approval of AS and

the PPP (Storck, Karpouzas & Martin-Laurent, 2017). The glyphosate as an AS is assessed by EFSA as unlikely to be carcinogenic (EFSA, 2015). On the other hand, the glyphosate as AS and PPP (along with other ingredients) is considered as potentially genotoxic and carcinogenic by the IARC (IARC, 2016). Therefore, there is some incoordination observed in the case of glyphosate authorization and its product approval.

It is also important to note that the EU makes the assessment to authorise a pesticide based on hazard rather than risk (Bozzini, 2017a). Hazard is anything that can cause harm while the risk is the likelihood of the hazard will cause harm. In the context of pesticide approval, there are cut off points in the beginning of the process where RMS will ban chemicals that are carcinogenic, mutagenic, toxic for reproduction, persistent, bioaccumulative and toxic for the environment (PBT), persistent organic pollutants, very persistent and very accumulative or endocrine disruptors (with some exception). On the other hand, the risk-based assessment may allow the above-mentioned chemicals for approval if the likelihood to cause harm is **deemed** to be low or negligible. Australia (APVMA) performs assessments *based on risks* to approve active constituents for use (APVMA, 2018f).

Both Australian and the EU regulation require feedback/comment from the public before approval of pesticides (*Agricultural and Veterinary Chemicals Code Act* 1994 (Cth) s. 13, Regulation EC 1107/2009). Currently, there is no research to support the effectiveness of having public participation in pesticide assessment. The public are the end-user of pesticides, it only makes sense that they become one of the stakeholders deciding if a pesticide should be approved and enter the food chain.

2.5.2 Regulations of pesticide residue in food

A small number of pesticides found in food items are called pesticide residues. Pesticide residues in food create a pathway of exposure in the general population. Consumers can get exposed to pesticides through dietary consumption. This can be regulated by setting the maximum residue levels of pesticides in food items to ensure that the levels of exposure among consumers remain safe.

Setting MRL in food is also a way to control if the good agricultural practice is in place when applying pesticides. MRLs are expressed in mg/kg and currently are not harmonized globally. MRL is defined as "the highest level of a pesticide residue that is legally tolerated in or on food or feed when pesticides applied correctly in accordance to Good Agricultural Practice" (CODEX Alimentarius FAO-WHO, 2018). The levels set should cover all types of consumers especially vulnerable populations, such as children, vegetarians and others.

2.5.3 Differences of MRL regulation in Australia and the EU

MRL data are required during registration in both Australia and the EU regulatory system for approval of a new active substance. In terms of approval of the MRL of pesticides in food, EU involves several authorities (EFSA, RMS and EC) where EFSA is responsible for the scientific evaluation of the application for approval and then EC will do the final approval. On the other hand, there is not much information about MRL setting processes shared publicly in Australia. The information that is publicly available on the APVMA website implies that evaluation of

data to determine MRL are done through peer review (APVMA, 2018f) and that MRLs are set by APVMA (APVMA, 2018d). No further detail, such as who is responsible for peer-review and how many authorities involved in the MRL setting process is provided by the APVMA (APVMA, 2018f, 2018d).

MRL enforcement and monitoring activities are done by the nation in the EU and the EFSA compiles, analyses and publishes the report. Australia is somewhat similar to the EU as food regulatory authorities in each state and territory are responsible to monitor and enforce the Food Standards Code (FSANZ, 2018a). However, the monitoring data in each of the states and territory in Australia is not published every year. FSANZ also does a national survey through the Australian Total Diet Survey (ATDS). As previously mentioned, pesticide residue monitoring is not done systematically and routinely in Australia as in the last ATDS, pesticides were not included as part of the chemicals surveyed in food. In contrast, the EU appears to have a closed-loop system where the food and feed commodities are monitored and reported every year. The reports of the EU pesticide residue monitoring are also publicly accessible for at least 5 years back in the EFSA Journal which confirms that there is monitoring of pesticide residue in food going on annually.

With all these differences and similarities, the MRL for apple and avocado (as examples) for several pesticides are presented in Table 7 and Table 8.

Table 8 Avocado MRL (mg/kg) set in Australia and EU (Asterisks (*) – data were set at the analytical limit of quantification/determination)

Pesticides	EU	Australia
Chlorpyrifos	0.01*	0.50
Permethrin	0.05	0.05
Cypermethrin	0.05*	0.01
Malathion	0.02	0.05
Glyphosate	0.10	0.05

Table 9 Apple MRL set in Australia and EU (Asterisks (*) – data were set at the analytical limit of quantification/detection)

Pesticides	EU	Australia
Chlorpyrifos	0.01*	0.50
Permethrin	0.05*	0.05
Cypermethrin	1.00	0.01
Malathion	0.02*	0.05
Glyphosate	0.10	0.05

Table 10 Summary of the comparison of some aspects between AU and EU pesticides regulatory system

Aspect	Australia	EU
Assessment for active constituent/product approval	Risk-based Active constituents and their products are regulated according to their risks which are the likelihood of being exposed to them and the potential effects of exposure.	Hazard-based The AS will be undergoing a cut-off process first. Chemicals that are carcinogenic, mutagenic, toxic for reproduction, persistent, bioaccumulative, toxic for the environment (PBT), persistent organic pollutants, very persistent and very accumulative or endocrine disruptors are not authorized and not going to the next stage of approval.
Authorities Involved	The APVMA makes assessments and does the approval and authorization of pesticides.	A clear separation of the roles between risk assessment and the approval and authorization of the pesticides. The EFSA conducts an independent scientific review of data provided by the applicant. The EC decides the approval of the active substance and the risks mitigation. The decision to authorize the PPP is made by the EU member states
Registration Period	None is set. A product is registered infinitely unless it is cancelled. Review of registration only happens when nominated.	Registration is set for not more than10 years. Review occurs every 10 years and in additional renewal programmes.
Pesticide Residue Regulation (MRL)	Nominated MRL is reviewed and set by APVMA. Enforcement and monitoring of MRLs in food for health- related concern is not done periodically and systematically.	Nominated MRL is reviewed by EFSA and EC decides to accept proposed levels. Monitoring of residue done by EFSA in food is reported annually.

2.6 Opportunities for improvement for Australian regulatory system

2.6.1 Pesticide use reporting system

There is no publicly available pesticide use data for specific individual pesticides in Australia. Currently, Australian pesticide use data can be postulated from the pesticides sale data. However, the sales report only categories of pesticides according to the group – herbicide, insecticide, miticide, and others. A comprehensive review conducted on pesticide use in Australia was published in 2002 by Radcliffe, (2002) reported the trends in the volume of pesticide use in the year of 1996-1999. The author also expressed difficulties in obtaining relevant data for the purpose of publishing the document. I would like to echo that which was suggested over almost two decades ago - there is a need for having this data available (Radcliffe, 2002).

In the US, there are several states that have the requirement to report pesticide use. As an example, California has been tracking pesticide use since at least 1950 (California Department of Pesticide Regulation, 2020). Both growers and commercial pest control operators are required to report the use of pesticides to the county agricultural commissioner (California Department of Pesticide Regulation, 2020). There are multiple data to be reported, including time and location of application, crops/commodity applied to, acre or units treated, application method, pesticides name and amount of pesticide applied. The data are publicly available where Department of Pesticide Regulation (DPR) summarises the breakdown of pesticide use, indexed by chemical (one volume) and indexed by commodity (in another volume) (California

Department of Pesticide Regulation, 2000). The full length and in-depth data can be purchased and some requests on specific data can be accepted too.

There are so many benefits of having pesticide use data that is available publicly. In exposure science especially, biomonitoring data can be related to the usage of any active constituents. Simply put, the source of exposure can be traced back to the actual usage of pesticide in a certain area or state. This is essential for building strategies to reduce pesticide exposure among the public. Pesticide use data may also assist in measuring the effectiveness of regulations set for pesticide control. For example, the increased pesticide residue level in certain food may be investigated by extracting pesticide use data from the system. Assessment based on data submitted by a pesticide manufacturer without any independent oversight creates an opportunity for a conflict of interest.

2.6.2 Assessment based on data submitted by pesticide manufacturer: a conflict of interest

As discussed above, to get active constituents or pesticide products approved, manufacturer/applicant will have to submit an array of data, including toxicity studies, to ensure the product does no harm to humans, animals and the environment. APVMA bases the assessment on industry generated toxicological study reports that hardly ever get the scrutiny of peer-review (e.g. through publication in scientific literature) (APVMA, n.d.). Applicants should be encouraged to use the studies they had published in peer-review journals in order to support their applications for approvals. (APVMA, 2018g). There are guidelines provided by the authority (the APVMA) on how the data should be presented as well as guidelines to ensure that the experiments conducted comply with the Organisation for Economic Co-operation and

Development (OECD) guidelines or similar. However, we cannot ignore the fact that unless the testing facilities are regularly independently audited for compliance and the study reports are peer-reviewed, the system encourages a conflict of interest which can lead to misinformation (provision of low-quality data or even biased data) to occur during the chemical registration process.

Chlorpyrifos had been in use in multiple countries since 1965 (US EPA, 2018). Its approval was renewed in the EU as recently as 2006 (European Commission, 2016). The relation of CPF exposure with neurodevelopmental toxicity was only discovered years after the approval and this was done by the independent academia (as examples (Rauh *et al.*, 2006, 2011, 2015). Mie, Rudén & Grandjean (2018) revealed that when the raw data from the original industry-funded studies conducted back in 1998-1999 was reviewed (Maurissen *et al.*, 2000) a number of discrepancies were discovered between conclusions drawn by the test laboratories and the actual observations pertaining to the neurodevelopmental toxicity test results for chlorpyrifos. These data were submitted as part of the reauthorization of chlorpyrifos in the US and the EU (the latest in 2015 risk assessment). It was suggested that the conclusions were withdrawn by pesticide producer "may be misleading" (Mie, Rudén & Grandjean, 2018). One of the issues brought up by Mie, Rudén & Grandjean (2018) was the industry-funded laboratory concluded that "there is no effect on brain morphology and behaviour were observed at low and medium dose level and multiple effects were identified at high dose level". This conclusion were drawn by the test laboratory by using the inappropriate method of calculation to demonstrate the absence of sensitive target region of the brain of the nursing rat pups. Re-analysis of raw data using the suggested calculation method has shown otherwise where all developmental neurotoxicity were present in each dose level (low, medium and high) tested. In addition, Tweedale (2017) suggested that relying on the manufacturers for declaring a product is safe to

use may not be wise because investigation on the pre-market toxicity studies of herbicide bentazon indicated that it has a greater hazard that it was claimed in the risk assessment (RA) during the pre-market era. This phenomenon of misinterpreting the data by the applicant has also seen in drug and medical device pre-market studies as reviewed by Lundh et al. (2018) where results are more favourable in industry-sponsored studies than the ones sponsored by other sources. Because the inherent risk of bias, in the future, it is highly recommended that these data originate from independent studies to ensure the risk to public from exposure to pesticides or chemical is properly understood and appropriately controlled.

2.7 Conclusion and Summary Points

This chapter presents a comparative analysis of the legislative and regulatory frameworks underpinning the use of pesticides in Australia and the EU from the perspective of controlling undue exposure among the general population.

1. The EU has a more robust structure and more rigorous processes to approve active constituents and pesticide products with multiple authorities involved in the review and approval to get these into the European market. Australian approval process involved a single authority, the APVMA.
2. MRL monitoring system in the EU is done systematically and reported every year while MRL monitoring in Australia is done sporadically.
3. Approvals of active constituents are based on *risk* in Australia while the EU regulators base their approach on *hazard* assessments.
4. Pesticide usage is not tracked but the usage can be very useful for Australia to monitor how much pesticides are used according to the area.
5. Both EFSA and APVMA relies on the data from the chemical industry to determine the toxicity and the safety of a product/active constituent. This may not be an ideal source because of the conflict of interest.
6. There is no legislative requirement for regular review and re-evaluation of registered chemicals in Australia. Re-registration is an administrative process without scientific review. The EU regulatory framework mandates reviews and scientific re-evaluation of all registered chemicals to occur regularly.

7. Scientific reviews of chemicals already approved for use in Australia only happen on an ad-hoc basis and are typically unacceptably slow.

CHAPTER 3: LITERATURE REVIEW

The comparisons of Australian pesticide regulation with the European Union were presented in **Chapter 2**. As there are no internationally harmonized laws and regulations to control pesticide use globally, there are many aspects in which the Australian regulation approach is different from the way the European Union (the EU) regulate pesticides. The first intention of a pesticide regulation system should be to protect people, animals and the environment. Monitoring of exposure is an imperative in order to achieve this. Generally speaking, the comparison presented in **Chapter 2** demonstrates that the EU regulatory framework allows for a more rigorous approach in approving, monitoring and reviewing pesticides. Hence, as a case study approach, this research investigated the level of chlorpyrifos (CPF) exposure amongst a South Australian urban population as a measure of response to the interim regulatory measures that were implemented in the year 2001-2002 (**Objective 2**). CPF's chemistry, toxicokinetics, mechanism of toxicity, health effects, current issues and biomonitoring approaches are reviewed in this chapter to establish the importance of monitoring exposure to this pesticide among the population.

3.1 Introduction

Chlorpyrifos (O, O-Diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate, CPF) (Table 11) is a broad-spectrum organophosphorus (OP) insecticide/acaricide/nematicide that is registered for use in over a 100 countries worldwide in both agricultural and urban settings (Dow AgroSciences, 2018). It has been used extensively throughout the world (Grube et al., 2011). CPF was first introduced by Dow Chemical Company in 1965 for non-agricultural markets; to control indoor pests and turfgrass and ornamental pests (ASTDR, 1997). It was later applied in the agricultural market in the 1970s. Due to its cost-effectiveness, CPF was considered a better substitute for persistent organochlorine pesticides (Testai, Buratti & Di Consiglio, 2010). Ever since then, CPF has been one of the most popular insecticides used for domestic and agricultural purposes.

Today, CPF use is being re-considered in many countries, including Australia, due to its ability to cause disruption to normal neural development (developmental neurotoxicity) in young children (Testai, Buratti & Di Consiglio, 2010). In Australia, CPF has been under the chemical review process since 1996 and yet a final decision/ruling has not been made. Despite this, some restrictions have been introduced because of the review (APVMA, 2019). In the past, before restrictions applied in the US and Australia, CPF was allowed for use by the general public at home and in the garden (NRA, 2000; US EPA, 2018). CPF was used widely in residential settings and home gardens to kill cockroaches, mosquitoes and other insect pests (Testai, Buratti & Di Consiglio, 2010). Today, there is no data on how much (in volume) CPF-containing formulations were used agriculturally or non- agriculturally globally or in Australia. However, the US EPA, estimates that approximately nine millions pounds were used in the US in 2012 in all market settings (Atwood & Paisley-Jones, 2017).

Table 11 Some of the chemical and physical properties of chlorpyrifos from Testai, Buratti and Di Consiglio (2010). Reproduced with permission.

Molecular weight	350.6
Empirical and structural formula	$C_9H_{11}C_{13}NO_3PS$
CAS registry number	2921-88-2
Melting point	41.5-42.5 °C
Boiling point	>300 °C
Vapor pressure	3.35 mPa at 25 °C
Density	1.51 g/ml at 21 °C
Partition coefficient	K_{ow}= 50 000

3.2 Toxicokinetics of Chlorpyrifos

CPF administered orally is well absorbed in the gastrointestinal tract compared to dermally applied dose (Nolan et al., 1984; Griffin et al., 1999). It is also well absorbed through the respiratory tract (Testai, Buratti & Di Consiglio, 2010). Studies with radiolabelled CPF demonstrate that it can cross the placenta and reach the foetus in pregnant rats (Abdel-Rahman et al., 2002). In humans, CPF and its metabolites were found in post-partum meconium (Whyatt et al., 2009) indicating that CPF crosses the placenta and reaches the foetus. Moreover, CPF and its metabolites have also been detected in the cord/newborn blood samples (Garfinkel *et al.*, 2005).

The CPF metabolism pathways are demonstrated in Figure 3. CPF undergoes metabolic activation via a desulfuration reaction mediated by CYP450 which leads to the formation of the toxicologically active metabolite, CPF-oxon. Hydrolysis of CPF and CPF-oxon, mediated by A-esterases, leads to detoxification and formation of 3,5,6-trichloro-2-pyridinol (TCPy).

TCPy is the main compound-specific metabolite. Other metabolites of CPF are dialkylphosphates (DAPs), metabolites common to most OP compounds (Nolan *et al.*, 1984).

TCPy concentration in plasma peaks at 6 hours after oral ingestion and at 24 hours after dermal absorption. The half-life of TCPy is similar, 27 hours, for both oral and dermal exposure routes. Urinary excretion of TCPy and DAPs appears to be the main route of CPF elimination as neither the parent compound nor the CPF-oxon was present in urine in both ADME (absorption, distribution, metabolism and excretion) studies done by (Nolan *et al.*, 1984) and (Griffin *et al.*, 1999).

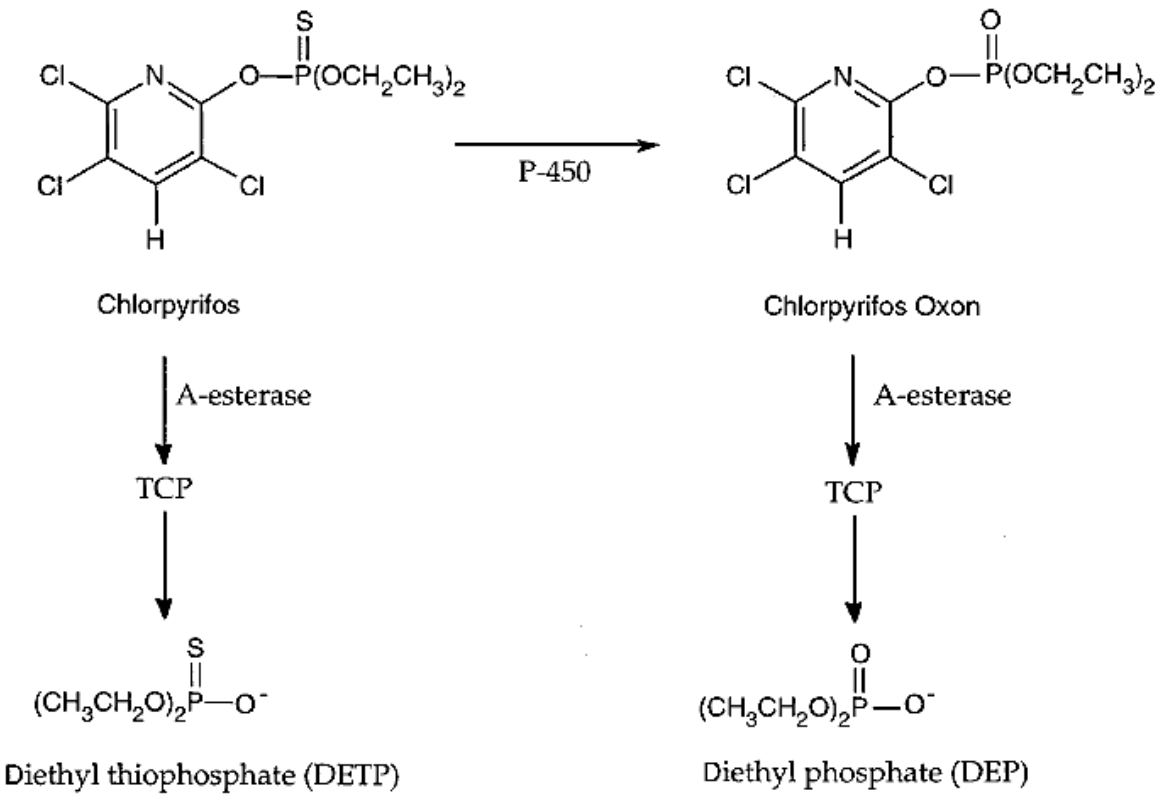

Figure 3 Metabolism of CPF from ASTDR (1997). Reproduced with permission.

3.3 Mechanism of CPF toxicity

The primary mechanism of action for OP pesticides is inhibition of acetylcholine esterase (AChE) (Morris *et al.*, 2014). AChE is an enzyme that catalyses the breakdown of acetylcholine into choline and acetic acid in the synaptic cleft (Matsumura, 1985; Vargas-Bernal, Rodríguez-Miranda & Herrera-Pérez, 2012). This process is to terminate signal transmission between neurons in the central and peripheral nervous systems (Vargas-Bernal, Rodríguez-Miranda & Herrera-Pérez, 2012). Inhibition of AChE leads to the accumulation of acetylcholine, resulting in overt stimulation of acetylcholine receptors (Figure 4).

CPF is a phosphorothionate OP with P=S which is transformed via desulfuration by CYP450 into CPF-oxon once absorbed into the body. CPF-oxon is the compound that has the highest potency to inhibit AChE (Timchalk, 2010).

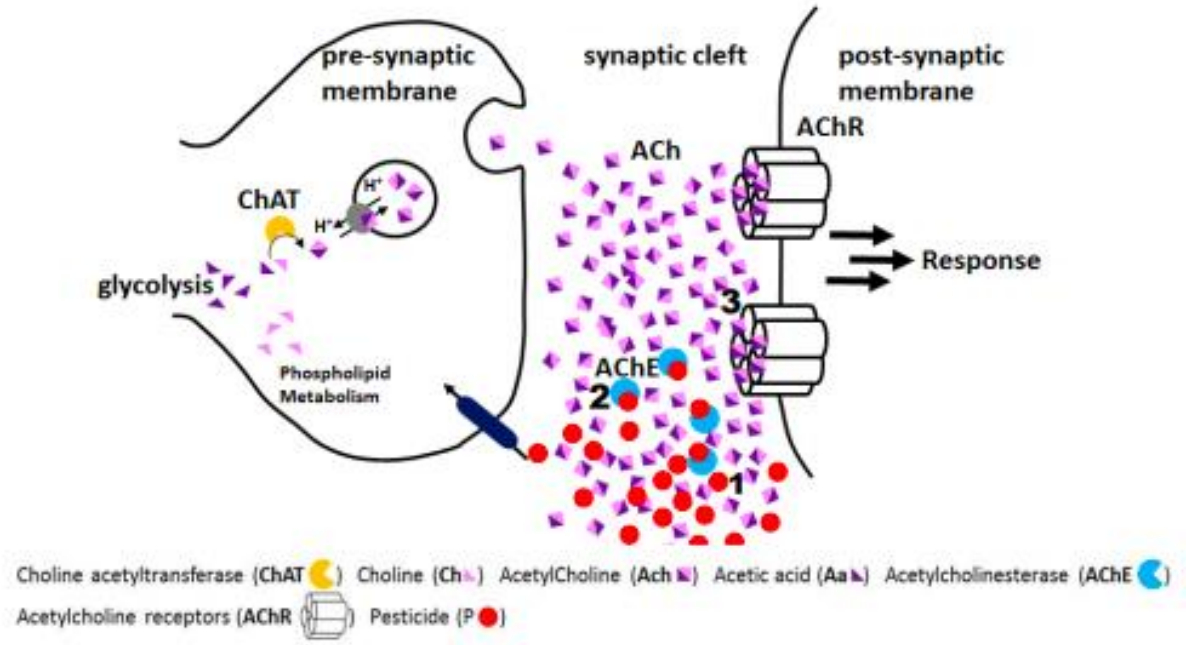

Figure 4 Inhibition of AChE by organophosphate pesticides by Vargas-Bernal, Rodrguez-Miranda and Herrera-Prez (2012). Reproduced with permission.

The clinical manifestation of acute, high-level exposure to CPF-oxon is well-defined. The overstimulation of both muscarinic and nicotinic receptors results in a mix of excitatory and inhibitory symptoms (Eaton *et al.*, 2008; Testai, Buratti & Di Consiglio, 2010). The symptoms and signs of acute OP pesticides poisoning (CPF included) are presented in Table 12 (Morris *et al.*, 2014). Individuals exposed to the high level of OPs may experience intermediate syndrome which develops a few days later (Senanayake & Karalliedde, 1987). The symptoms of this syndrome include weakness of the respiratory system, neck and proximal limb muscle (*reviewed in* (Suratman, Edwards & Babina, 2015)). The next syndrome in people exposed with high level of OPs is OP induced delayed polyneuropathy (OPIDP). OPIDP is caused by inhibition of neuropathy target esterase (NTE) (Capodicasa *et al.*, 1991).

Table 12 Signs and symptoms of acute OP compounds poisoning (from (Morris et al., 2014)). Reproduced with permission.

Muscarinic parasympathetic	Muscarinic sympathetic	Nicotinic Neuromuscular	Central Cholinergic effects
Hypersalivation	Sweating	Fasciculation	Confusion
Diarrhoea		Hypercontraction	Agitation
Loss of bladder		Weakness	Vomiting
control		Paralysis	Coma
Bronchorrhoea			Seizures
Rhinorrhoea			Centrally mediated apnoea and cardiac arrest
Lacrimation			
Miosis			
Bradycardia			
Hypotension			
Bronchospasm			

On the other hand, the mechanisms of action involved in chronic low-level exposure to CPF (or other OP pesticides) are multiple, independent of AChE inhibition and are not fully understood. There is a substantial body of epidemiological evidence demonstrating significant associations of chronic low-level exposure to CPF and several health effects (reviewed in Section 3.5 in this chapter). Research to understand these relationships, as well as the mechanism of the toxicity is still on-going.

3.4 Monitoring of CPF exposure

In exposure science, exposure assessment is done as part of risk assessment (Figure 5) and this ultimately aids in making decisions on how to manage the risk. This is in line with the definition of exposure science by *Journal of Epidemiology and Exposure Science*: *"*a study of human contact with chemical, physical and biological agents occurring in the environments. It advances knowledge of the mechanisms and the dynamics of events either causing or preventing adverse health outcomes" (Barr, 2006). It is an essential element in risk assessment because it will fundamentally establish the link of the source of exposure with health outcome.

The goal of a developed exposure assessment is to characterize 1)potentially exposed population, 2) potential pathway/s of exposure and 3) potential dose of exposure (Cohen Hubal *et al.,* 2000). It is usually done using direct or indirect measurement methods or a combination of both (Lioy & Weisel, 2014). Direct exposure measurement is "conducted by examining the contact of the person with the chemical concentration in the exposure media over a period of time*"* (Sheldon, 2010). Some examples include the collection of personal air or diet samples or biological samples (for example, urine, blood and saliva) used to measure chemical concentration over a period of time. While indirect exposure measurement usually uses the information of the location of exposure, time and how the exposure occurs and takes samples from those locations to estimate exposure (Sheldon, 2010; Lioy & Weisel, 2014).

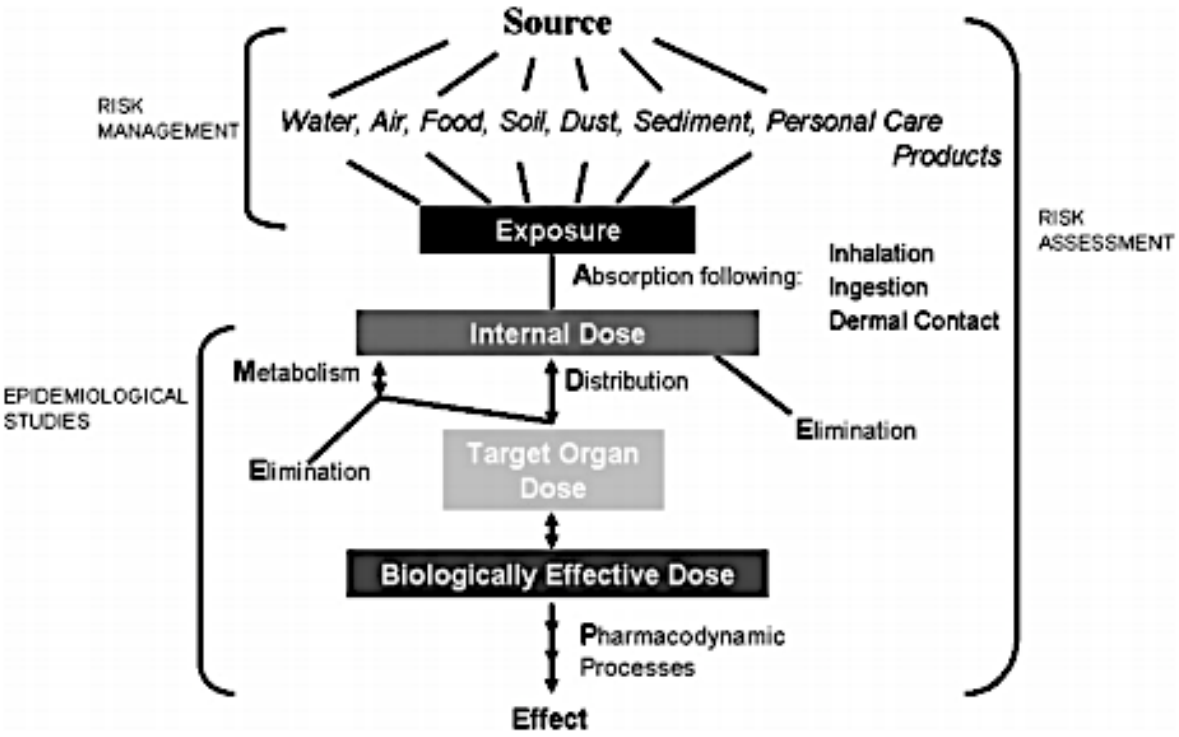

Figure 5 Exposure effect continuum for an environmental chemical by Angerer et al. (2006). Reproduced with permission.

3.4.1 Biomonitoring studies to estimate exposure to CPF

Biomonitoring, the direct method for measuring exposure, is one of the tools needed to generate the data for exposure assessment as it provides the measure of the body burden of the toxicant and/or its metabolites in a biological matrix (Lioy & Weisel, 2014). In pesticide exposure studies, questionnaire survey and environmental monitoring are often included in addition to biomonitoring measurements. This literature review is mainly focusing on biomonitoring studies done to estimate exposure to CPF.

Biomonitoring (a contraction of 'biological monitoring') is an assessment of human exposure by means of laboratory measurement of the chemical in question, its metabolites or its reaction

product(s) in human urine, blood, saliva, milk or other tissue taken in individuals (Needham, Calafat & Barr, 2007). Biomonitoring data presents total body burden and it will not indicate the route of exposure (ingestion, dermal absorption or inhalation) nor the source of exposure of the chemicals. There are three types of biological monitoring (Table 13): (1) biological monitoring of the internal dose, (2) biological monitoring of effective dose and (3) biological monitoring of effects. In monitoring CPF exposure, 3,5,6-trichloro-2-pyridinol (TCPy), diethyl phosphate (DEP), diethyl thiophosphate (DETP) in urine and CPF and CPF-oxon in blood are biological indicators of dose exposure, while measuring the activity of AChE in blood is a type of biological indicators of effect.

There are different purposes of biomonitoring of pesticide exposure as reviewed in Needham, Calafat and Barr (2007). Firstly, biomonitoring study will measure the internal dose of the pesticide of interest. In NHANES for instance, TCPy internal dose is compared each year to study the trend of exposure of CPF among the population. Secondly, internal dose measurement can be connected to the clinical manifestation of a disease or health effect as it was usually done in epidemiological studies. Finally, internal dose measurement can also be used in depicting the exposure pathway.

Table 13 Definition of three types of biological monitoring (Aprea, 2004)

Type of biological Monitoring	Measurements
Biological indicators of dose or exposure	The measurement and assessment of chemicals or its metabolites in biological tissue taken in an individual
Biological indicators of effect	Measurement of early changes caused by exposure such as enzyme activity or micronuclei
Biological indicators of effective dose	Measurement of the product of the chemicals or its metabolites to specific cellular receptors such as DNA and proteins

3.4.2 Selection of biomarkers and matrices for biomonitoring of CPF exposure

CPF pathways of metabolism are described in the earlier section and in Figure 3. In brief, DEP and DETP are both non-specific metabolites that are part of general dialkylphosphates (DAPs) metabolites of OP pesticide class. DAPs metabolites are the biomarkers to assess exposure to OP pesticides as a class and DAP measurements in biological samples alone would not help to identify specific OP pesticides. TCPy is the specific biomarker of exposure to CPF and CPF-methyl (Table 14).

The selection of biomarker/s for a study is generally based on different reasons. When a biomarker is selected, we must ensure that we consider the right biological matrices because not all biomarkers are present in each matrix. Advantages and disadvantages of both urine and blood samples were discussed in Barr and Angerer (2006). In brief, the major drawbacks of blood sampling are that venepuncture needs to be done (except umbilical cord) to withdraw blood and this comes with associated discomfort and risks to the participants. This does not happen when collecting urine as a sample. Moreover, urine sample collection is higher in

volume compared to blood thus lower concentrations of target compounds can be detected in the analysis. In choosing between blood and urine, the target population would be one of the factors to consider when selecting a biomarker to understand the exposure of a toxicant in some cases. For children, as an example, urine would be a convenience sample collection compared to blood collection. Also, there are multiple analytical methodologies well-developed and published for urine analysis of TCPy and DAP metabolites. DAPs, however, are also metabolites for multiple OP pesticides whereas TCPy is also a product of chlorpyrifos-methyl metabolism.

Table 14 Evaluation of CPF biomarkers. (Adapted from (Barr & Angerer, 2006)*) Reproduced with permission.*

Evaluation Criteria	CPF	CPF-Oxon	TCPy	DAP (DEP, DETP)	AChE	PON
Specificity of marker to exposure	Specific	Specific	For CPF, CPF-methyl and it is preformed in the environment	For other DE OPs and preformed in the environment	For other OP and Carbamate pesticide exposure	NA
Matrix for measurement	Blood	Blood	Urine	Urine	Blood	Blood
Another source of this biomarker in matrix	NA	Environmental oxon	Preformed in the environment	Preformed in the environment	Carbamate	NA
Stability	Not stable	Not stable	Stable	Stable	Stable	Stable

3.4.3 Target population of biomonitoring studies of CPF exposure

The target populations of studies conducted on biomonitoring of CPF can be categorised into adults (occupational groups and non- occupational), pregnant mother/infant pairs, and children.

Studies in pregnant mother/infant pair and children

There are numerous published cohort studies investigating exposure to CPF in pregnant mothers and their infants (mother/infant pair studies) (Table 15). The chemical exposure of mothers and infants during pregnancy and in early postnatal period can be assessed using samples of whole blood, urine (mothers and children), plasma and serum of umbilical cord blood and meconium (infants).

CPF has been demonstrated to cross the placenta in several animal and human studies (Abdel-Rahman *et al.,* 2002). Low level CPF exposure also has shown to have impact on brain development in animal studies (Tang, Carr & Chambers, 1999; Chakraborti, Farrar & Pope, 1993; Middlemore-Risher, Buccafusco & Terry, 2010). Rauh and colleagues (2012) reported that the impact of CPF exposure shown in animal brain triggered a series of studies examining "whether purportedly "safe" exposure levels" had similar outcomes to those in animals. In addition, children that have been exposed to CPF in the prenatal stage were shown to have negative neurodevelopmental effects. Potential associations of health effects with CPF exposure was explored in large cohort studies in Table 15 and discussed in Section 3.5. The health effects investigated were commonly birth effects and neurological related health effects. In some studies, biomonitoring was done in pregnant mothers in order to investigate internal dose and to compare the levels measured to levels reported in other biomonitoring studies (for example National Health and Nutrition Examination Survey (NHANES)).

Table 15 Some studies conducted for large cohort in investigating chlorpyrifos exposure or organophosphorus pesticides among pregnant mothers and infant

Study, Location, Year	Author, Year	Target Population	Matrix and Biomarker of CPF Exposure Measured in matrix	Matrix and Concentration
Centre for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) Salinas Valley, California (US) From 1999- 2001	(Huen *et al.*, 2012)	n mothers at delivery = 234 n newborns = 256	CPF in maternal cord blood plasma CPF in umbilical cord plasma	Chlorpyrifos in plasma (newborns) = 0-1726 ng/mL Chlorpyrifos in plasma (mothers) = 0-1385 ng/mL
	(Eskenazi *et al.*, 2004)	n pregnant woman = 488	TCPy in urine	Median TCPy in urine = 3.3ug/L
	(Young *et al.*, 2005)	n mothers = 381	Total DEs in urine	Average pregnancy median (DEPs in urine) = 21nmol/L Post Delivery median = 27 nmol/L
	(Castorina *et al.*, 2010)	n pregnant women = 601	TCPy in urine (2 samplings)	Median urinary TCPy (13 weeks of gestation) = 2.1 µg/L Median urinary TCPy (26 weeks of gestation) = 3.2 µg/L
	(Eskenazi *et al.*, 2007)	n mothers-children pair = 447	TCPy in urine (mothers)	Average maternal urinary total DEP (during pregnancy) = 81.5nmol/L

			Total DEs in mothers and infant urine	Median maternal urinary TCPy (during pregnancy) = 3.54 µg/L GM urinary total DEP in children (6 months) = 45.5 nmol/L GM urinary total DEP in children (12 months) = 59.5 nmol/L GM urinary total DEP in children (24 months) = 70.9 nmol/L
	(Marks *et al.*, 2010)	Mothers and children pair n mothers =348 n children at 3.5 years = 290 n children at 5 years =320	Total DEs in urine	GM mothers' total DEP in urine =17.7nmol/L GM children's total DEP at 3.5 years in urine= 7.0 nmol/L GM children's total DEP at 5 years in urine =7.2 nmol/L
	(Castorina *et al.*, 2003)	n mothers = 446	DEP and DETP in urine	Median urinary DEP =1.1 µg/L Median urinary DETP = 0.9 µg/L
	(Raanan *et al.*, 2015)	n mothers = 359 n children at 5 years old =344 n children at 7 years old = 347	Total DEs in urine	Mean maternal urinary total DEP = 24 nmol/L *Mean total urinary DE (children 0.5-5 years) = 259 nmol/year/g
	(Eskenazi *et al.*, 2014)	n mothers-children pair = 343	Total DEs in urine	Total DEP in urine was not shared.

	(Bradman *et al.*, 2005)	n pregnant mothers = 600	Total DEs in urine	Mean prenatal sample 1 (urinary total DEP) = 16.7 nmol/L Mean prenatal sample 2 (urinary total DEP) = 20.7 nmol/L Mean postpartum (urinary total DEP) = 16.7 nmol/L
	(Bradman *et al.*, 2011)	n children-mothers pair = 460	Total DEs in urine	GM urinary total DEP in children (6 months) = 8.6 nmol/L GM urinary total DEP in children (12 months) = 14.2 nmol/L GM urinary total DEP in children (24 months = 8.4 nmol/L
	(Quirós-Alcalá *et al.*, 2011a)	n children-mothers pair = 274	Total DEs in urine	GM urinary total DEP in children (6 months) = 10.4 nmol/L GM urinary total DEP in children (12 months) = 10.7 nmol/L GM urinary total DEP in children (3 and ½ years) = 6.6 nmol/L GM urinary total DEP in children (5 years) = 7.6 nmol/L

	(Sagiv *et al.*, 2018)	n mothers-children pair = 601	Total DEs in urine	GM urinary total DEP = 20.3 nmol/L
Columbia Centre for Children's Environmental Health (CCCEH), New York (US) 1997-1998	(Rauh *et al.*, 2006)	n children = 254	CPF in umbilical cord plasma	Chlorpyrifos level in umbilical cord plasma = Undetectable – 63 pg/g
	(Whyatt *et al.*, 2004)	n mother-children pair = 314	CPF in umbilical cord plasma	GM Chlorpyrifos level in umbilical cord plasma = 4.0 pg/g
	(Perera *et al.*, 2003)	n mother-children pair =263	CPF in maternal blood plasma and umbilical cord plasma	Mean plasma CPF = 7.5 pg/g
Mother, Child and Environment Study Israel Established in 2012	(Ein-Mor *et al.*, 2018)	n mothers = 273 n children = 107	Total DEs in urine	Maternal urinary total DEP = 21 nmol/L Neonatal urinary total DE = 9 nmol/L
The Generation R Study Netherland 2004	(Ye *et al.*, 2008)	n mothers = 100	TCPy in urine	Urinary TCPy = 0.2 µg/L
The Mount Sinai Children's Environmental Health Cohort Study Mount Sinai, New York (US) 1998-2002	(Engel *et al.*, 2011)	n mother-infant pairs = 360	Total DEs in urine	Urinary DE levels were not shared

	(Berkowitz *et al.*, 2004)	n mother-infant pairs = 404	TCPy in urine	Median TCPy urinary = 7.6 µg/L And 11.5 µg/g
Health Outcomes and Measures of the Environment Study (HOME) Cincinnati, Ohio (US) 2003-2006	(Rauch *et al.*, 2012)	n mother-infant pair = 306	Total DEs in urine	Median urinary DEP=17.7 nmol/L
	(Yolton *et al.*, 2013)	n mother/infant pairs=350	Total DEs in urine	GM maternal urinary diethyl phosphates=9.4nmol/g
	(Donauer *et al.*, 2016)	n mother-infant pairs=327	Total DEs in urine	Median maternal urinary diethyl phosphates =21.1 nmol/g

1. AUC = area under the curve (using the area under the curve from 5 measurements made during childhood to summarize DAP concentration over time.
2. Total DEs = total diethyl phosphates metabolites which are diethyl phosphate (DEP), diethyl thiophosphate (DETP) and diethyl dithiophosphate (DEDTP).

Occupational and para-occupational studies

Occupational groups have been extensively studied for pesticides exposure including organophosphorus pesticides, which covers CPF exposure as well. As mentioned in the above (Table 14), the most specific biomarker of CPF exposure would be TCPy. Biomonitoring studies that investigate metabolites other than urinary TCPy do not directly inform the extent of CPF exposure alone but also other organophosphorus pesticides applied at work. Therefore, it will not be discussed in this review.

Occupational group biomonitoring studies for exposure to CPF (urinary TCPy as biomarker) have been done among the pesticides applicators (Callahan *et al.*, 2014; Hines & Deddens, 2001; Crane *et al.*, 2013; Singleton *et al.*, 2015; Farahat *et al.*, 2011; Ismail *et al.*, 2017), farmers (Wang *et al.*, 2016), farmworkers/farmers (Wang *et al.*, 2016; Scher & Sawchuk, 2008), pest control workers (Hines & Deddens, 2001; Fenske & Elkner, 1990), workers at CPF manufacture (Albers *et al.*, 2010; Burns *et al.*, 2006) (Table 16). Occupational groups are often compared with non-occupational groups as a reference to confirm the exposure is occupational. The level of biomarker before application is often used as a baseline as well. The urinary TCPy level was found to be statistically inversely correlated with the blood butyryl cholinesterase (BuChE) and acetylcholinesterase (AChE) activities (Farahat *et al.*, 2011)

Factors that are associated with CPF occupational exposure were investigated as well. Dermal route of exposure was found to be accounted for about two-thirds of the estimated absorbed CPF dose during structural control treatments (Fenske & Elkner, 1990). In the same study, consistent use of PPE (chemical resistant gloves, long-sleeve shirts and/or chemical resistant workpants) would reduce dermal exposure during the structural control treatment. Besides, using respirators during the structural control treatment while working in enclosed spaces

would reduce respiratory exposure as well. In another CPF exposure study among the pest control workers, the minutes of CPF applied and whether the applicator treated the enclosed crawl spaced was found to be the determinants of airborne exposure to CPF (Hines & Deddens, 2001). Urinary TCPy levels among CPF termiticide applicators was determined by 1)day-of-the-week, 2)the CPF air concentration one or two days before urine collection, 3)minutes of CPF applied one or two days before the urine collection, 4)enclosed space treated (yes/no) and 5) commercial space treated (time-weighted) (Hines & Deddens, 2001). Applicators in the farm performing spraying application of CPF liquid mixture had significantly higher GM levels of TCPy in urine (day-1) than those using granular products with in-furrow or over the row application (Thomas *et al.*, 2010). The quantity of CPF formulation applied, the application duration, and the number of spray tanks applied positively associated with the absorbed CPF dose from the occupational application of applicators on rice farms in Ghana (Atabila *et al.*, 2018).

Table 16 Some CPF occupational exposure studies in the literature

Location, Year of Collection	Reference	Sample	Result
Egypt **April 2010 to January 2011**	(Callahan *et al.*, 2014)	n pesticide applicators = 38 n non applicator = 24	Concentration of urinary TCPy of applicators was higher than the non-applicators. The mean cumulative of urinary TCPy (AUC) of the applicators after spraying was 33, 217.6 (SD= 49 179.3) while the mean cumulative for non-applicators was 3290.8; SD = 3994.9.
Egypt **April 2010 to January 2011**	(Callahan *et al.*, 2017)	n = 43 adolescent pesticide applicators n= 38 non applicator	Concentration of urinary TCPy increased during CPF application. The strongest predictor was found to be the total hours applying CPF (semi-partial $r_2 = 0.32$), and total hours in the field applying other pesticides (semi-partial $r_2 = 0.08$). Wearing clean clothes to work was associated with lower concentration of urinary TCPy
China **May 2013**	(Wang *et al.*, 2016)	n adult farmers = 20 n urban adult = 15	Pesticide spraying activities increased the urinary TCPy levels of the adult farmers.

			Urinary TCPy level of farmers increased to 7 times on the first day of spraying and then decreased to three times higher on the third day of spraying than the level of urinary TCPy before spraying activity. CPF metabolic efficiency to TCPy increased as the exposure increased.
North Carolina (US) **March to July 1998**	(Hines & Deddens, 2001)	n applicators = 41	Range of urinary TCPy for applicators was from 9.42 to 1960 mg/g creatinine. Significant determinants of urinary TCP levels 1) day-of-the-week, 2)the chlorpyrifos air concentration one and two days before urine collection, 3)minutes of chlorpyrifos applied one and two days before urine collection, 4)enclosed crawl space treated (yes/no), and 5)commercial structure treated (time-weighted).
Egypt **April 2010 to January 2011**	(Crane *et al.*, 2013)	n applicators = 57 n non applicators = 38	Throughout the CPF application, the applicators demonstrated increased TCPy concentration and BChE depression than the non-applicators.

Egypt **July 2008**	(Farahat *et al.*, 2011)	n applicators = 14 n technician = 12 n engineer = 12	Average urinary TCPy levels is this order: Applicators > technician >engineers There was a statistically inverse correlation between urinary TCPy and blood BuChE and AChE activities.
Egypt **2010 and 2011**	(Ismail *et al.*, 2017)	n applicators = 46 n non-applicators = 38	Urinary TCPy level increased during application. Deficits in Neurobehavorial performance were associated with elevated pesticide exposure
Michigan (US)	(Albers *et al.*, 2004)	n CPF manufacturing workers = 66 n referent workers = 74	CPF manufacturing workers had significantly higher urinary TCPy level and lower average BuCHE.
Egypt **Summer 2008**	(Singleton *et al.*, 2015)	n applicators = 14 n technicians = 12 n engineers = 12	Urinary TCPy levels were significantly higher than the baseline and related to blood BuChE and AChE inhibiton.

It has also been demonstrated that workers can bring home pesticides that they use at work, which leads to exposure among the family members (Curwin, 2006). The take home pathway (para-occupational pathway) of CPF exposure was assessed with environmental sampling and biological sampling (Table 17). The para-occupational pathway of CPF exposure was assessed by analysing urinary TCPy and other general OP metabolites too. The urinary TCPy was detected in children of farmworkers even when CPF has been banned in the US for at least three years at the time of the sample collection (Arcury *et al.*, 2007). The urinary TCPy level in farm children is significantly higher when their fathers applied CPF prior to sample collection than those children where CPF was not recently applied (Curwin *et al.*, 2007). Environmental sampling was undertaken by analysing house dust (Simcox *et al.*, 1995; McCauley *et al.*, 2003; Butler-Dawson *et al.*, 2016; Bradman *et al.*, 2007; Curl, Fenske & Kissel, 2002; Curwin *et al.*, 2005), vehicle dust (Curl, Fenske & Kissel, 2002; Thompson, Coronado & Grossman, 2003; Coronado *et al.*, 2006), floor and surface wipe (Bradman *et al.*, 2007; Curwin *et al.*, 2005), indoor and outdoor air samples (Bradman *et al.*, 2007; Curwin *et al.*, 2005). The level of CPF in house dust was found to be significantly lower in reference homes than farmworkers/farmers households (Simcox *et al.*, 1995; Butler-Dawson *et al.*, 2016). Vehicle dust of farmworkers consistently have higher CPF concentration than non-farmworkers vehicle dust sample (Coronado *et al.*, 2011; Thompson *et al.*, 2014). The median level of total OP pesticide (CPF included) residue detected in house dust samples from play areas of home was significantly associated with the number of people have high pesticide contact at work (McCauley *et al.*, 2003). When male workers waited for two hours before changing cloth after coming home from work, the mean levels of total OP (CPF included) of house dust was significantly higher than the mean levels of total OP when male workers changed within two hours after returning home from work (McCauley *et al.*, 2003)

Table 17 Some CPF para-occupational studies in the literature

Location, year of collection	Reference	Sample size	Sample collected	Results
Washington (US) 1992	(Simcox *et al.*, 1995)	n farming families =26 n farmworkers = 22 n non farming families = 11	Household dust Soil	CPF concentration in household dust were significantly higher than in soil. CPF concentration in household dust were significantly lower in reference homes than in farmer/farmworkers homes.
Oregon (US) 1998	(McCauley *et al.*, 2003)	n agricultural families = 24	Household dust	The median level of total OP pesticide (CPF included) residue detected in house dust samples from play areas of home was significantly associated with the number of people have high pesticide contact at work. When male workers waited for two hours before changing cloth

				after coming home from work, the mean levels of total OP (CPF included) of house dust was significantly higher than the mean levels of total OP when male workers changed within two hours after returning home from work
Pacific Northwest (US) 2008-2011	(Butler-Dawson *et al.*, 2016)	n agricultural house = 116 n non-agricultural house = 47	Household dust	CPF in dust were detected more frequently in agricultural homes than in non-agricultural homes.
North Carolina (US) July to August 2004	(Arcury *et al.*, 2007)	n farmworker children = 60	Urinary TCPy	Urinary TCPy were present in their urine sample (detection rate: 83.3%). Median of urinary TCPy level detected was the highest than other pesticides.
California (US) June to September 2002	(Bradman *et al.*, 2007)	n farmworker children = 20	House dust Indoor air Outdoor air Surface wipe Toy wipe Cotton socks Union suits Urinary DAPs	CPF were detected in house dust, outdoor air, indoor air, surface wipe, toy wipe, cotton socks. Indoor and outdoor air for CPF were strongly correlated.
Washington (US) 1999	(Curl, Fenske & Kissel, 2002)	n farmworker = 213 n young child = 190	Household dust Vehicle dust Urinary DAPs	This research supports the hypothesis that the take-home exposure pathway

				contributes to the pesticide contamination in farmworkers home.
Washington (US) **1999**	(Thompson, Coronado & Grossman, 2003)	n farmworkers = 571	Household dust Vehicle dust Self-reported pesticide exposure	Percentage of house dust above the LOQ was 18% while the percentage of vehicle dust above LOQ was 26%.
Washington (US) **2005-2006**	(Coronado *et al.*, 2006)	n farmworkers = 218	Household dust Vehicle dust	The percentage of house dust and vehicle dust detected were the same as above.
Washington (US) **2005-2006**	(Thompson *et al.*, 2014)	n farmworkers = 100 n non-farmworkers = 100	Household dust Vehicle dust	Farmworkers had higher level of pesticide residue in household dust and vehicle dust.
Iowa (US) **2001**	(Curwin *et al.*, 2005)	n farm homes = 20 n non-farm homes = 19	Air Surface wipe Household dust	CPF was detected most frequently in air and wipe samples. CPF was detected more often in farm homes than in non-farm homes. Take home pathway may be an important source of home contamination.

Iowa (US) **2001**	(Curwin *et al.*, 2007)	n farm children = 66 n non-farm children = 52	Urinary TCPy	CPF absorbed dose is higher in farm children than in non-farm children.
Iowa (US) **2001**	(Curwin *et al.*, 2006)	n farm household = 25 n non-farm household = 25		Urinary TCPy was higher in farm fathers and farm mothers compared to non-farm mothers and fathers. Urinary TCPy in farm children and non-farm children were not significantly different. When CPF was applied by their fathers before sample collection, urinary TCPy was significantly higher than those children that their fathers did not recently applied CPF. TCPy urinary level was positively associated with the CPF levels in dust (not significant).
	(Huen *et al.*, 2012)	n mothers blood= 234 n umbilical cord = 256	Mother's blood Umbilical cord Urine DAPs	CPF detected in plasma of both mothers and newborn blood.

North Carolina (US) **June – October 2010**	(Arcury *et al.*, 2014)	n migrant farmworkers house = 176	Household dust	CPF was detected in household dust but not associated with the camp characteristic.
California (US) **1999**	(Harnly *et al.*, 2009)	n homes in agricultural area = 197	Household dust	"CPF agricultural use on agricultural field were significantly associated with 83% increases in dust concentration for each kg applied per day, near participant homes, in the month or season prior to sample collection"
California (US) **2006**	(Quirós-Alcalá *et al.*, 2011b)	n urban home in Oakland = 13 n agricultural home in Salinas = 15	Household dust	There were no differences in pesticide concentration in dust between urban and agricultural homes. CPF concentration in urban home is lower by 40-80% than in agricultural home.
Washington (US) **2005-2006**	(Smith *et al.*, 2016)	n farmworkers families = 100 n non-farmworkers families = 100	Household dust	OP pesticides concentration in thinning and harvest were higher than in the non-spray season. CPF concentration in farmworkers house dust

				samples were 9.8 times higher than in non-farmworkers home.

3.4.4 Some issues related to the use of urinary biomarker to estimate exposure

Pre-formed metabolites in food and environment.

When interpreting biomonitoring results, we have to be conscious about the fact that environmental degradation may lead to the formation of the same metabolites as human metabolism and that these pre-formed metabolites can be absorbed into the human body. The levels of biomarker detected in biological matrices may be caused by direct ingestion/administration (without metabolism) of pre-formed metabolites. Although TCPy metabolism following absorptions not fully investigated in humans, animal studies showed oral administration of TCPy excreted 100% in rat (Timchalk *et al.*, 2007) and 90% in sheep (Bakke & Price, 1976). Therefore, it is likely that the administration of TCPy in human is followed with excretion of substantial amounts of unchanged compound in urine.

When entering the environment, CPF will undertake several pathways of degradation (*reviewed in* Eaton *et al.*, 2008). One of the products of environmental degradation of CPF is TCPy. The extent of environmental degradation of CPF into TCPy is not fully understood and research reports are contradictory. Morgan *et al.* (2005) reported TCPy measured in solid food samples at the median level estimated to be 12-29 times higher than the parent compound. On the other hand, mean TCPy levels were found to be lower than mean CPF level in raw vegetables (for instance spinach (TCPy=0.009mg/kg vs CPF=1.04 mg/kg), yet after cooking, some vegetables have elevated TCPy levels (mean TCPy in spinach after cooking is 0.023 mg/kg) possibly due to CPF degradation with heat application (Randhawa *et al.*, 2007).

Similarly, TCPy too was not found in other uncooked material, e.g. orange juice (Radford *et al.*, 2018). Other than in food, CPF also degraded in the environmental media to TCPy and it has been detected in indoor dust, soil, indoor air and outdoor air (Wilson *et al.*, 2003). In summary, although TCPy metabolite is safer than the parent compound (*reviewed in* (Eaton *et al.*, 2008)), the biomonitoring data should be read with caution taking account ingestion of the pre-formed metabolites in food and environment.

Other factors to consider in biomarker selection.

To specifically measure CPF uptake, one will need to choose to measure CPF in plasma or serum because CPF is not eliminated unchanged via urine. The convenience of the sample collection, the vulnerability of the target population and the toxicokinetic of a compound need to be taken into consideration when designing a biomonitoring study. Large scale biomonitoring study such as NHANES (CDC, 2017) collected urine samples because this approach is non-invasive and easy to collect. There are also other limitations involved, which are the availability of developed analytical methods and the availability of standards in the market. In brief, in designing a biomonitoring study, there are a lot of factors to be considered before deciding the right biomarker and matrix.

3.5 Human health associations with exposure to chlorpyrifos

In environmental health and toxicology studies, controlled experiments on the effect of CPF exposure on human population is not possible to be undertaken although it has been done in the past to investigate its ADME. Therefore, fundamental toxicity effects are usually investigated in animal and laboratory studies. In humans, the health effects of exposure to any environmental contaminant are best investigated in epidemiological studies, such as case-control studies, cross-sectional studies, and prospective cohort studies.

CPF exposure association with health effects has been investigated using direct and indirect measures of exposure and effect using tools like interviews, questionnaires, as well as clinical and biomarker assessment of participants. Several reviews have been conducted on health effects of exposure to OPs in general and CPF in particular (Eaton *et al.*, 2008; Koureas *et al.*, 2012; Mostafalou & Abdollahi, 2017; Reiss *et al.*, 2015). In summary, CPF exposure has been associated with the following health effects:

a. Birth outcomes

CPF can cross placenta as demonstrated in animal and human studies when mothers were exposed during pregnancy (Abdel-Rahman *et al.*, 2002; Garfinkel *et al.*, 2005). In animal studies, when exposed during gestation, rat pups were reported to have a decreased weight and postnatal weight gain (*reviewed by* Eskenazi, Bradman and Castorina, 1999). In human epidemiology studies, there were inconsistent results where some studies suggested association with negative birth outcomes while some proving opposing conclusion. CCCEH cohort reported negative correlation between CPF cord blood with birth weight and birth length of

African American (n=116; p=0.018) and Dominican population (n=146; p=0.26) (Perera *et al.*, 2002). An extension of this study reported CPF cord plasma inversely associated with birth weight (n=314; p=0.03) (Whyatt *et al.*, 2004). No associations were observed with CPF levels in maternal serum (n=138, p=0.268) and cord serum (n=148, p=0.408) with any birth effects such as birth weight, birth length and head circumference in one study (Barr *et al.*, 2010). When levels of CPF in maternal urine were categorized as above the limit of detection (n=404,p > 0.05) (Berkowitz *et al.*, 2004), no association of exposure with birth weight, birth length and head circumference was reported. However, when both urinary maternal TCPy and serum paraoxonase (PON1) were taken into account together, a small significant decrease of head circumference was observed. In the same cohort, higher concentration of total DEP in maternal urine and slower PON1 activity were associated with lower birth weight (n=404; p=0.042) (Wolff *et al.*, 2007). CHAMACOS study of the agricultural population, conversely, failed to show association between prenatal maternal urinary TCPy and total urinary DEP with foetal growth or length of gestation (n=439, p>0.05) (Eskenazi *et al.*, 2004). Maternal urinary DEP in the HOME study (n=306; median=17.7 nmol/L) of not necessarily agricultural population was lower than in CHAMACOS study (n= 488; median=22 nmol/L) and there were no associations with any birth outcome (p>0.05) (Rauch *et al.*, 2012). Nevertheless, maternal urinary DEP concentrations were associated with shorter period of gestation in susceptible infants with lower PON1 activity in Mexican-American women (n=436, p<0.05) (Harley *et al.*, 2011) while Shanghai birth cohort suggested association of increased log-transformed DEP levels with decreased length of gestation (n=91, p=0.001) (Wang *et al.*, 2012). CCCEH cohort reported lower CPF in blood sample and no relationship was seen between birth length and birth weight with cord plasma CPF and diazinon among infants born after 1/1/01 (p=0.03) (Whyatt *et al.*, 2004). This impact supported the regulatory effort to ban CPF from residential use in the US in 2000 (US EPA, 2002).

b. Neurodevelopment effects in infant after prenatal exposure.

Negative neurodevelopment outcomes have been reported in children exposed to CPF either prenatally or in early childhood or both. Neurodevelopmental outcomes were investigated with estimating prenatal exposure and its association with an indication of neurodevelopment impairment. Some studies investigated OP exposure in general, which will not be considered in this section because of its non-specificity. In CCCEH cohort (n=254), Rauh *et al.* (2006) observed significant correlation between prenatal subacute exposure and impaired cognition ($p=0.05$), motor function ($p=0.002$), ADHD ($p=0.018$) and developmental problems ($p<0.05$). There was also evidence of deficits in working memory ($p<0.05$) and reduced IQ ($p<0.05$), at the age of seven as a result of prenatal exposure to CPF (n=265, Rauh *et al.*, 2011; n=335, Horton *et al.*, 2012). More recently, there were also reports of the association between prenatal exposure with childhood tremor among the eleven year old children (n=263, $p<0.05$, Rauh *et al.*, 2015). Boys ($p<0.05$) who were exposed to higher levels of CPF had suggestive association with increased ADHD index while girls ($p<0.05$) who are exposed to the middle level of CPF has increased attention problem (n=187, Fortenberry *et al.*, 2014). Children of mothers who lived near agricultural area and were exposed to CPF during pregnancy (in the second semester) showed an association with increased risk for ASD (autism spectrum disorder) (n=486, Odds Ratio=3.3, Shelton *et al.*, 2015).

c. Endocrine disruptor

CPF exposure has been reported to cause alterations in adult rats' mammary gland via endocrine disruption mechanisms and was suggested to be a risk factor for breast cancer development (*reviewed in* Rodgers *et al.*, 2018). There is a reduction of circulating levels of estrogen, progesterone and luteinizing hormone in adult rats exposed to No Observed Adverse

Effects Level (NOAEL) and Acceptable Daily Intake (ADI) of CPF ($p<0.05$, Ventura *et al.*, 2016) .

d. Cancer

CPF is classified as Class E ("*Evidence of Non-Carcinogenicity for Humans*") by US EPA based on the report dated 1993 (US EPA, 2017a) and recommended as "medium priority" for IARC monograph by the advisory group recently (IARC, 2014). While animal studies show no carcinogenic effects of CPF exposure, an epidemiology study demonstrated that CPF use among agricultural workers with a family history of prostate cancer statistically linked to prostate cancer in the Agricultural Health Studies (AHS) (n=42 948; OR=1.65, 95% CI: 1.02, 2.66, Alavanja *et al.*, 2003). CPF use among agricultural workers also was statistically linked to lung cancer (n pesticide applicators=57 284; n spouses of farmer applicators= 32, 333; P =0.03 in Alavanja, Hoppin & Kamel, 2004; n pesticide applicators=54 383; p=0.002 in Lee *et al.*, 2004) in other AHS cohort. CPF use was also associated with significant increase in the risk of breast cancer among spouses of private pesticide applicators in the AHS cohort ($n \geq 10$; RR=1.41; 95% CI 1.00 to 1.99; Lerro *et al.*, 2015). Other studies in the AHS cohort also suggested association of CPF exposure with rectal cancer (n=93; p=0.008; Lee *et al.*, 2007) and the risk of glioma among male farmers in Nebraska, USA (n=10; OR=22.6, 95% CI 2.1-191.7; Lee *et al.*, 2005). Finally, higher exposure to diethyl (DE) OPs (which indicated by higher the level of DEP in urine) was associated with a significantly higher risk of childhood acute leukaemia among children in the Shanghai cohort (n=258; $p<0.05$; Zhang *et al.*, 2015).

e. Sperm quality and male reproductive toxicity

The evidence of the effects of OP exposure on semen quality is suggestive as reviewed in Perry, 2008; Martenies & Perry, (2013). TCPy levels in urine samples had suggestive borderline

statistical association with sperm concentration and motility among non-occupationally exposed population (n=330; p=0.09 for [each]sperm concentration and motility; Meeker et al., 2004), DNA damage in sperm (percentage DNA tail: p=0.004 and tail distributed moment: p=0.03) (n=260; Meeker et al., 2004) and reduced testosterone level (n=336; Spearman correlation coefficient=0.3; Meeker et al., 2006). Sperm concentration was significantly lower among non-occupationally exposed men with higher DETP levels in urine in a reproductive cohort study in China (n=18; absolute sperm concentration difference (low vs high exposure)=-1.0 with 95% CI -1.8, -0.2; Perry et al., 2007).

f. Other effects

Residential pesticide exposure and cases of well water contaminated with CPF and other pesticides were associated with higher risk of Parkinson disease (OR=1.87; 95% CI: 1.05-3.31, Gatto *et al.*, 2009; n=64; OR=2.6 95% CI:1.3-5.4, Manthripragada *et al.*, 2010). CPF exposure was also associated with wheeze among farmers and commercial pesticide applicators (n=486; OR=2.40, 95% CI: 1.24, 4.65 Hoppin *et al.*, 2006).

3.6 Biomonitoring studies were done in Australia for CPF exposure

There are very few published biomonitoring studies conducted in Australia to study CPF exposure (Table 18). Urinary TCPy were investigated as part of a general population pesticide biomonitoring study in Brisbane (Heffernan *et al.*, 2016) and as part of children exposure study in South Australia (Babina *et al.*, 2012) and in Queensland (Li *et al.*, 2019). Urinary TCPy in

Australian children in Babina *et al.*, 2012; Heffernan *et al.*, 2016; Li *et al.*, 2019 were reported to be higher in the US (CDC, 2019). In addition, exposure to CPF among termite control workers in Western Australia was also studied with biomonitoring of urinary TCPy (Cattani, 2004). Finally, Johnstone (2006) conducted organophosphorus exposure studies of agricultural workers in Queensland by examining DEPs metabolite in urine. As of March 2020, there have been no large cohort studies or periodically national biomonitoring studies conducted to study the population exposure to CPF or any pesticide in Australia.

Table 18 Chlorpyrifos biomonitoring studies conducted in Australia

References	Population	Biomarker level	Key Findings
(Babina *et al.*, 2012)[a]	Children (3-6 years); n = 340 South Australia, 2003-2006	TCPy GM urban = 21.5 µg/g GM peri-urban=27.1 µg/g GM rural = 16.3 µg/g	Percentage more than LOD in urban population: 92.2% The GM TCPy level reported for urban children is seven times higher than the TCPy level in urine of 6-11 year old US children in the year 2001-2002.
(Heffernan *et al.*, 2016)[b]	Children and adult (0->60 years); n=24 Queensland, 2012/2013	TCPy GM= 23.0 ng/mL Children (0-4 years) GM = 23 ng/mL	The TCPy reported is higher than the urinary concentration reported in children in Spain (6–11 years, *n* = 125, GM 3.36 ng/mL (Roca *et al.*, 2014a)
(Li *et al.*, 2019)[b]	Children (0-5 years); n=20 Queensland, 2014/2015	TCPy GM= 9.7 ng/mL	This level is 50% lower than reported in 2012/2013 study in Queensland in the 0-4 age group (Heffernan *et al.*, 2016).
(Cattani, 2004)[a]	Termite control workers; n=19 Western Australia, 1998/1999	TCPy Median pre-application= 230 µg/g Median post application = 208 µg/g	The levels of TCPy pre and post application does not significantly vary. The range level of TCPy clearly indicated that there were exposure of CPF among the workers and this reflected the work practice and the use of control measure during work.

a- Creatinine corrected TCPy ; b-Pool samples

3.7 Current discussion on CPF exposure

3.7.1 CPF toxicity beyond AChE inhibition

Cholinergic effects of OPs (CPF included) in mammals are well defined and widely documented. However, there are some clinical manifestations of exposure to OPs that are that

cannot be explained by AChE inhibition alone (Voorhees *et al.*, 2017; Terry, 2012). For example, neurodevelopmental toxicity effects of in utero exposure to CPF occurs at lower concentrations that could cause AChE inhibition in the foetus or the mother (US EPA, 2016). In other words, there are other targets of OPs toxicity being discussed in the literature. Some of the potentially relevant and important non-cholinergic targets of OP toxicity are neuroinflammation and oxidative stress (*reviewed in* (Costa, 2018)). Both neuroinflammation effects and oxidative stress effects of CPF were reported in a number of animal studies (for example Ma *et al.*, 2013; Hernandez *et al.*, 2015; Tian *et al.*, 2015) and both have been shown to play a major role in neurodegenerative diseases such as Alzheimer's and Parkinson's disease. The molecular mechanisms of these effects, especially for CPF, are not fully understood and hence further research is required.

3.7.2 Interaction of CPF with other OPs and other classes of pesticides

In the field, applicators or agricultural workers often use more than one pesticide at a time. For instance, application of pyrethroids (PYR) often followed with OPs because OP pesticide would act as an inhibitor of the esterases involved in the metabolism of PYR thus potentiating the insecticidal efficacy of PYRs (Okeke, 2018). In other words, OP pesticides are the synergists of PYR toxicity in target and non-target organisms. Okeke (2018) examined the interaction of both PYR and OPs pesticide among adolescent applicators and found there was a significant decrease of PYR metabolites with the increase of TCPy. A study investigating co-exposure to CPF and another OPs (profenofos - PFF) in cotton workers, reported that the "relative exposure of CPF and PFF are highly correlated" (Singleton *et al.*, 2015). This raises questions on how does the co-exposure to multiple pesticides change the biomarker levels, the

AChE inhibition, toxicity and metabolism of the chemicals involved and, most importantly, how does it affect human health. However, the interaction of CPF with other OPs is beyond the scope of this research.

3.7.3 CPF ban and restriction in Australia and other countries

Chlorpyrifos is among one of the most controversial and debated pesticides globally. It has been banned in multiple countries due to its health effects, especially its potential to elicit neurodevelopmental delays among children and/because of prenatal exposure of foetus and newborn/children. The restrictions have been applied in the US, the EU, and Singapore, other than in Australia (Table 19). Anyone can nominate a chemical (active substance, product or approved label) to be re-considered in Australia (APVMA, 2017d). Any news, discoveries or any regulations imposed related to how to control CPF exposure in other countries can spark the public interest on the topic of how CPF or any pesticides should be restricted in Australia. The APVMA also take into account regulatory decisions from counterpart authorities in other countries including the US (APVMA, 2017d).

Table 19 CPF status and restriction applied as of December 2019

Country	CPF status and restriction applied as of December 2019
Australia	CPF is currently under reconsideration in Australia since 1996 (Refer to Section 3.7.4)
USA	CPF remains registered as it undergoes the review of registration that is due in October 1, 2022 (Refer to Section 3.7.3.1)
Singapore	CPF is not permitted to use for any anti-termite soil treatment (National Environment Agency Singapore, 2009).
Thailand	CPF ban is delayed until June 1, 2020 (Yuvejwattana, 2019)
EU	CPF is undergoing evaluation for pesticide approval under the EU's peer review system. However, the European Food Safety Authority (EFSA) released a statement that based on the human health assessment done, CPF does not meet the approval criteria as stated in Article 4 of Regulation (EC) No 1107/2009. This approval criteria are applicable to human health (EFSA, 2019a)

3.7.3.1 Petition to cancel CPF registration in the US

In the US, CPF has gone through a lot of changes in regulations following the periodical re-registration and risk assessment process and petitions, and law amendments (Table 20) (US EPA, 2018). The US EPA is the primary body/agency that regulates pesticides in the US according to several federal statutes. When Food Quality Protection Act (FQPA) signed into law in 1996, CPF was chosen as the first pesticide to be reassessed for food tolerances and other criteria (Clune, Ryan & Barr, 2012). Following the finalised risk assessment done to comply with FQPA, there was a need to modify CPF uses to meet the new standards for health and environment protection, especially to protect children (US EPA, 2018). Carol M. Browner, the EPA Administrator back then explained that "children are not little adults." She then added that the children's body systems are still developing and thus they are far more susceptible to toxicant risks (Walker, 2000). The US EPA and CPF manufacturers reached an agreement to

cease all home use of CPF and also restricted CPF use on apples and grapes and banned its usage on tomatoes (US EPA, 2018)

Following the ban or restrictions announced following the risk assessment done by US EPA, the use of CPF at home declined dramatically in the US. Before the ban and restrictions was introduced in 2000, the EPA estimated 11 million pounds of CPF were applied in non-agricultural settings (i.e. residences, school, parks, golf courses) (US EPA, 2006). After the ban, data provided by Dow Agrosciences (the CPF manufacturer) to Eaton *et al.*, (2008) showed that non-agricultural settings use in 2002-2006 was half of that in 1998-2001. However, it is important to note that the US share in global agricultural and non-agricultural settings was 16% and 26% respectively in the years 2002 & 2006. The relative use of CPF in agricultural settings increased from 60% in 1998-2001 to 97% in 2002-2006. These numbers were also provided by Dow Agrosciences to Eaton *et al.,* (2008).

Given all the substantial evidence that CPF can cause harm to neurodevelopment, there are demands for CPF tolerance revocation and cancellation of CPF registration by both Pesticides Action Network North America (PANNA) and National Resources Defence Council (NRDC) (September 2007) (NRDC, 2007) and the Environmental Justice (September 2016). NRDC petition claimed that CPF registration should be cancelled because to be registered under Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) a chemical must comply with the clause "will perform its intended function without unreasonable adverse effects on the environment". Moreover in the Food, Drug and Cosmetic Act (FDCA), the tolerance should be revoked if the residue level in food is not safe. The US EPA also agreed with this statement where there is a growing body of literature supporting the non-existence of safe tolerance level of CPF exposure and thus proposed to "revoke all CPF tolerances" in 2015 (US EPA, 2015a).

Nevertheless, in March 2017, Scott Pruit, the EPA Administrator newly appointed by Donald Trump denied NRDC and PANNA 2007 petition to include or review some claims in the next registration review in Oct 2022 (US EPA, 2017b). In the latest news, August 2018, the US Ninth Circuit Court of Appeals ordered to ban CPF in 60 days and then, the Department of Justice asked the Court to reconsider this. The current political climate in the US seems to interfere with the well-established scientific review processes and principles.

Table 20 History in the regulation of CPF in the US (Adapted from (Centner, 2018)*). Reproduced with permission.*

Year	Action
1965	Introduction of CPF by Dow.
1996	FQPA 1996 signed into law – requires comprehensive risk assessment.
1997	CPF indoor usage deemed unsafe.
2000	EPA and US manufacturer in agreement to stop manufacture CPF by the end of 2000. All usage in areas that children has potential to be exposed with CPF will be phased out (Walker, 2000).
2007	NRDC and PANNA petition to cancel CPF
2015	EPA was not able to conclude that the risk of aggregate exposure to CPF usage met the safety standard of FFDCA.
2016	EPA concluded that the uncertainties remain but they cannot deny there are neurodevelopment effects to children at low-level exposure.

3.7.3.2 CPF reconsideration in Australia

As previously described in Section 2.3.2, any agvet chemicals may be nominated to be reconsidered by the APVMA or anyone from the public. CPF was one of the chemicals prioritized in 1995 which marked the beginning of the Chemical Review Program by the APVMA, then known as the National Registration Authority (NRA) (APVMA, 2015b). CPF is used in Australia to protect a wide range of crops and to control various pests in agricultural settings, termites in the house, and pests in home gardens.

The chemical review of CPF was initiated in Australia in 1996 because of concerns over its toxicity to the environment and human health as well as occupational health and safety-related issues (APVMA, 2019). CPF (the active constituent), registered CPF-containing products, and the associated label approvals for products containing CPF were nominated and then were prioritized in 1995 as the first group "designated to review". After the scope of the review was finalized, in December 1996, a notice of reconsideration was sent to the stakeholders to start the review of this pesticide (APVMA, 2015b).

The end in mind of a chemical review is to set the final regulatory decision whether or not to have the chemical registered and approved for use in Australia (**Chapter 2**). For CPF and its products, the scope of the assessment was set for toxicology, occupational health and safety (OHS), maximum residue levels (MRL), and environment. APVMA (NRA then) published the draft of CPF review for public comment in January 2000 (NRA, 2000). In September 2000, *Chlorpyrifos Interim Report* was shared to the public which summarised the outcomes of the review of different dossier components (toxicology, chemistry, agricultural, OHS, environmental). More importantly, the NRA introduced interim regulatory measures for CPF (Table 21) for public consultation. These recommendations were implemented between 2000 and 2001 (APVMA, 2009). Other than the interim regulatory measure, the outcome of the 2000 report was to review residue data to confirm the temporary MRL set for several plants and commodities.

In August 2009, the APVMA released a preliminary review findings report on additional residue data (APVMA, 2009). The report was mainly about the results of supplementary residue data assessment, the review of the updated toxicology report and the proposed recommendations. In addition, this report highlighted the examination of the validity of

selecting the AChE inhibition as toxicological endpoints for CPF. The toxicological risk assessment conducted as part of this preliminary review findings report established that for determining the acceptable daily intake (ADI) and no-observable adverse effect level (NOAEL), the plasma cholinesterase inhibition, being the most sensitive effect, was the selected the toxicological end-point. Despite the decision, the conclusion drawn in the 2000 report remained unchanged. The rest of the report was about the assessment of MRLs in food and commodities. In summary, the report was mainly an affirmation of the decision made in 2000 and further evaluation of MRLs set for food.

Table 21 Regulatory interim measures introduced at the earliest stage of CPF reconsideration process (NRA 2000)

Recommendations	Details of the proposed recommendations
First aid and safety direction.	First Aid Instruction No changes for first aid instruction. Safety direction Some amendment of the current first aid entries and addition of new first aid entries.
Home Garden and indoor use of certain CPF products	No more emulsifible concentrate and/or liquid concentrate CPF product with concentration more than 50g/L for home garden and domestic pest control approved for use Label to warn the product is not for householders were recommended for all emulsifiable concentrate and liquid concentrate
Label warnings for occupational health and safety	Warning statement in labelling for re-entry period for greenhouses, cotton chippers, field crops, tree crops and vines. Warning statement in labelling for pre-construction and post-construction termite control as well as general pest control.
Label warning for environmental protection	Label warnings were recommended for termiticide products and agricultural products to avoid run-off and drift after application.
Label statements associated with residues and maximum residue limits.	Some changes recommended for cotton and grapevine leaves and major animal feeds.
Changes to MRL standard	MRL values for various commodities were labelled as temporary subject to evaluation of further data.

Another supplementary toxicology assessment report on developmental and behavioural toxicology of CPF published in 2017 (*Reconsideration of Chlorpyrifos: Supplementary Toxicology Assessment Report*) that concluded no evidence indicated potential

neurodevelopmental effects reported at or below doses that inhibit acetylcholinesterase (AChE) activity (APVMA, 2017c). To date, the process of CPF review is still on-going with interim regulatory measures implemented and still on-going to finalize data for re-establishment of permanent maximum residue levels.

3.8 Summary

The toxicity of CPF and other OPs extends beyond just AChE inhibition. The cellular and molecular mechanisms leading to the health effects observed in populations with chronic low dose exposure to CPF are not fully understood. What we know today is that the exposure to CPF is associated with neurodevelopmental delay, birth outcomes/effects, cancer, endocrine disruptor, sperm quality and male reproductive toxicity. These effects occur not only in occupational populations but also in non-occupational populations, especially mothers, infants and children. Research on prenatal exposure to CPF and its impact on infants and children are widely investigated.

Because of the health effect association with exposure to CPF, it is imperative to provide controls on the use of the chemical and on the extent of exposure in the general population. To control exposure among the population, CPF is restricted and banned in several countries. In **Chapter 2**, it has been noted that there some regulation features in Australia that are not at par as the EU. This raises questions on the effectiveness of the overall regulatory system in AU to protect the population from the exposure to pesticides. In Australia, CPF is currently undergoing a reconsideration process since the year 1996 (Section 3.7.4). As part of this process, APVMA has introduced several interim regulatory measures in the response to the

risk assessment process of CPF in the year of 2000 (Table 21). However, there is no data to examine if the regulatory measures introduced are effective to control CPF exposure among the population.

Biomonitoring study can be undertaken to learn about the exposure of populations to environmental contaminants. It is also noted that there is not any national biomonitoring program going on in Australia like the ones conducted in the US (NHANES). Patterns and trend of pesticide exposure are not known in Australia because there is no periodic and systematic biomonitoring study conducted nationally. In other words, there is a lack of information on the extent of pesticide (CPF) exposure among the Australian population and thus little can be said on how effective the regulation in controlling exposure to pesticide (CPF). In conclusion, there is a need for biomonitoring studies of pesticide (CPF) exposure in investigating pesticide exposure among the Australian population. Biomonitoring data can aid in learning whether or not the pesticide policy and regulations are effective in controlling exposure among the population. In specific to CPF pesticide, biomonitoring study in examining CPF exposure among the population will tell whether or not the regulatory measure introduced (Table 21) as part of CPF reconsideration is effective in controlling CPF exposure.

3.9 Strategies to fill the Gap in Research

Generally, there is not much information on the extent of pesticide exposure among the Australian population. Australia as a country does not do any periodic and routine biomonitoring studies to examine the population exposure to any pesticides. Therefore, the information or scientific data on whether or not the pesticide policy implemented for Australia is effective to control exposure among the population is lacking. We do not know how much are the Australian population is exposed to pesticides.

Exposure to CPF has been demonstrated to be associated with several health effects. CPF in Australia was and is still under review by the APVMA in Australia since it was nominated in 1995. In the earliest stage of the reconsideration process, there were several regulatory measurements introduced by the APVMA as the response of this review process undertaken for CPF (Table 21). Although there are several studies on biomonitoring of CPF among different populations in Australia, there is not any that focuses on the effectiveness of the regulatory measure in controlling CPF exposure among the population.

Investigation of pesticide exposure is often done through exposure assessment whether in the direct or indirect method. The reason direct method, biomonitoring is selected in this research is that I am comparing the exposure of CPF investigated with the ones that were done at the earliest stage of the new regulatory measurement introduced (Babina, 2007). To investigate whether the regulatory measurement successfully controls the CPF exposure, the level of CPF exposure among the population in this research will be compared with the level of CPF exposure that was investigated in the earliest stage of the introduction of the regulatory measurement back in the year 2003-2006.

3.9.1 Study Design

The research conducted in this thesis was a small-scale biomonitoring study that answers the research question regarding the effectiveness of regulatory measure to control the exposure of chlorpyrifos among the Australia population. It was originally part of a study to investigate take-home pathway of pesticide exposure among families of pesticide handler and families of the non-pesticide handler. Hence, the recruitment process was samples collected from research participants consist of adults and children with urine along with house and vehicle dust sample. The result and discussion of the pathway of pesticide exposure research will not be presented in this thesis.

3.9.2 Ethics approval

Ethics approval for this research project was granted on 2 November 2015 by Southern Adelaide Clinical Human Research Ethics Committee (Reference number: 291.15 - HREC/15/SAC/248). Approval from the Department of Education and Child Development (DECD) South Australia is required when conducting research and evaluation with the staff of the department and students from schools, kindergarten and child-care centers. Researchers were also required to get Child Related Employment Screening by the Department of Communities and Social Inclusion (DSCI) before working or collecting samples from children and a police clearance for other activities related to collecting samples, interview and site visits. All approvals were sought before any of the sample collection activities began.

During sample collection, participants and the guardian/parents of children were given the overview of this research and the procedure involved. Upon agreement of participation, written informed consent was provided by all research participants before data collection. Children were represented by guardian/parents.

3.9.3 Subject recruitment

The recruitment process for this project was flexible and dynamic. It was done on trials and error basis. Because this study was part of a study to investigate the pathway of pesticide exposure among the families of pesticide handlers, I was looking for pesticide handler families and non-pesticide handler families that live in South Australia to participate (for both control and target population). The age of children was proposed to be at 2-6 years at first but was later set to 2-10 years. To get participants, there were several methods got approved by the ethics committee. However, overall, the participation rate was very low and thus, the convenience sampling method was the most effective ways in getting we had to amend ethics approval multiple times by suggesting few other ways to get participation. At the end of the recruitment process of this research, participants were sought from pest control businesses, orchards, not-for-profit organization, farmers/farmworker associations, and kindergarten/schools.

3.9.4 Biomonitoring of CPF Exposure

As previously mentioned, the exposure assessment were conducted through a biomonitoring study of CPF Exposure. Since the subjects recruited has children, it was best to take urine sample because of its invasiveness and also convenience. The biomarker selected for this biomonitoring study was urinary TCPy because it is relatively specific than other diethyl phosphate metabolites. More importantly, TCPy was selected so that the levels of exposure can be compared with the study conducted in 2003-2006 (Babina, 2007; Babina *et al.*, 2012) .

3.9.5 Analytical method to measure TCPy in urine

To analyse TCPy in urine, GCMS technique was selected. The rationale of choosing GCMS and the method development process in this research was detailed in **Chapter 4**.

3.9.6 Statistical analysis

3.9.6.1 Method development to analyse TCPy in urine

While developing the GCMS method to analyse urinary TCPy, the addition of derivatization agent (MTBSTFA) was required to make the compound of interest volatile. Some derivatization parameters require optimization are 1) time to react; 2) temperature; 3) volume of MTBSTFA. Response surface method was used to estimate the optimal conditions to derivatize TCPy, Through Unscrambler software, a series of experiments was set using Box Behnken design. ANOVA was performed to see which parameters are statistically significant.

3.9.6.2 Analysis of urinary TCPy data

Data screening were initially conducted before any further statistical analyses. Stem-and -leaf plots were applied to visualize the shape of the distribution of the data set. With this, outliers are also detected and decided if it is going to be included in the data set. Each outliers data is further investigated of what possibly be the cause of the data to differ from other data point.

The analytical method developed is capable to analyse unconjugated TCPy only. TCPy excreted in animal studies (Bakke & Price, 1976) consist of 80% glucuronide -TCPy and 12% free TCPy. Therefore the assumption applied was that 1)the free TCPy detected is 12% of the total TCPy and 2) non-detected TCPy means there is no conjugated TCPy as well.

In addition, the result of TCPy level in urine were not corrected for creatinine. The estimated total TCPy will be creatine adjusted according to creatinine levels published by (Adeli *et al.*, 2015). This will then make the data of this study were comparable with data published by Babina (2007).

Levels of estimated total TCPy were presented in range, percentage of sample detected above LOD and in percentiles. There were no statistical test done to see if the analytical method developed in this research was different from the ones done by Babina (2007). The data comparison with (Babina, 2007) is of qualitative nature.

CHAPTER 4: THE DETERMINATION OF URINARY TCPY USING GC-MS WITH MODIFIED QUECHERS EXTRACTION

As discussed in the literature review chapter, it has been suggested that CPF exposure is linked with neurodevelopmental deficits in children, cancer, poor sperm quality, poor birth outcomes and endocrine disruption (**Chapter 3**). As a result, CPF use has been restricted in some countries (Singapore, South Africa, India, Sweden). In Australia, CPF exposure has been of interest to NRA (APVMA now) since 1995 (APVMA, 2015b). The APVMA reviewed the risks of CPF in the year 2000 and subsequently released a report titled *The NRA Review of Chlorpyrifos* that restricted CPF use at home and in the agricultural sector (see Section 3.7.3.2 for further details). The recommendations of this report were implemented between the year 2001-2002 (APVMA, 2019). Shortly after the report was written, in 2003-2006, Babina and colleagues showed that children in South Australia (SA) have been widely exposed to CPF along with other OPs and pyrethroid (PYR) (Babina *et al*., 2012). This was during the earliest stage of the implementation of CPF use restriction in Australia. The levels reported were higher than their peers in Germany and the US (Babina, 2007). One of the objectives (**Objective 6, Chapter 5)** of this thesis was to determine if there had been a decrease in the extent of CPF exposure as a result of the regulatory measures that were introduced as in Table 21 from *The NRA Review of Chlorpyrifos* report (NRA, 2000). To achieve that objective, it was decided to measure the levels of the CPF urinary metabolite (3,5,6-trichloro-2-pyridinol (TCPy) (**Objective 3**) as reported by Babina (2007). The instrumentation used by that study was not available in this study, thus an analytical method to analyse TCPy in urine needed to be developed and validated (**Objective 4**). This chapter describes the process to develop the analytical methodology to analyse TCPy in urine.

4.1 Determination of TCPy in urine

As discussed in **Chapter 3**, CPF exposure has been estimated by undertaking the analysis of CPF and its metabolites (DEP, DETP and TCPy) in urine, blood, hair, and meconium. TCPy is not the most specific metabolite as it also a product of CPF-methyl (IPCS, 1975) and trichlorpyr (US EPA, 1992) urinary excretion. However, measurement of TCPy in urine was chosen in this study because i) the target population consisted of children and taking blood samples may have been more problematic and ii) the availability of analytical standards in the market. It was also thought that withdrawing blood from people may impact participation rate negatively.

There are no methods for direct measurement of TCPy in urine without separation or sample pre-treatment. Prior work on the detection and measurement of TCPy in urine has been achieved with various separation techniques including GC-MS, GC-MS/MS, LC/MS-MS, capillary GC/MS and immunoassay (Table 22). LCMS is the method of choice as determination of TCPy is generally straightforward without any modification of the compound needed. It also has a lower detection limit than that of GC-MS. This was the method used by Babina (2007). An LCMS system was not available for this research and, thus TCPy analyses in urine needed to be done with GCMS. GCMS is an instrument that works very well in the detection of volatile compounds, thus sample pre-treatment is often required.

In its native form TCPy is not very volatile (Li *et al.*, 2014), and thus poorly detected in GCMS. For this reason, a derivatization approach was required to chemically alter the TCPy into a form that had properties more amenable to the GCMS analysis technique and thus improve its detection. There are two reagents commonly used to derivatize TCPy for GCMS analysis in

the literature which are N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and N-(tertbutyldimethyl)-N-methyltrifluoroactamide (MTBSTFA). In this project, I added MTBSTFA as a derivatization agent before injection into the GCMS. This produced a more volatile derivative that allowed better detection limits.

MTBSTFA is the favoured derivatization agent for TCPy compound as it used extensively for the same analysis and separation technique in urine (Hines & Deddens, 2001; Koch & Angerer, 2001; Wilson *et al.*, 2004), duck muscle (Li *et al.*, 2014), blood (Brzak *et al.*, 1998) and rat saliva (Smith *et al.*, 2012). The primary derivatization reaction involves silylation reaction where the active hydrogen compound was substituted with tert-butyldimethylsylil (TBDMS) moiety from MTBSTFA (Figure 6) and thus the formation of TBDMS-TCPy compound. The same reaction also applies to the internal standard compound chosen for this analysis when MTBSTFA was added.

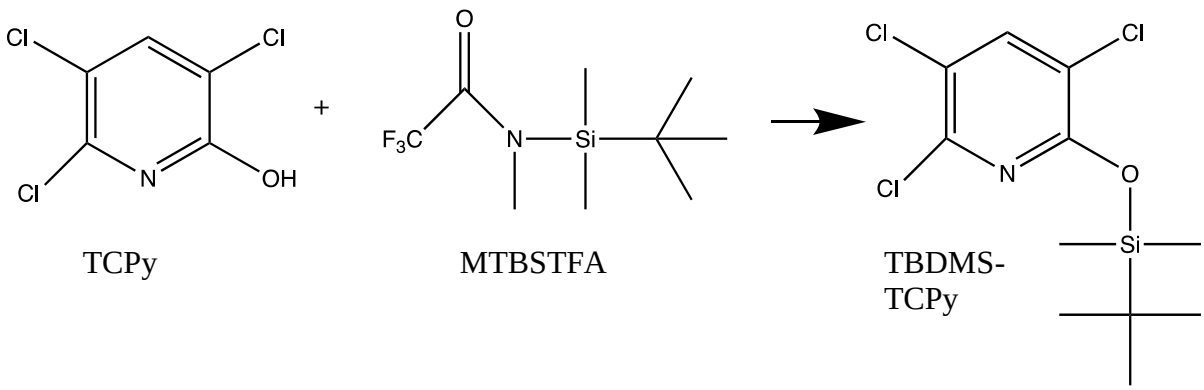

Figure 6 Silylation reaction of TCPy with MTBSTFA (derivatization process)

4.2 Sample preparation for urinary TCPy analysis

Before analysis, TCPy is usually extracted from the urine matrix with liquid-liquid extraction or solid phase extraction (SPE) (Table 22). However, analysis of multi-residue pesticide analysis in food has been notably conducted more recently with QuEChERS extraction. QuEChERS extraction was first developed to extract multi-class of pesticide from fruit and vegetables (*reviewed in* Rejczak & Tuzimski (2015)) and has been successfully applied for any other matrices such as soil (Caldas *et al.*, 2011; Yu *et al.*, 2016), water (Amelin, Lavrukhin & Tret'Yakov, 2013) and urine (Usui *et al.*, 2012; Otake & Hanari, 2018; Roca *et al.*, 2014a). Other than pesticides metabolites, QuEChERS extraction for urine has been done for other compounds as well such as drugs (Salimiasl *et al.*, 2012), bisphenol A (Correia-Sá *et al.*, 2018) and lipids (Bang, Byeon & Moon, 2014). Fundamentally, QuEChERS method involves the addition of an immiscible organic solvent into an aqueous homogenized sample, along with NaCl (to inhibit the capability of water in the matrix to dissolve solvent and the analyte) and anhydrous $MgSO_4$ (to absorb water from the matrix) (Anastassiades *et al.*, 2003). Roca *et al.* (2014) validated a method with liquid-chromatography tandem with high resolution mass spectrometer (LC-HRMS) along with QuEChERS extraction of a group of pesticides (OPs, PYRs, phenoxy herbicides and chloroacetanilide herbicides) from urine. This was the first publication using the said extraction method for TCPy in urine samples. This method not only covers multiple pesticides analysis (simultaneous) in urine, but it also does not need derivatization for the TCPy and other compounds because it was done with LC-HRMS. The percent of recovery of TCPy, particularly, is 72% with 15% of RSD. Consequently, in this project, extraction of urinary TCPy was done with QuEChERS with the intention to include other pesticides metabolites and parent compound in the future project.

This chapter presented the experiments done to achieve the objective of having a validated analytical method to analyse urinary TCPy. The development of the analytical method is divided into 1) setting of GCMS instrument parameters, 2) QuEChERS extraction method and 3) method validation.

Table 22 Some analytical method developed to analyse urinary TCPy

Reference	Sample preparation	Detection	Performance
(Dowling *et al.*, 2005)	Extraction with 1-chlorobutane and derivatization with MTBSTFA	GLC-MS	LOD= 3-5 ppm
(Burns *et al.*, 2006)	Extraction with Toluene and derivatization with MTBSTFA	GC/MS	LOD= 0.2 µg/L
(Bicker, Lämmerhofer & Lindner, 2005)	Extraction with Ethyl acetate/acetonitrile	LC–ESI–MS/MS	signal to noise ratio=0.25 mg/L
(Shackelford *et al.*, 1999)	C_{18} solid phase extraction	Immunochemical method using trichloropyidinol RaPID Assay Kit	LOD =0.89 ng/mL
(Chuang *et al.*, 2004)	GC-MS=extract with chlorobutane ELISA kit = sample clean-up after hydrolysis	GC-MS and ELISA	GC-–S - LOQ = 6.0 ng/mL ELISA = 37.5ng/mL
(Koch & Angerer, 2001)	automatic steam distillation followed by solid-phase extraction on a polystyrene-divinylbenzene copolymer	Capillary GCMS	LOD = 0.05 µg/L
(Olsson *et al.*, 2004)	SPE	LC/MS	LOD= 0.4 ng/mL
(Babina, 2007)	SPE	LC/MS-MS	LOD = 0.2 µg/L
(Fortenberry *et al.*, 2014)	Method from (Olsson *et al.*, 2004)	HPLC/MS	LOD= 0.1 ng/mL

4.3 Materials and methods

The overall process to determine TCPy in the urine samples collected from the population in this study is presented in Figure 7. The methodology for each of these steps is described below.

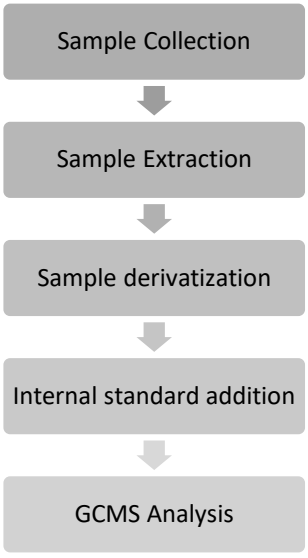

Figure 7 Overall process to determine urinary TCPy for the sample collected from the population of this study

4.3.1 Sample collection

78 first morning urine samples were obtained between July 2017 and May 2018 through convenience sampling from 55 adults and 23 children living in (and around) metropolitan areas of Adelaide, South Australia. Further description of the population is in Section 3.9.3 and

Section 1123.9.4. Briefly, the population of this study consisted of the general population and agricultural workers. None of the research subjects reported having used CPF at home or work. Samples were de-identified, labelled and then stored frozen at -20 ◦C until analysis. Adult urine was used for testing and validation of method development. All samples were collected according to Ethics approval number 291.15-HREC/15/SAC/248.

4.3.2 Chemicals and reagents

Standard 3,5,6-trichloro-2-pyridinol (TCPy) (analytical grade, neat), MTBSTFA (purity >97%), 3,5-dichlorophenol (analytical grade), magnesium sulphate anhydrous and sodium chloride were all purchased from Sigma Aldrich. Analytical grade solvents ethyl acetate (LC-MS grade), acetonitrile, acetone and dichloromehtane (DCM) were from Chem-Supply.

4.3.3 TCPy standard solution preparation

Stock standard solutions of TCPy and 3,5-dichlorophenol were prepared by diluting analytical standards in ethyl acetate to yield a concentration of 1000 µg/ml each. The stock TCPy standard was further diluted to 10 µg/ml and subsequently to 1 µg/ml in ethyl acetate. All standard and internal standard solutions were kept in sealed glass vials and stored in 4◦C refrigerator and used within 2 months.

4.3.4 Sample preparation

The procedure employed to determine TCPy in urine is presented in Figure 8. The optimized condition to extract TCPy in urine was tested for different parameters (volume of urine, extracting solvents, volume of extracting solvents, number of extraction washes, mass of sodium chloride (NaCl) and magnesium sulphate ($MgSO_4$)). The final conditions used are as follows: $MgSO_4$ (750 mg) and of NaCl (750 mg) were first added in a centrifuge tube followed by extraction solvent (2ml of 1:1 dichloromethane (DCM): acetone) and urine (5 ml). The tube was then vortexed for 20 seconds then centrifuged for 15 minutes to separate the aqueous and organic solvents. The organic layer was then removed and placed in a vial. The extraction was repeated one more times with the addition of 1ml of extraction solvent. The two extracts were combined (placed in the same vial) and then the solvent removed by drying under nitrogen.

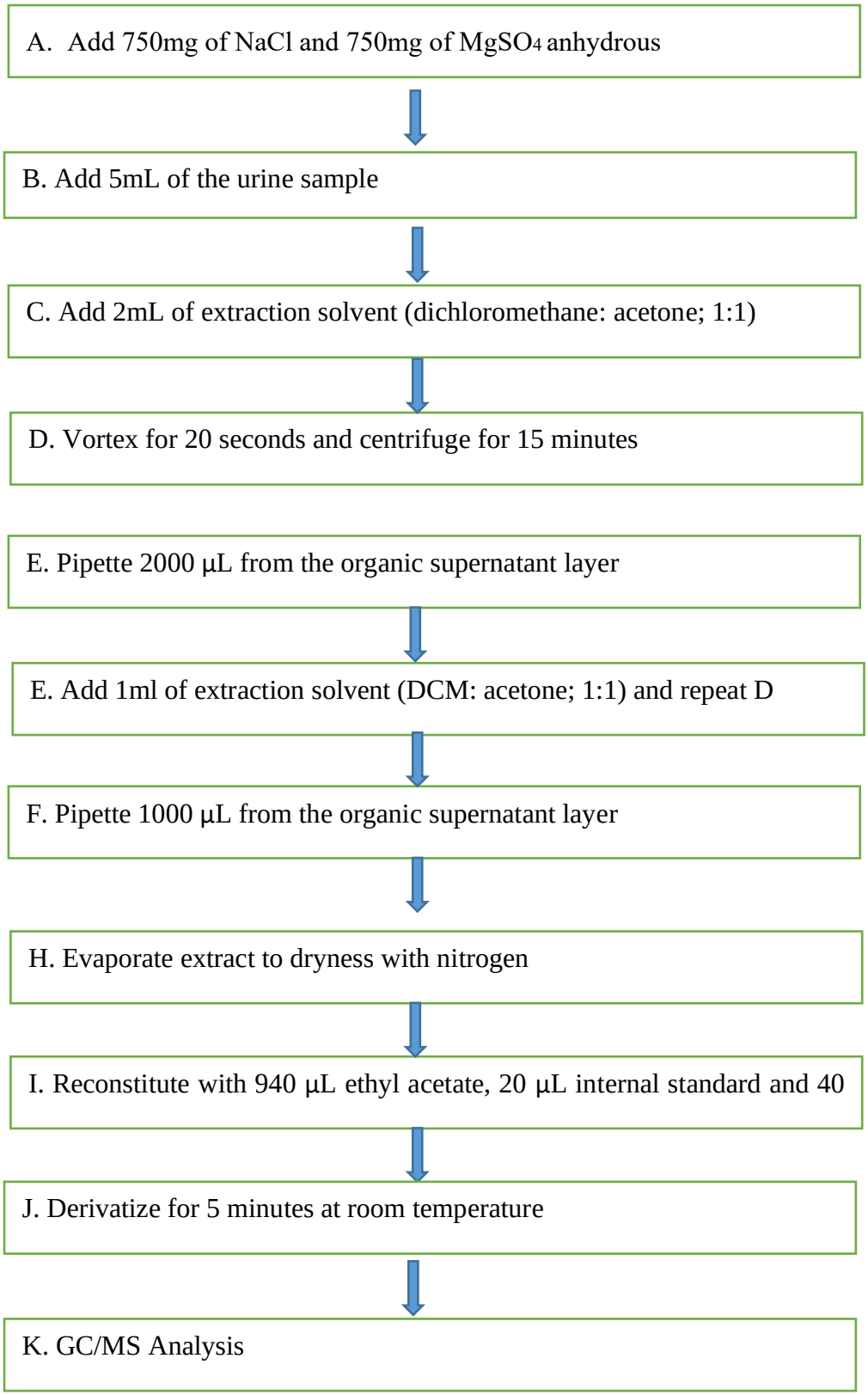

Figure 8 Schematic description of the analytical method adapted for TCPy determination in urine with GCMS and modified QuEChERS

The optimum condition of TCPy derivatization with MTBSTFA was tested for some parameters. Derivatization time (5, 20 and 40 minutes), temperature (20°C, 30°C and 40°C) and volume (10, 25 and 40 µL) were tested for optimal sylilation reaction (Figure 6). The final conditions were as follows: the dried extract was reconstituted with 940 µL of ethyl acetate, 20 µL internal standard and 40 µL derivatization agent (MTBSTFA). The vials were then left to derivatize for 5 minutes in room temperature and were sent for GCMS analysis.

4.3.5 Gas chromatography separation

A GC system Agilent Technologies 7890A was used throughout the study. The column used was a HP-5MS 5% Phenyl Methyl Siloxane (30m x 250 um x 0.25 um i.d) from Agilent Technologies. GCMS condition for urinary TCPy detection was achieved by testing several parameters such as split time, purge flow, inlet temperature, oven temperature and head pressure. The final conditions were as follows: 1 µL of sample injected into 225 °C injector in spitless mode. The column oven temperature was 40 °C initially for 2 minutes. The temperature was then increased to 300 °C at a rate of 20 °C min and then kept constant for 3 minutes. Carrier gas Helium was used throughout with flow rate 1.33ml/min at a pressure of 10.4 psi.

4.3.6 Mass spectrum detection

The MS system used was an Agilent Technologies 5975C inert XL EI/CI MSD with Triple-Axis detector with an electron impact ion source in 70 eV. The transfer line was set at a constant

temperature of 280° C while the ion source and quadrupole temperature were held constant at 230 °C and 150 °C.

4.3.7 Data interpretation

The quantification ion selected for TBDMS-TCPy was 254 m/z with 219 m/z for TBDMS-3,5-dichlorophenol (Figure 9). The ratio of ion 254 m/z and ion 219 m/z was used throughout the interpretation of data. The results from GCMS run of each sample were first interpreted by extracting ion 254 for TBDMS-TCPy at retention time 10.2 min and ion 219 of the internal standard at retention time 10.8 min (Figure 10). The peak area of both ions were retrieved and was calculated for its ratio (TBDMS-TCPy peak area to IS peak area). These data calculations were done using Microsoft Excel.

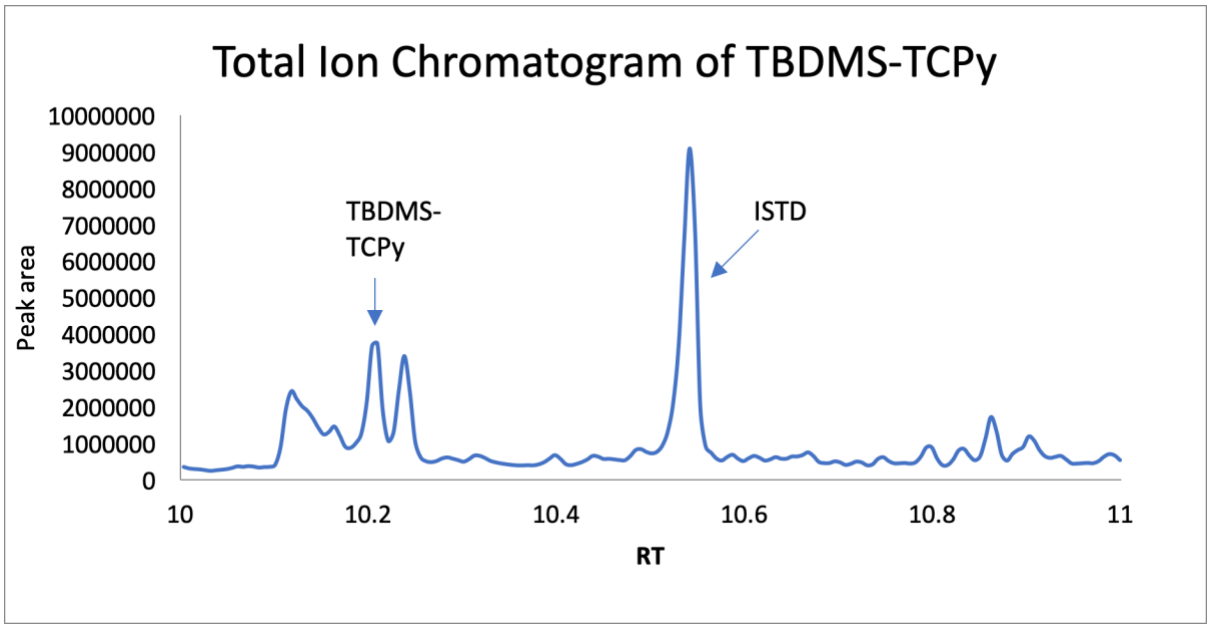

Figure 9 Total Ion Chromatogram of TBDMS-TCPy extracted from urine spiked with 1µg/ml

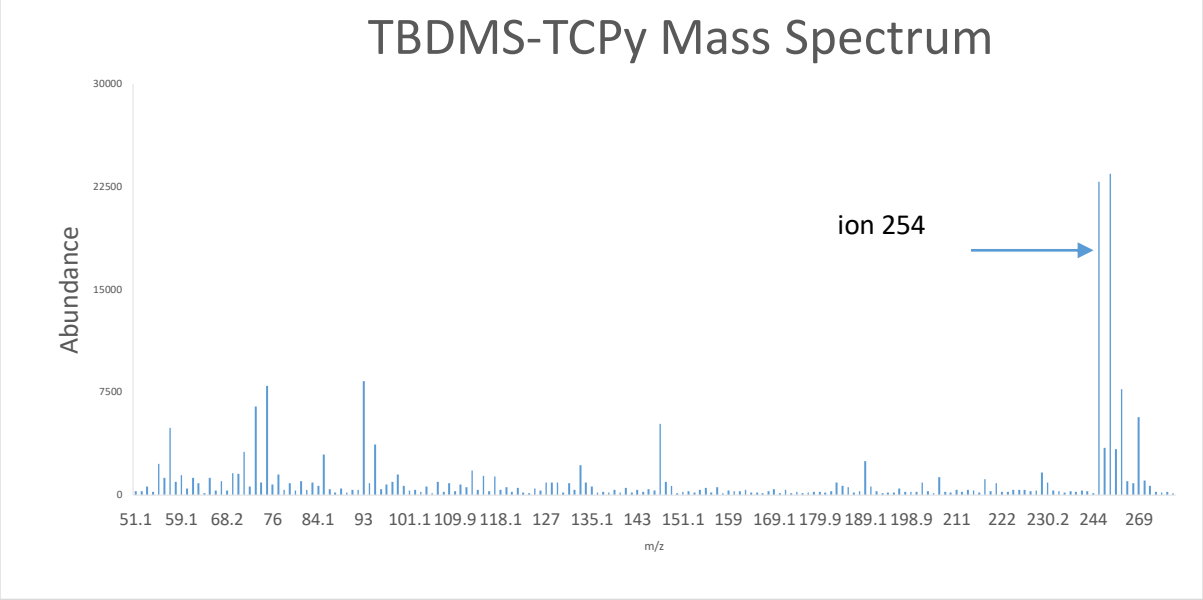

Figure 10 TBDMS-TCPy mass spectrum (extracted from urine)

4.4 Result and discussion

4.4.1 Sample derivatization

The silylation reaction of TCPy is shown in Figure 6. MTBSTFA was found to be a suitable derivatization agent for TCPy as proven in many other studies. The derivatization parameters which are 1) time to react, 2) temperature and 3) volume of MTBSTFA were tested with 0.1µg/ml TCPy standard. Optimization of derivatization condition experiment was undertaken using a Box Behnken Design developed through Unscrambler® software. Initially, there were other pesticides/metabolites (TCPy included) in this method development (2,4-dichlorophenol, MCPA, and chlorpyrifos). Therefore, the decision made was in consideration of other compounds as well. According to the response surface (Figure 11) the response of TCPy was the best at 5 minutes reaction time and 40 µL volume MTBSTFA. The temperature was not a

significant variable in this experiment (ANOVA statistical test with p-value more than 0.05). Thus the lowest temperature was selected. Ultimately, the condition is chosen to derivatize TCPy was using 40µL of MTBSTFA in room temperature for 5 minutes.

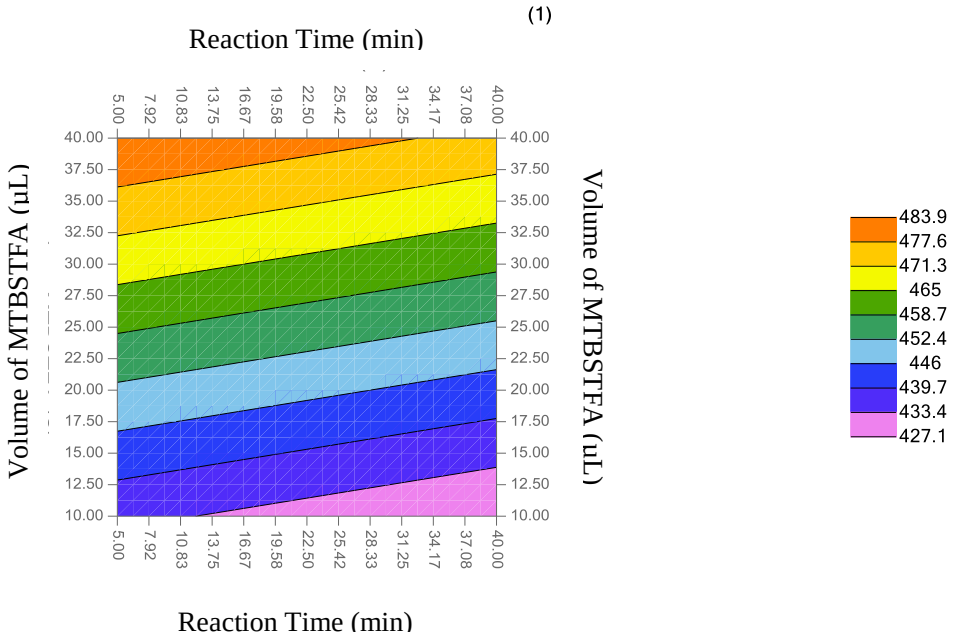

Figure 11 Response surface of TCPy peak area with MTBSTFA volume and reaction time as variables

4.4.2 Sample extraction

The population of this study may not be exposed to CPF at high concentration. Therefore, the aim was to detect urinary TCPy at as low as possible concentrations. 1ml of urine was the volume of sample used initially. However, 1ml of urine only detected concentration as low as 0.1 µg/ml. To lower the linear range of TCPy detected with this method, 5ml volume of the sample were added instead of 1ml. Spiking the same concentration of TCPy into both 5ml and 1ml of urine does not yield the same mass of TCPy. As an example, spiking both 1ml and 5ml of the same concentration of TCPy (0.005 µg/ml) ended up having 0.05 µg and 0.025 µg of TCPy mass respectively (Table 23). After extraction, 2000 µl of extracts were dried with nitrogen. At this point, 5ml of urine sample still had higher mass than 1ml of urine. This

increases the chance of GCMS detection in comparison with samples that have lower mass of the target compound.

Table 23 Comparison of TCPy mass of 1ml and 5ml of urine samples

Volume of urine (µL)	Volume TCPy standard spiked (µL)	Spiked concentration (µg/mL) from 1µg/ml standard	Mass added (µg)	Volume of extracts solvent dried (µL)	Mass of TCPy in ext residue (µg)
1000	5	0.005	0.005	2000	0.0075
5000	25	0.005	0.025	2000	0.0167

4.4.3 Choice of extraction solvents

An investigation was undertaken to determine the ideal extraction solvent for the QuEChERS extraction of TCPy from urine. In order to achieve maximum extraction, the TCPy should dissolve more easily in the extraction solvent than in the matrix solvent (in this case water for urine). Typical water-immiscible solvents such as diethyl ether, MTBE (methyl term-butyl ether), dichloromethane, ethyl acetate are used in liquid-liquid extraction, however, the salt in QuEChERS also allows the use of solvents such as acetone and acetonitrile.

Initial studies used acetonitrile as the extraction solvent like other published QuEChERS extraction methods. Acetonitrile has been used in the first QuECHERS study (Anastassiades *et al.*, 2003) of multiresidue pesticides extraction from fruits and vegetables. However, acetonitrile as extraction solvent of urinary TCPy from GCMS analysis did not work in this study because the signal of TBDMS-TCPy was not repeatable and reproducible. This is was

possibly caused by the miscibility of acetonitrile with water that may have left residual water in the extracts. Water may slow down the derivatization process or completely stop the process completely. As a result, there might be some fractions of TCPy that were not properly derivatized and thus not volatile enough for GCMS to detect. The presence of water seemingly resulted in irreproducible signals.

Ethyl acetate was tested too for urinary TCPy as extraction solvent. Ethyl acetate also does not produce reproducibility and repeatability of the signal of TBDMS-TCPy. This might have occurred due to the volatility of ethyl acetate that may have evaporated off TCPy and thus affected the efficiency of the TCPy extraction from urine. This, in turn, presented inconsistent signal for TBDMS-TCPy during GCMS analysis.

Finally, DCM and acetone (1:1) was experimented as an extraction solvent. DCM: acetone (1:1) has been validated as an extraction solvent of TCPy from sludge previously that was paired with the clean-up process with Florisil (Díaz-Cruz & Barceló, 2006). DCM: acetone (1:1) as an extraction solvent produced a reproducible result for TBDMS-TCPy GCMS signal and thus chosen as the extraction solvent as part of the QuEChERS technique. Extraction of 0.09 µg/ml TCPy from urine with DCM: acetone (1:1) had RSD of 11% while extraction with ethyl acetate had RSD 42% (Table 24).

Table 24 The value of RSD% of recovery of 0.09 µg/ml TCPy extracted from urine

Solvent	RSD%
Ethyl acetate	42%
DCM:acetone (1:1)	11%

To increase the percentage of recovery, replicate extraction was performed. Single extraction of 0.08 µg/ml of TCPy spiked in urine has a percentage of recovery of 76% while double extraction of 0.08 µg/ml TCPy has improved the percentage to 90% (Table 25).

Table 25 Percentage of recovery of single extraction and double extraction of DCM: Acetone (1:1) solvents

Extraction	Percent of recovery±SD
Single extraction	76% ± 0.07
Double extraction	90% ± 0.02

4.4.4 3,5-dichlorophenol as an internal standard

The internal standard 3,5-dichlorophenol was selected because both 3,5-dichlorophenol and TCPy have a similar functional group (-Cl) and (-OH). There were three conditions tested to determine the time to add ISTD in order to have a repeatability and reproducibility signal. 3,5-dichlorophenol is a compound that has hydroxy group hence it will get derivatized with MTBSTFA forming TBDMS-3,5-dichlorophenol. This added another variation in sample preparation and have caused poor repeatability. Therefore, there were multiple attempts made to reduce the variations that may have been caused by the addition of this ISTD to the sample.

4.4.4.1 An attempt to add internal standard after derivatization of TCPy by autosampler

When analysing extracted TCPy from urine (and then derivatized), we found that sometimes the internal standard signals were not giving consistent result. In a pool of samples that were spiked with different concentration of TCPy, at times, there was one of the vials that have a peculiar signal of the internal standard by the GCMS. This sample usually has a magnitude lower signal of internal standard than other samples that were prepared within the same batch of the urine sample. To eliminate human error, we trialled having the autosampler to inject the internal standard after derivatization for every analysis. This means that there was no manual addition of internal standard at all. However, this technique did not demonstrate good repeatability too of the internal standard as well as the target analyte. At times, TBDMS-TCPy detected was not linear as the concentration increased. The problem could be the injection syringe itself because sometimes, there were bubbles introduced during GCMS injection. Therefore, the autosampler injection effort for this study could not be implemented. Another reason could be the residue of water in the extracts that could be interrupting or stopping the derivatization process of 3,5-dichlorophenol itself. Although the use of DCM: acetone (1:1) solvent managed to solve this problem, the addition of 3,5-dichlorophenol through autosampler after derivatization was not tested after the said extraction solvent was introduced in the sample preparation process. In sum, the addition of 3,5-dichlorophenol was done per status quo i.e. before derivatization process after DCM: acetone (1:1) was used as extraction solvent.

4.4.5 GCMS system and operating conditions

The goal of a GCMS method development is to have a reproducible signal of the target compound along with the best chromatogram characteristic. 1µg/ml of TCPy standard and 0.2 µg/ml internal standard was injected into GCMS to identify the retention time (RT) and characterization m/z ion to several parameters as well the chromatogram of both compounds. The characterization ion (ion 254 for TBDMS-TCPy) was identified according to numerous analytical methods developed in the literature (Table 22) to analyse TCPy with GCMS that used MTBSTFA as derivatization agent.

Each changed variable was tested by injecting sample five times each. RSD% of TBDMS TCPy to TBDMS-3,5-dichlorophenol ratio were measured each time and the accepted condition had RSD % below 10%. The best conditions to inject TBDMS-TCPy for this experiment were having purge flow to split vent of 250ml/min for 1.5 minutes, the inlet temperature of 225 C, turning off septum purge flow and the inlet head pressure of 10.4 (Condition E in Table 26). The finalized settings of both GC and MS instrument for all analysis were maintained constant as in Table 27.

Table 26 Condition of GCMS changed to get the best characteristic of the chromatogram and reproducible signals.

	Condition A	**Condition B**	**Condition C**	**Condition D**	**Condition E**
Split flow	0.75 min	**0.5 min**	**1 min**	**1.5 min**	1.5min
Purge Flow	15ml/min	**250ml/min**	250ml/min	250ml/min	250ml/min
Inlet Temperature	200 C	**250 C**	250 C	250 C	225 C
Septum Purge Flow	On	On	On	On	**Off**
Head Pressure	8 psi	8 psi	8 psi	8 psi	10.4 psi

Table 27 GCMS settings for this method developed to analyse TCPy in urine

GC **Agilent Technologies 7890A GC system**	Column	HP-5MS 5% Phenyl Methyl Silox (30m x 250 um x 0.25 um i.d)
	Carrier Gas	Helium
	Flow rate	1.33 ml/min
	Injection Mode	Splitless
	Solvent Delay	8 minutes
	Transfer Line Temperature	280 C
	Oven temperature program	Initial temperature: 40 C for 4min Ramp 20 C/min Final temp: 300 C for 3 min
MS **Agilent Technologies 5975C inert XL EI/CI MSD with Triple- Axis detector**	Ionization Mode	EI
	Ionization Energy	70 eV
	Scan Range	50-550 m/z
	Ion Source Temperature	230 C
	Quadrupole Temperature	150 C

4.4.6 Method validation

4.4.6.1 Calibration curve and linear range

Calibration curve and linear range sample were prepared with spiking blank urine sample with 7 concentrations (0.005,0.008, 0.01, 0.015, 0.02, 0.03, 0.04 µg/ml) of TCPy and then extracted according to QuEChERS extraction described previously. The peak area ratio of the target analyte to internal standard was calculated to obtain regression equation and correlation coefficient. The response was linear across this range of concentration, with an R_2 value of 0.97 (Figure 12).

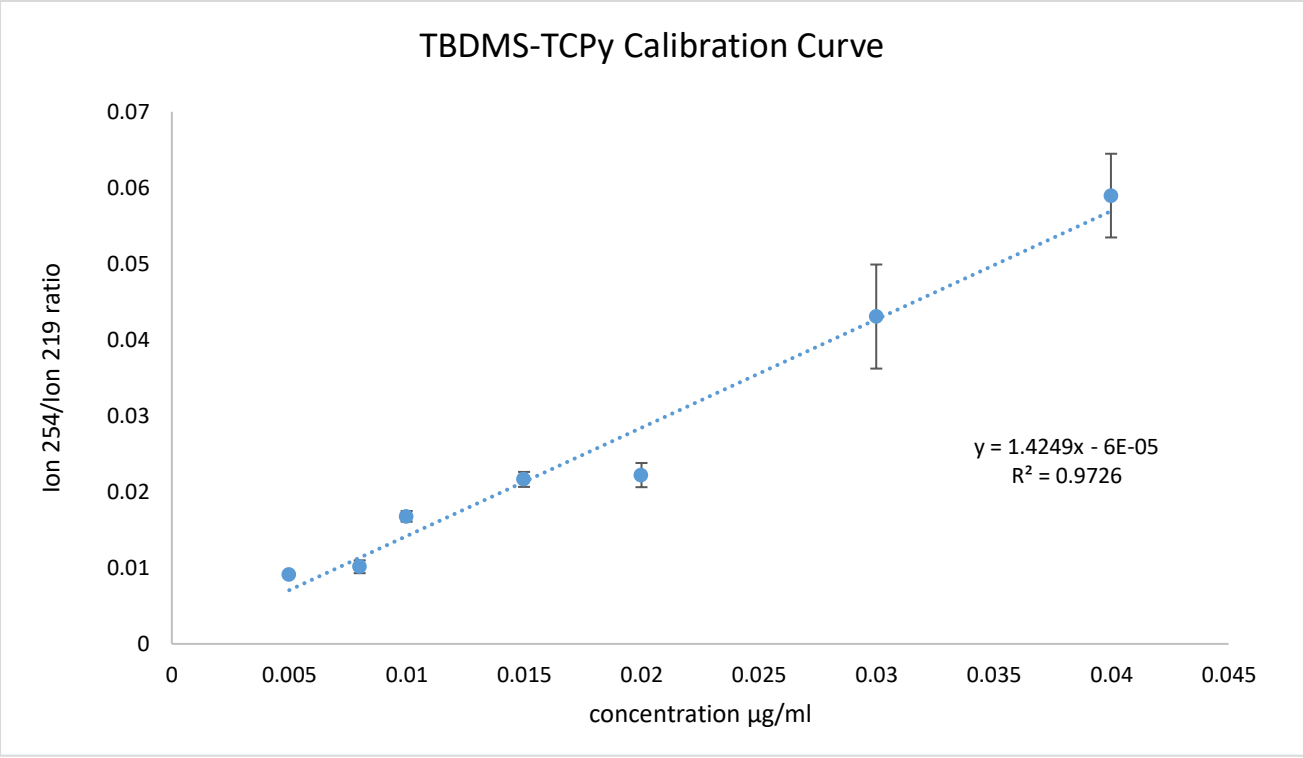

Figure 12 An example of TBDMS-TCPy calibration curve

4.4.6.2 Recovery experiment

Recovery experiments were conducted to study the effectiveness of QuEChERS extraction. Recovery of TCPy was evaluated by spiking 0.03 µg/ml and 0.09 µg/ml TCPy standard into urine samples with three replicates each (Table 28). Each fortification sample was extracted with the final QuEChERS method and then was analysed with GCMS. Along with those samples, the reference samples of both concentrations were prepared by adding 0.03 µg/ml and 0.09 µg/ml TCPy after extracting and drying process as described before. The reference samples were also analysed with the same GCMS settings. The peak area ratio of the analyte to internal standard of both spiked and reference samples was compared and calculated. Recovery of 0.03 µg/ml and 0.09 µg/ml spiked TCPy were 83% and 90.5% respectively.

Table 28 Recovery for 0.03 µg/ml and 0.09 µg/ml of TCPy spiked

Spiked Concentration µg/ml	Average Recovery (%)	RSD (%)
0.03	83	19
0.09	90.5	7.8

4.4.6.3 Repeatability, reproducibility, limit of detection (LOD) and limit of quantification (LOQ)

Repeatability of this method was assessed by spiking 0.09 µg/ml of TCPy in urine (n=3) within the same day (Table 29) and reproducibility was assessed by preparing the same spiked sample in three different days (Table 30). Limit of detection (LOD) was calculated a signal to the ratio of 3 while LOQ was calculated using a signal to the ratio of 10.

Table 29 Repeatability and reproducibility data of this method

	Within days (n=3)	Between days (n=3)
Average percentage recovery (%)	90.5	101
Standard deviation	7.8	11.2
RSD (%)	8.6	11

Table 30 LOD, LOQ ,linear range and regression equation of this method

Regression equation	y= 1.4249x – 6 x 10-5 R_2= 0.97726
Linear range (µg/ml)	0.005 – 0.04
LOD (µg/ml)	0.001674
LOQ (µg/ml)	0.005073

4.5 Suggestion for future work

This analytical method based on modified QuEChERS extraction with GCMS detection (Teoh, 2015) and was first tested with urinary TCPy analysis. For this chapter, the analytical method presented here was successfully developed for free (non-conjugated) urinary TCPy detection. The parameters and variables tested and optimum chosen is presented in Table 31.

Table 31 Summary of parameters tested and optimum chosen

Parameters and variables	Range trialled	Optimum chosen
Volume of urine	1ml, 5ml	**5ml**
Extracting solvent	Ethyl acetate, DCM: acetone, acetonitrile	**DCM: acetone; 1:1**
Volume of extracting solvent	1ml, 3ml	**3ml**
Number of extraction washes	1 time, 2 times	**2 times (double extraction)**
Mass of NaCl and $MgSO_4$	75 mg, 150 mg and 750 mg	**750mg**

This method are not able to determine total TCPy in urine for examining CPF exposure. This is because of the absence of a step to de-conjugate any conjugated TCPy metabolites in urine. During metabolism of CPF, TCPy formed are subsequently conjugated (via phase II metabolism) and then excreted in the urine in the form of unconjugated TCPy and other conjugated TCPy (including glucuronide conjugates) (Bakke & Price, 1976). Therefore, this method and research as a whole may not able to fully estimate the exposure of CPF nor CPF-methyl of the population because 80% of TCPy were not isolated from urine. There are two methods to release conjugated TCPy which are acid hydrolysis and enzyme hydrolysis. Enzyme hydrolysis is widely used and said to increase recovery percentage (Nolan *et al.*, 1984)This method also does not have any purification steps.

The method developed in this study emphasized that residue of water influenced derivatization of the target compound hence affected the GCMS signal. In future, this problem should be addressed first before anything else. It is important to ensure all reagents are dry beforehand. As for water content in urine, increased effort is recommended in varying the amount of NaCl and $MgSO_4$.

Although using DCM: acetone as extraction solvent has possibly solved this issue, it is important to note that DCM is not the safest solvent there is. Another immiscible solvent should be tested in replacement of DCM.

This method has higher LOD value (less sensitive) compared to other method and this may be caused by the absence of purification step. Adding purification steps would be a great start to reduce the limit of detection of this method in addition to hydrolysis steps.

Finally, these are some other recommendations of items that should be done to assist in ensuring the repeatability and productivity of GCMS instrument.

a. The septum was changed frequently.

b. Before the analysis of standards and samples, column baking is done for at least 30 minutes.

c. Ensure there was no defect of the syringe by injection the same sample 5 times. The ratio and RSD% was calculated.

d. Injecting blank solvent and blank extracted urine.

e. Quality control samples were injected every 5 samples to make the known concentration samples obtained the same signal.

f. Calibration curve was made for every 15 runs of sample during sample analysis.

4.6 Conclusion

In conclusion, a method to analyse urinary free (unconjugated) TCPy with modified QuEChERS coupled to GCMS detection system was developed and tested. The method was tested for its linearity, repeatability, recovery and limit of detection and quantification. In order to serve the purpose of the study which is to estimate CPF exposure in human, the method requires further work, primarily adding hydrolysis step to isolate the conjugated TCPy from urine.

CHAPTER 5: EFFECT OF 18 YEARS OF REGULATORY RESTRICTIONS ON CPF USE IN AUSTRALIA ON THE LEVELS OF URINARY TCPY IN A SAMPLE OF URBAN SOUTH AUSTRALIAN POPULATION - A CASE STUDY

This chapter attempts to explore the effectiveness of regulatory measures that were introduced during the reconsideration process of this pesticide 18 years ago in controlling CPF exposure in Australia (**Objective 7**). Urine samples were collected and analysed for the CPF metabolite TCPy using the analytical method developed in **Chapter 4** (**Objective 5**),The result of urinary TCPy analysis in this study was then compared with those from a study conducted in the year 2003-2006 (Babina, 2007; Babina *et al.*, 2012)when the restriction of CPF use was initially introduced to the general public (**Objective 6**).

5.1 Introduction

CPF was nominated to be reconsidered by APVMA (at the time, National Regulation Authority or NRA) in 1995. This process of CPF reconsideration was described previously in Section 3.7.3.2. In the year 2000, the NRA published a report titled *"The NRA Review of Chlorpyrifos"* where several interim regulatory measures were proposed to be implemented (Table 21). Among the measures proposed by the NRA that would have a direct impact on the public health were the following:

i. Emulsifiable concentrate (EC) and/or liquid concentrate (LC) CPF product with concentration more than 50g/L for home garden and domestic pest control NO LONGER approved for use

ii. Labelling to warn people that the product is not for household use was recommended for all emulsifiable concentrate and liquid concentrate

iii. Registration for all EC and LC CPF products in amounts greater than 50g/L that did not have a clear warning that the product is not for householders was cancelled.

iv. CPF products (at a concentration above 5%) that can be applied inside the building for crack and crevice treatment should have an appropriate label that it can only be applied inside buildings for crack and crevice treatment.

v. The existing MRL values of CPF for some commodities became temporary until appropriate data were submitted and evaluated. (in *Table 7X* in *The NRA review of Chlorpyrifos)*

These interim regulatory measures along with others were implemented in the year 2001-2002 (APVMA, 2019). The NRA also considered that "there should be no adverse effects on public health from the continued use of CPF in Australia" based on the implementation of the proposed regulatory measure as described above (NRA, 2000). CPF bans and restrictions are not exclusive to Australia only. For example, CPF was banned for residential use in the US in 2000 (US EPA, 2006) as a result of stringent safety standard to protect children set by the Food Quality Protection Act (FQPA) 1996 (**Chapter 3**). Elsewhere, CPF has also been banned in Singapore (National Environment Agency Singapore, 2009), banned for household and garden use in 2010 in South Africa (Department of Agriculture Forestry and Fisheries Republic of South Africa, 2017) and restricted as plant protection product in Europe (EU Legislation 540/2011).

The impact of these restriction on CPF for home garden and indoor use (Table 21) in terms of the use of CPF in Australia is unknown. This is because Australia still does not have a thorough and unified system to track the amount and type of pesticides applied each year (Radcliffe 2002;**Chapter 2**). Nevertheless, the number of CPF-containing products reported as registered

on APVMA Public Chemical Registration Information System Search (PUBCRIS) did not decline steadily after the introduction of the regulatory measures as a result of the CPF reconsideration process (Table 21). Instead, the number of registered products fluctuated (Table 32). As of March 2020, there were 84 CPF-based products and 30 CPF active constituents registered as reported on PUBCRIS (APVMA, 2018e). These 111 products were registered for agricultural area/use with different crops such as apple, avocado, bean, banana and beetroot. CPF is also registered for non-agricultural area which are home garden or garden lawn/use, base of building wall/fence/rockwork, domestic area – outdoors, buildings- around, bowling garden, golf green turf, tennis court, commercial buildings/houses/factories under construction, for cattle and dairy cattle lactating and ornamental nursery plant. This suggests that the restrictions of CPF use does not have impact to the amount of products registered in Australia. The impact of the NRA's interim regulatory measures introduced in response to CPF reconsideration process (Section 3.7.3.2) to control CPF exposure among the general population is also not known as Australia does not conduct systematic and routine population exposure (biomonitoring) studies (**Chapter 3**). There are, however, small scale CPF biomonitoring studies conducted among termite control worker (Cattani, 2004), agricultural workers (Johnstone, 2006) and the general population of Brisbane (Heffernan *et al.*, 2016; Li *et al.*, 2019). All these studies conducted in Australia is presented in Section 3.6.

Table 32 Number of CPF products approved in APVMA PUBCRIS

Date	Number of products and active constituents approved in APVMA PUBCRIS
3 December 1996[a]	80 products and 8 active constituents
September 2000[a]	161 products and 20 active constituents
Mid of August 2009[a]	85 products and 16 active constituents
Early Feb 2018[b]	111 products and 28 active constituents
End of March 2020	84 products 30 active constituents

a. From CPF: Preliminary Review Findings Report on Additional Residues Data (APVMA, 2009) ; b- PUBCRIS (APVMA) (Accessed in Feb 2018)

5.1.1 Measuring exposure to CPF in the general population

One of the ways to measure the effectiveness of APVMA regulatory actions in controlling CPF exposure could be to examine the extent of exposure to CPF among the general population. Humans get exposed to toxicants from multiple routes of exposure and sources. Therefore, estimating exposure in human populations is never a trivial process. Biomonitoring is one of the ways to assess the absorbed dose of CPF and the extent of exposure in a given population. CPF biological monitoring involves measuring biomarkers of exposure, effect and susceptibility in urine and blood and hair (**Chapter 3**). The effectiveness of CPF and other OPs restriction in Israel and the US has been demonstrated in biomonitoring studies. Clune, Ryan & Barr (2012) and Ein-Mor *et al*. (2018) measured urinary DAP levels in pre and post restriction of OP pesticides to see if there was a decline of OP exposure in US and Israel respectively. Both studies suggested that the decline levels of urinary DAPs among the population may have been caused by the restrictions applied to OP pesticides use.

5.1.1.1 Chlorpyrifos metabolism

CPF metabolism involves formation of diethyl phosphate (DEP), diethyl thiophosphate (DETP) and 3,5,6-trichloro-2-pyridinol (TCPy) (Sams, Cockery & Lennardz, 2004) (**Chapter 3**). TCPy is a specific metabolite of exposure to both CPF and CPF-methyl and triclopyr. DEP and DETP are non-specific metabolites reflective of exposure to a wide range of diethyl OP pesticides. Therefore, urinary TCPy is often a biomarker of choice for CPF exposure assessment in occupationally (Berent *et al.*, 2014), para-occupationally (Arcury *et al.*, 2007), and non-occupationally exposed groups (Lu *et al.*, 2008).

5.1.2 Exposure to CPF in the general population of Australia

In Australia, high concentration CPF products (those containing more than 50g/L CPF) are no longer accessible for public use since 2001-2002 (Table 21, NRA, 2000). Before this era, there were no CPF monitoring studies done for non-occupational group. There are however, some pesticide monitoring studies done among the occupational group; agricultural workers (Johnstone, 2006) and termite control workers (Cattani, 2004). A pesticides biomonitoring study was done among a Brisbane population after the restriction era (Heffernan *et al.*, 2016). In 2003-2006, a study of South Australia preschool children showed that there was a widespread exposure to OP, CPF in particular, and pyrethroid insecticides among the study population (Babina *et al.*, 2012). The levels of TCPy measured in the 2003-2006 study population were higher than among their peers in the US and Germany (Babina, 2007; Babina *et al.*, 2012).

To fulfil O**bjective 5, Objective 6 and Objective 7** of this thesis which are: 1)to undertake analysis of urine samples to assess CPF exposure among the selected populations and 2) to compare the exposure of CPF in this research with the analysis done during the earliest stage of the with the 2003-2006 data and 3) to discuss the effectiveness of the Australian regulatory approach in controlling exposure to CPF, a biomonitoring of CPF exposure among a sample population in SA was conducted.

There were two assumption applied in this case study.

- Assumption 1 - the 2003-2006 study by Babina *et al.* (2012) was done at the earliest phase of the interim regulatory measures when CPF products were slowly being phased out of residential use, but were still widely available to the public. *Therefore, the exposure sources for the general population were varied and many and the extent of the CPF exposure should be assumed to be significant and widespread at the time.*

- Assumption 2 - the regulatory measures implemented as part of the reconsideration process (NRA, 2000; APVMA, 2009), meant that the general public could no longer access high concentration (less than 50g/L) CPF for home and indoor use in Australia (except for crack and crevice treatment). Today, high concentration (more than 50g/L) CPF products are rarely seen in the shop shelf. Commercial pest control operators are still allowed to use CPF as "quasi-domestic" for termite control. CPF can still be used for termite control in the pre- and post- construction scenario but it is classified as Restricted Chemical Product. Besides, CPF is still used extensively in agriculture. *Therefore, currently the sources of CPF exposure for the general population are likely limited to diet, the environment and, on rare occasion from domestic use of lower concentration product and the extent of exposure should be expected to be lower.*

In this chapter, an investigation of (1) the frequency of detection (FOD) of urinary TCPy and/or (2) the levels of urinary TCPy detected in urine samples collected from the general population in SA compared with the results of Babina and colleague (Babina, 2007). While the results may not be representative of the Australia population, they can give an indication of CPF exposure among the SA population over recent years and give general idea whether the regulatory measures set to protect the public have had an impact on actual exposure measures.

5.2 Materials and method

5.2.1 Sample size calculation

To test the differences between exposed and non-exposed population, the formula (Wang & Chow, 2007) of comparing two proportions below was applied to determine the minimum sample size (n). The expected proportion of the population with urinary TCPy detected and otherwise was 0.92 (92%) and 0.78 (7.8%) respectively. This was based on the FOD of urinary TCPy among urban group reported by Babina *et al.*, (2012). Given a 95% confidence level and 80% power, the minimum sample (n) needed for both groups was **2**.

$$N = (Z_{\alpha/2}+Z_{\beta})2 * (p1(1-p1)+p2(1-p2)) / (p1-p2)_2 \qquad (1)$$

where $Z_{\alpha/2}$ value is 1.96 and while value for Z_{β} is 0.84. P1 represents the expected sample proportion of exposed group while P2 represents non-exposed group.

5.2.2 Subject Recruitment and ethics approval

The subject recruitment and ethics approval protocol were described in Section 3.9.3. As previously mentioned in Section 3.9.3, convenience sampling was used to recruit participants in this study. Briefly, this study received ethical approval from the Southern Adelaide Clinical Human Research Ethics Committee (Reference number: 291.15 - HREC/15/SAC/248). In addition, approval was sought from the South Australian Department for Education because this is a requirement set by the department when conducting research and evaluation with staff and students from kindergartens, child care centres and schools. Before working with children, a Child Related Employment Screening by the Department of Communities and Social Inclusion (DSCI) was obtained for the researchers as well. Finally, a police clearance was also obtained for the researcher that was going to meet with participants at home.

Following securing all relevant approvals, contacts were made with the principals and directors of schools and kindergartens with phone calls providing a brief introduction of the research. Some schools' leadership gave a positive response while some declined to permit us to approach the school communities. Also, information flyers were also distributed at public places such as at universities and public libraries. Any individuals or families who expressed interests to participate were briefed on the objectives and methodology of the research and once they agreed to participate, an appointment was made for interview and sample collection. In addition, possible candidates were approached through pest control businesses, orchards and agricultural workers associations/non-governmental organizations and research institute since January 2016. Contact information was obtained from Yellow Pages directory for pest control business, schools and orchards. Calls and emails were sent to organizations, schools and businesses. Pest control businesses and orchards were first approached with phone calls.

The description of the "urban" population in this study matched with the urban population described in Babina *et al.* (2012) geographically. However, unlike the study by Babina *et al.* (2012), this study aimed to focus on adult/child pairs from the same household. This was because this research was part of the study that investigates para-occupational exposure among the family members of workers. Therefore, the population of this research consist of a mixture of adult and children. Participants were fully informed of the background of the research and written consent was provided to research if they agree to participate (APPENDIX 1). Children's consent was obtained from their parents/guardians.

5.2.3 Sample collection

Urine sample collection took place from July 2017 until May 2018. The first morning void urine samples were collected by the study participants and then handed to the researcher. During collection, label code, date and time were noted down by the researcher. Urine samples were placed in a cooler box and quickly transported to the lab and stored at -20◦C in a freezer.

Adult participants also had an interview with a researcher for a survey questionnaire. Questionnaires consisted of demographic information and questions about pesticide use at home. Pesticide handlers were also asked on personal protective equipment (PPE) used at work, safety training in handling pesticides, and hygiene practices at work in addition to pesticide use at home. As previously mentioned, because this study was part of para-occupational research, specific data of pesticide handlers specific was not presented.

5.2.4 Laboratory Analysis

The details of the analytical method applied in analyses of urinary TCPy were described in **Chapter 4**. Briefly, this study conducted analyses of TCPy metabolite in urine to measure the exposure to CPF among the population. TCPy metabolite is specific to CPF and TCPy levels in this study were to be comparable with TCPy levels measured in the urban group of the study population conducted in 2003-2006 (Babina *et al.*, 2012). Urine was chosen as the matrix because TCPy is excreted 90% of the administered dose (ASTDR, 1997) and also because it is relatively convenient for participants to provide urine samples other than blood or any other biological samples.

5.2.5 Data analysis

Stem-and-leaf plot was used to display the basic statistic of data set visually. The geometric mean of free TCPy concentration was not calculated because the frequency of detection was less than 60% (Clune, Ryan & Barr, 2012).

5.2.6 Estimation of Total TCPy

As mentioned in **Chapter 4**, the urine samples were analysed for the presence of free (unconjugated) TCPy only. In vivo studies showed that TCPy excreted in the urine in rats consists of 80% of glucuronide-TCPy and only 12% of free TCPy (Bakke & Price, 1976). To estimate the total TCPy in this study, free TCPy detected was assumed to be 12% of the total TCPy. In addition, non -detected free TCPy were also assumed to not have conjugated urinary

TCPy. In other words, we assumed that non-detected free TCPy meant that there were no TCPy detected in urine. The estimated total TCPy level is to be compared with levels of TCPy among the urban population in Babina *et al.* (2012). Babina *et al.* (2012) presented creatinine adjusted TCPy levels (in microgram TCPy per gram creatinine: µg/g). We estimated creatine adjusted total TCPy for this study using age- and gender-specific population median creatinine levels published by Adeli *et al.*, (2015) and presented in Table 33 and Table 34. Finally, the frequency of detection (FOD) will also be compared with the FOD of TCPy detection reported for 2003-2006 study (Babina *et al.*, 2012; Babina, 2007). It is important to note that both methods had different LODs (limit of detection) and thus there were no statistical comparison done to test the differences of both analytical methods developed. The comparison presented here is of semi-qualitative rather than quantitative nature. Finally, as CPF metabolizes rapidly in the human body, the presence of TCPy metabolites in urine may indicate recent exposure.

Table 33 Creatinine levels from Canadian Health Survey for Male from Adeli et al. (2015)

Age	Lower limit mg/dL	Upper limit mg/dL	Median mg/dL
3-5	14.7	151.6	63.3
6-11	13.6	195.7	86.0
12-13	21.5	214.9	124.4
14-29	19.2	305.4	147.1
30-79	14.7	294.1	134.6

Table 34 Creatinine levels from Canadian Health Survey for female from Adeli et al.(2015)

Age	Lower limit mg/dL	Upper limit mg/dL	Median mg/dL
3-5	14.7	151.6	63.3
6-11	13.6	195.7	86.0
12-13	21.5	214.9	124.4
14-29	19.2	305.4	147.1
30-79	12.4	229.6	79.2

5.3 Results

The subject recruitment was conducted as in Section 5.2.2. Some parents were very interested in having their children participate in this research because they were very keen to find out the levels of pesticides in their children only. However, their children ended up not being part of the research subject because they thought that participation of children only without the parents will not be accepted. There were also candidates that thought that handing their urine samples to researchers may involve exposing their personal health status (for example kidney function) and decline to participate on the grounds of concern for their biometric privacy.

Some business representatives refused to convey information to workers by stating *"not interested"* and *"we are currently very busy".* There were no research subjects recruited from pest control businesses and agricultural workers' association/NGO. Support from schools, child care centres and kindergartens was limited too.

Recruitment from orchards and research institute yielded 23 participants, while 55 participants came from kindergartens, flyer distributions and existing participants' network. The final study population comprised of 55 adults and 23 children from 32 urban SA households (Table 35). According to the calculation of minimum sample needed in Section 5.2.1, we managed to get more than 2 samples needed to do the analysis needed. There were nine samples that either did not get to analysed due to low sample volume or questionable sample quality.

There were 33 samples from households that at least had one agricultural worker/researcher. There were six participants who worked as pesticide handlers who reported that their work does not involve CPF, CPF-methyl and triclorpyr. The remaining of the adults in the study population reported not using pesticide at work. 21 out of 33 households reported 'use of pesticides' either indoor or outdoor. The reported 'pesticide use' in our study population included indoor and outdoor pest spray, head lice shampoo, products used for pets, herbicides for garden, ant killer, mosquito coil, sulphur and copper-based pesticides and spider spray. None of the participants in this study reported that they were using CPF, CPF-methyl nor triclopyr at home or at work. Therefore, the presence of TCPy in their urine samples indicated that they may be exposed to CPF from food or the wider environment.

Table 35 Demographic data of the study population

Number of Subject	Adult	55
	Child	23
Mean Age ± SD	Adult	40.7 ± 11.6
	Child	5.9 ± 2.5
Gender of Adults	Female	28
	Male	27
Gender of Children	Female	10
	Male	13
Reported use of pesticides at home (from 33 households)		63%

5.3.1 Analysis of level of free TCPy in urine of SA population and estimation of total TCPy

Stem-and-leaf plot revealed there were two outliers of urinary TCPy levels data with concentrations of 146.83 µg/g and 596.32 µg/g (Figure 13). These two outliers were two times and eight times higher than the next value, respectively.

0	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0.75
1	4.82 7.40 9.27
2	2.43 3.39 4.04 6.29 7.77
3	1.82 2.62 3.91 6.24 7.00
4	
5	0.00 3.17 8.50
6	9.53
7	
8	
9	
10	
11	
12	
13	
14	6.83
….	
59	6.32

Figure 13 Stem and leaf plot of urinary TCPy level (µg/g), n =69, Leaf unit = 1.00

Free TCPy were detected in 18 out of 67 analysed samples and 17 of them were above the limit of detection (LOD). Overall, the concentration of free TCPy in this urine sample was ranged from 0 – 0.0094 µg/ml (Table 36).

Table 36 TCPy levels from this study and Babina (2012)

	% above LOD	Range	P50	P75	P95
Free TCPy[1] (µg /mL)	25%	0 - 0.009	0	0.001	0.005
Total TCPy[2] (µg/g)	25%	0 - 69.53	0	12.288	46.088
TCPy levels in 2003-2006 of urban population[3] (µg/g)	92.2%	< LOD- 217.9	12.5	24.8	71.1

1- Analysis of free urinary TCPy with GCMS method in Chapter 4
2- Estimation of total urinary TCPy (Section 5.2.6)
3- TCPy level in urine in 2003-2006 study (Babina, 2007; Babina *et al.*, 2012).

5.4 Discussion

The levels of free urinary TCPy as a biomarker of CPF exposure were presented in this chapter. CPF is an active substance that is currently undergoing a chemical reconsideration process in Australia at the time of this thesis is written. In the earliest stages of this on-going reconsideration process, several interim regulatory measures were introduced (Table 21) to control the exposure of CPF among the general population in Australia. The estimation of CPF exposure among the Australian population should be vital to the decision-making process during the review. Our study appears to be the only one attempted to explore how the exposure may have changed. To our knowledge, no attempts have been made to conduct monitoring of the population exposure dynamics in the 18 years since the regulatory measures were introduced in Australia.

There were two data points with values two times and four times higher than the next point. The outliers were identified as 4-year-old female and male respectively. Unfortunately, there was not any further investigation done to ascertain the possible other factors that might have

caused higher levels of TCPy than the rest of the datasets. The adults who live in the same households had non-detected urinary TCPy. It is generally known that children have different behaviour and physiology than adult thus pathway and magnitude of exposure are different. However, on closer look, both outliers were considered to be analytical artefacts and were excluded from further data analysis.

While some research subjects in this study reported using pesticides at work, none of them reported using CPF specifically. As such, the study population is considered to be non-occupational. As previously mentioned, non-occupational populations in Australia are likely to be exposed to CPF through diet and environment.

Creatinine levels, urine volume, genetic polymorphism, clinical disease, medication, alcohol use, nutritional status (National Research Council, 2006) and day-to-day occupation/activities are the variables that may need to be accounted for when doing an interpretation of biomarker levels among individuals in a biomonitoring study. The concentration of urine among individuals varied and this may be a source of misinterpretation of biomarker output on each individual. The urinary metabolite measurement should, therefore, be standardised for each urine sample as possible to minimise the effect of urine dilution across the study group. The first step is to ensure that the urine samples are collected in the same manner by all subjects. In this study, all participants were asked to provide first-morning void sample.

The results of urine metabolite measurement in biomonitoring studies are often expressed as per gram of creatinine. This is because the creatinine level itself or creatinine excretion rate can be a source of variability (Barr *et al.*, 2005). Children and the elderly excreted less creatinine and also has a lower output of urine (National Research Council, 2006). Ideally, the

levels of the biomarker are better adjusted with the individuals own level of creatinine. However, limited resourcing available to this study precluded us from conducting creatinine measurements for each sample. The levels of TCPy in this study were corrected mathematically using age and gender stratified population-based mean creatinine levels reported by the Canadian Health Survey (Adeli *et al.,* 2015).

Other than age group, race/ethnicity and sex are also variables in creatinine excretion rate. Genetic polymorphism, clinical disease, medication, alcohol use and nutritional status are among other variables that could have been used to analyse the variability of the TCPy level in urine among the population in this study.

5.4.1 Comparison of this study with children exposure study in South Australia (year 2003-2006).

Babina *et al.*, (2012) measured TCPy and other metabolites in children age 2.5-6 years old living in urban, peri-urban and rural of South Australia in the year 2003-2006. This was when the implementation of the interim regulatory measures (Table 21) at the earliest stage. Personal communication with the author confirmed that a wide range of CPF-based products were sold to the public for use inside homes and in the gardens at the time (Figure 14 and Figure 15). However, it is important to note that the population of the previous study is not equivalent to this study. The target population in the 2003-2006 study was children aged 2.5- 6 years old (n=115) while this study only involved 23 children out of 78 samples. In the same environmental setting, children have more potential sources and different level of pesticides exposure than the adult. Children spend more time closer to ground and CPF tend to be

distributed on the lower ground because it is heavier than air. Moreover, children have a higher breathing rate than adults and thus pesticide exposure through the inhalation route is greater. Therefore, it could be expected that children's biomarker level can be expected to be higher.

The method applied by Babina *et al.* (2012) was different from our method (Table 37). This is because of the absence of a significant step of hydrolysis to isolate the conjugated TCPy from urine before extraction. Babina *et al.* (2012) used β-glucuronidaze type H-1 to release glucuronide and/or sulfate conjugated TCPy. The methods also differed in the analytical instrument employed. We used GC/MS while Babina *et al.* (2012) used LC/MS-MS. Analysis of TCPy with GCMS requires derivatization to increase thermal stability and volatility for better separation and hence, better detection. There may be some part of the compound that did not get derivatized and left undetected. On the other hand, Babina *et al.*, (2012) applied a more sensitive method (LOD = 0.2 µg/L) using LC/MS-MS system. With GC/MS analysis, this study has a less sensitive method (LOD = 1.67 µg/mL). The FOD is usually increased with better (lower) detection limit because of the ability to detect lower concentration. This could be the reason that this study has a lower FOD. However, if we focusing on P95 alone, the concentration of TCPy urinary of Babina *et al.*, (2012) is almost as twice as the value of P95 in this study. Furthermore, it is important to note that despite the lower sensitivity of the analytical method, the level of TCPy analysis in this study is still lower than what reported by Babina *et al.*, (2012). This demonstrated that the population of this study may, in fact, have lower exposure to CPF than the population in Babina *et al.*, (2012) study.

Although the results of free urinary TCPy alone in this research are not enough to categorically to confirm whether the interim measures successfully reduced the exposure of CPF exposure among the said population but the restriction of CPF use among the public might have been

one of the determinants of lower the exposure of this pesticide of the Australian public. With limitations to the analytical method (as presented in detail in **Chapter 4**) and extrapolation was done to our data (estimated total TCPy and creatinine correction), this may be an indication that perhaps the CPF exposure of the population residing in metropolitan South Australia is declining.

Table 37 Comparison between the method applied in this study and Babina et al. (2012)

	This study	**Babina (2007)**
Instrument	GC-MS	LC-MS/MS
Extraction	QuEChERS	SPE
LOD	1.67 ug/L	0.2 ug/L
Isolation process	present	absent

Figure 14 Chlorpyrifos were still sold at store for residential and garden use back in 2006 (1). Image photographed by Kateryna Babina in 2006 (reproduced with permission).

Figure 15 Chlorpyrifos were still sold at store for residential and garden use back in 2006 (2). Image photographed by Kateryna Babina in 2006 (reproduced with permission).

5.4.2 Restrictions of CPF in USA and its impact on TCPy urinary level

Other than Australia, CPF in the US also has similar restrictions on use. There is no nation other than the US who has been doing systematic and routine biomonitoring studies on CPF exposure. Therefore, the result of this study should also be compared with CPF exposure biomonitoring trends in the US.

CPF (and diazinon) was voluntarily removed by registrants from residential settings except for some product with child-resistant packaging as part of the *Food Quality Protection Act (FQPA)*

requirement (US EPA 2018). Correspondingly, CPF usage in all market has been declined from 11-16 million pounds in the year 2001 (Grube *et al.*, 2011, p.17) to 5-8 million pounds in the year 2012 (Atwood and Paisley-Jones, 2017 p. 18).

National Health and Nutrition Examination Survey (NHANES) documented urinary biomonitoring data of multiple pesticides including TCPy to estimate exposure of CPF and CPF-methyl among the population of the US (CDC, 2019). The accessible data for TCPy urinary level was before the restriction (the year 1999-2000 and 2001- 2002) and after 7 years of restriction (the year 2007-2008 and 2009-2010) (Table 38). When comparing the data between the pre- and post- FQPA 1996, the FODs demonstrated that there were still widespread of CPF exposure among the US population. However, the decline of GM throughout suggests that the exposure among CPF or CPF-methyl indeed decreased. The value of 95th percentile after 7 years has lessened two times than before too.

The FOD of the NHANES results of pre and post restriction is not decreasing over time. This may have been caused by the unequal analytical methodology of pre and post of restriction era. The LODs, particularly of the later has decreased and thus the FODs is expected to increased. For this reason, the FOD does not drop as expected despite the reduction of CPF use. Furthermore, the sample preparation when analysing sample with GC-MS/MS (for pre-restriction era) involved derivatization. This process of forming volatile compound to suits GC-MS condition may have caused variations as in this study. On the other hand, the process of sample preparation before injecting into HPLC-MS (for post restriction era) is more efficient as it is simpler. In brief, the method of analysis in the previous two surveys is not as sensitive as the other. Hence, there might be an underestimation of the frequency of urinary level of TCPy detected.

There is no clear breakdown on the proportion of the NHANES 1999 and 2001 sample population as to occupational exposure. In 2007 and 2009, only 1 and 2 percent of the sample population respectively worked in agriculture, forestry and fishing industry but there is no information on what the proportion of respondents worked in pest control industry. Thus, we could not fully conclude if the sample population in each year of the NHANES study is equal or if the occupation had contributed the percentage of the FODs. To conclude, there may be a declined of CPF exposure with the median but with the more sensitive and efficient analytical method the FODs are increased. To sum up, the restriction and ban of CPF in the US have seemingly reduced the exposure of CPF as shown in TCPy levels in the NHANES study and this is consistent with our study.

Table 38 NHANES data taken from Fourth National Report on Human Exposure to Environmental Chemicals (CDC 2019)

Survey	**Range of TCPy concentration (ug/L)**	**GM µg/L**	**Range of age**	**%FOD**	**Pesticide use at home (%)**	**Agricultural worker (%)**	**Analytical method - LOD**
NHANES 1999-2000	0.28-180	1.77	6-59 years	87%	22.9%	Not shared	GC-MS/MS LOD- 1ug/L
NHANES 2001-2002	0.28 -79.59	1.72	6-150 years	75%	18%	Not shared	GC-MS/MS LOD- 1ug/L
NHANES 2007-2008	0.07 - 98.15	1.29	6-150 years	88.6%	10.2%	1%	SPE HPLC Heated Electrospray Ionization Tandem MS LOD of TCPy: 0.0001ug/ml
NHANES 2009-2010	0.07 to 26.1	0.779	6-150 years	80.4%	8.7%	2.3%	SPE HPLC Heated Electrospray Ionization Tandem MS LOD of TCPy: 0.0001ug/ml

In conclusion, the presented study appears to be the only one to attempt exploring how the exposure may have changed since the introduction of the interim regulatory measures during the reconsideration process of CPF in Australia. To our knowledge, no attempts have been reported by the regulatory authorities or other researchers to monitor the dynamics of CPF exposure in the 18 years since the regulatory measures were introduced in Australia. Our study suggests that the extent of exposure to CPF among the general population in metropolitan (urban) settings in South Australia has declined since the introduction of the interim regulatory measures, which restricted CPF use at home/garden. However, this interpretation was done with a few assumptions applied. In future studies, it is suggested that the analytical method is developed without derivatization (with LCMS as an example) and the addition of isolation (de-conjugation) process.

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CHAPTER 6: GENERAL DISCUSSION

Pesticide regulations are shown to be effective in controlling pesticide exposure to the population and pesticide use. However, initial review of Australian pesticide regulations revealed that 1) the Australian Total Diet Study (a national pesticide residue, contaminants and other substances monitoring in food conducted by FSANZ) does not include pesticides frequently (with the last one happening when in the year 2011 at the time this thesis was written), 2) there are some pesticides approved in Australia that are no longer approved in the European Union (as presented in Table 2) and 3) there are no systematic routine biomonitoring studies investigating pesticide exposure among the general Australian public.

With this, the objectives of this study were set as the following:

1. To review the Australian pesticides regulatory framework and compare and contrast the Australian system with the European Union system.
2. To do a review of chlorpyrifos and its status in Australia including exploring the availability of the data on the extent of CPF exposure in the Australian population.
3. To select a biomarker for CPF exposure assessment.
4. To develop an analytical method to measure the selected biomarker (TCPy) in urine samples.
5. To compare the exposure of CPF in this research with the analysis done during the earliest stage of the implementation of the restriction on CPF use among the public.
6. To discuss the effectiveness of the Australian regulatory approach in controlling exposure to CPF.

Objective 1: To do a review of Australian pesticides regulatory framework and compare and contrast the Australian system with the European Union system.

With the lack of research done to measure the effectiveness of Australian pesticide regulatory system in protecting the public from pesticide exposure, an attempt was made to compare the Australian and European Union systems. Comparisons were made based on the review of publicly available information published on-line by the relevant authorities in Australia and the EU and on the available peer-reviewed literature **(Chapter 2)**. In brief, EU pesticide regulations apply a more stringent approach in the assessment to authorize a pesticide. Pesticides that are hazardous (according to Annex II) is not approved and will not be assessed in the first place. While in Australia, pesticides will be assessed based on their risks – the likelihood of being exposed to the pesticides and the potential of effects of exposure to the pesticides. In the EU, renewal/reconsideration is required after 10 years of registration. In contrast, the reconsideration process of a chemical is not prescribed at certain time intervals in Australia. Rather, reconsideration is only initiated when new scientific information on a registered chemical causes changes in other jurisdictions or causes enough public concern. At this point, the regulator, APVMA, initiates a review process (reconsideration) to ascertain whether to consider the new scientific information as an addition to the existing known risks to human health, environment, crops or trade (APVMA, 2017d).

As for pesticide residues in food or produce, monitoring done by FSANZ through ATDS (FSANZ, 2012) is not undertaken routinely and systematically. The last time pesticides were included as one of the contaminants tested for in food was in 2011. They were overlooked in the latest ATDS (24th Australian Total Diet Study) conducted across 2014-2016 (FSANZ, 2014). Although it appears that the states and territories are responsible for monitoring and enforcement of level of pesticides in food are within acceptable limit (FSANZ, 2012), there is

only one publicly available report of monitoring done by the states and territories prepared by Western Australia (Department of Health Government of Western Australia, 2015). There were no other reports available from the remaining states and territories. The "National Residue Survey" conducted by Department of Agriculture and Water Resources does food testing in some (not all) commodities, however this survey is undertaken for trade purposes rather than for health protection (Department of Agriculture and Water Resources, 2019). In 2018-19 survey, only 31 plant products (26 grains and 5 horticulture) and 19 animal products were surveyed for various agvet chemicals, including CPF (Department of Agriculture Australian Government, 2019). The compliance rate was high ranged from 88-100% but there are many more plant products were not tested for pesticide residue (Department of Agriculture Australian Government, 2019). To sum up, the exposure of Australian general population to pesticide (CPF especially) is not known because of the lack of monitoring program/studies.

Objective 2*: *To do a review of chlorpyrifos and its status in Australia including exploring the availability of the data on the extent of CPF exposure in the Australian population.

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Literature review revealed that the toxicity of CPF is beyond acetylcholinesterase inhibition. The exposure to CPF is associated with neurodevelopmental delay (Rauh et al., 2006, 2011, 2015; Horton et al., 2012; Fortenberry et al., 2014; Shelton et al., 2015), birth outcomes/effects (Perera et al., 2003; Whyatt et al., 2004; Barr et al., 2010; Berkowitz et al., 2004; Eskenazi et al., 2004; Rauch et al., 2012; Harley et al., 2011; Wang et al., 2012; Wolff et al., 2007), cancer (Alavanja et al., 2003; Alavanja, Hoppin & Kamel, 2004; Lee et al., 2004, 2007, 2005; Zhang et al., 2015; Lerro et al., 2015), endocrine disruptor (Ventura et al., 2016; *reviewed in* Rodgers et al., 2018), sperm quality and male reproductive toxicity (Perry, 2008; Martenies & Perry, 2013; Meeker *et al.*, 2004b, 2004a, 2006; Perry *et al.*, 2007) (**reviewed in Chapter 3**). For many reasons, CPF is also banned and restricted in several countries (**Chapter 3**). In Australia,

CPF was first nominated to be in the reconsideration process since the year 1995. The reconsideration process is still on-going to date since the commencement of the review in 1996 (APVMA, 2019). As previously mentioned, during this reconsideration process, there were several interim regulatory measures introduced in the effort to control the exposure of CPF among the public (Table 21). However, no data that confirm that this effort has been effective in reducing the exposure to CPF among the public. This is because there are not enough CPF biomonitoring studies conducted to investigate pesticide exposure in Australia. There are, however, very few published CPF biomonitoring studies conducted among Queensland population (in the year 2012/13 for Heffernan *et al.*, 2016; in the year 2014/15 for Li *et al.*, 2019), South Australia (in 2003-2006 for Babina *et al.*, 2012), and termite control workers (Cattani, 2004). The biomonitoring studies conducted for the general population suggested that the Australian population has higher levels of TCPy metabolites than the US (Babina, 2007; Heffernan *et al.*, 2016; Li *et al.*, 2019) , Spain (Heffernan *et al.*, 2016) and Canada (Li *et al.*, 2019) (refer to Table 18 in **Chapter 3**). While the biomonitoring study conducted for termite workers in Western Australia suggested that the range of levels of TCPy urinary levels 'clearly' showed exposure to chlorpyrifos in the workers and this might be attributed to the work practices and use of control measures (Cattani, 2004). However, these few studies could not validate the trends and pattern to CPF exposure among the Australian public.

Objective 3: To select a biomarker for CPF exposure assessment

Biomonitoring is one of a way to assess human exposure that is by measuring biomarker of CPF exposure in biological tissue. Urine is a non-invasive sample that suits all population (children or adult). Collecting urine samples may encourage participation instead of having

have to withdraw an amount of blood from potential research subject, especially children. Urine sample collection is convenient to both researcher and research subject because it can be independently done by the subject. This is contrary to blood withdrawal where only appropriately trained personnel are able to perform the sample collection.

With urine selected as the biological matrix, TCPy was the biomarker of exposure selected for this research because it is the major compound-specific metabolite of CPF (**Chapter 5**). In many other studies of estimating CPF, urinary TCPy has been the most popular biomarker of CPF exposure among pregnant woman (Fortenberry *et al.*, 2014), occupational (Rodríguez *et al.*, 2006) and non-occupational group (Koch, Hardt & Angerer, 2001). To estimate CPF exposure, biomonitoring was chosen where TCPy were analysed in urine (**Chapter 4**).

Objective 4: To develop an analytical method to measure the selected biomarker (TCPy) in urine samples.

An analytical method was developed to analyse TCPy in urine. The method used QuEChERS as extraction technique in conjunction with GCMS for separation and detection (**Chapter 4**). Ideally, an LCMS system would have been used, as TCPy is not particularly suited for GCMS analysis, however, LCMS was not available for the study. Thus a derivatization step was incorporated to convert the TCPy into a more volatile derivative (TBDMS-TCPy) for analysis. The method was capable of analysing free (TCPy) in urine, but did not characterize phase II metabolized TCPy (eg TCPy-glucuronide). In terms of this study, the LOD of this study is more than two times higher than most studies. Whilst the detection limit was higher than some studies, it was sufficiently low enough to detect TCPy at the levels expected in based on prior work reported by Babina (2007).

In this study, the level of free TCPy measured is likely to underestimate the total TCPy (including metabolites). To address this, we estimated the total TCPy (*i.e*. free + metabolite) of the population of this study by taking into account literature data on TCPy metabolism in animals as human data was not available. We extrapolated our data based on two assumptions: (1) total TCPy level in urine were adjusted on the assumption the free TCPy level in this study is 12% of total TCPy in urine as demonstrated in animal study (Bakke & Price, 1976); (2) urine samples that had non-detected free TCPy did not have TCPy levels above detection limits. Future work would also conduct creatinine analysis, to allow for variation in urine concentration. This study took this into account by applying a correction (urine dilution adjustment) using median age and gender-specific creatinine levels reported in the Canadian Health Measures Survey (Adeli *et al.*, 2015) as there was no creatinine analysis done for the samples in this study.

With all the limitation of the developed method, interpretation of results must be done with caution. Thus we have focused on the number of detections rather than absolute concentrations. Results can then be compared with prior work.

Objective 5: To compare the exposure of CPF in this research with the analysis done during the earliest stage of the implementation of CPF use restriction among the public.

With these premises, the frequency of detection (FOD) of urinary TCPy among urban population of children and adult in SA is lower by 76% than the previous study in 2003-2006 when the regulatory interim measures were first introduced (Babina, 2007; Babina *et al.*, 2012). The median level of this study was 0 µg/g (non-detected) while in Babina (2007) the median level was 12.5 µg/g. This apparent decline in exposure levels can reasonably be attributed to the restrictions applied to the CPF use in the home as part of the APVMA's reconsideration

process of CPF. The Australian restrictions were similar to the restriction introduced in the US in 1999-2000. From the national biomonitoring study (NHANES) data, the level of TCPy (GM) among the US population has declined 50% in 2009-2010 compared to the urinary TCPy levels in the US population before the residential use of CPF was restricted in the US (in 1999-2000) (CDC, 2019).

Objective 6: To discuss the effectiveness of the Australian regulatory approach in controlling exposure to CPF.

Overall, this study demonstrated that there is a decline of CPF exposure of urban SA population in 2017/18 compared to the 2003-2006 study. The Australian population is still exposed to CPF as it is still used extensively in agriculture and the main pathway of exposure in the general population is likely via CPF residue in food. Besides, CPF is still applied for termite control in residential settings in pre- and post-construction.

The regulatory decision to ban access of the public to high concentration CPF products (more than 50g/L) seemingly have reduced the exposure among the urban population in SA. The direct quote from the first report published from the CPF reconsideration process (*The NRA Review of Chlorpyrifos*),*"*It is generally regarded that liquid formulations containing chlorpyrifos at 50g/L are acceptable in terms of their compliance with the NRA guidelines*"* (NRA, 2000). With this, high concentration CPF products (more than 50g/L) is not accessible to the public anymore although there are other sources of CPF exposure from the environment. Today, the urban population still can still buy CPF products containing concentrations less than 50g/L, however, when the researcher went to some supermarkets and hardware stores in South Australia, the products are rarely seen on the shop shelves.

CONCLUSIONS

This research aimed to investigate exposure to CPF among a sample of urban population in South Australia after the implementation of interim regulatory measures that were introduced during the CPF reconsideration process (Table 21).

1. Based on the biomonitoring studies conducted in investigating the CPF exposure of an urban population in South Australia and despite the limitation presented to the analytical method and the extrapolation of data done, it can be concluded that CPF exposure among this population has decreased after 18 years implementation of the interim of regulatory measures that were introduced for public health.

2. Based on findings in (1), it may be possible that the restriction of pesticide use applied 18 years back for public health has contributed to decreasing the exposure to CPF among the urban population in South Australia. This raises questions on what is the level to CPF exposure of populations in other states.

3. Based on the review conducted for the Australian pesticide regulatory system, there are two main findings that this research would like to highlight which are a) the absence of systematic pesticide residue monitoring in food and b) the absence of national biomonitoring program conducted routinely to investigate the Australian public exposure to pesticides.

The findings of this study are valuable in investigating the effectiveness of Australian pesticide regulatory measures as well as Australian pesticide policy as a whole. It also offers a fraction

of information needed to understand the trends and pattern of pesticide exposure of the whole Australian population.

LIMITATION OF THIS STUDY

This study only took one sample from each participant. Kissel *et al.*, (2005) however, demonstrated that the first morning void urine is the best predictor of estimated total daily excretion (for TCPy) compare to urine sample taken other time of day. CPF is a compound that rapidly metabolized thus any levels detected is an indication of recent exposure. There is no information on the variability intra or inter individual of this population because of the single sample collection. The trends of CPF exposure cannot be inferred because of the single sample collection together with limitations of the analytical method applied.

As previously mentioned in **Chapter 3**, CPF also degraded into TCPy in the environment. TCPy levels are found in food samples (Morgan *et al.*, 2005), raw and cook vegetables (Randhawa *et al.*, 2007) and indoor dust, soil, indoor air and outdoor air (Wilson *et al.*, 2003). Wilson *et al.*, (2003) suggested TCPy in food may suffice to produce detectable level for urinary biomarker. Thus, the TCPy levels in urine may not only come from CPF absorption but also directly from the exposure to preformed TCPy from several environmental media. In future studies, we may want to measure the TCPy level in the subjects' immediate environment too.

Finally, the sample size of this study is small and limited to urban South Australia population. This limited our ability to predict the effectiveness of the interim regulatory measures implemented for CPF chemical review for the whole Australian population. There was another study conducted in Brisbane, Queensland of urinary pesticide metabolites including urinary TCPy of the population. However, comparison cannot be made with this study because the

reported results were presented in different units (ng/mL) It is also inferred that the exposure of both populations is vastly different. APVMA pesticide database 118 (PUBCRIS)[4] revealed that Queensland has more products (five) registered for use than in South Australia. This may or not explain the higher usage of CPF in Queensland than in South Australia and the potential of dietary exposure among the population. These five products are approved for use for cotton, banana, sugarcane, maize, sorghum, and sunflower. Banana, sugarcane, and cotton are grown in Queensland only, not in South Australia. Moreover, APVMA is only overseeing the regulation of agvet chemicals up to and including the point of retail sale. The state and territory government assumed to take control over monitoring the use of these chemicals after retail activity. Each state may have a different approach to control the usage of pesticides. In practice, at a state level, there is no record keeping of total volumes purchased against volumes used and the extent of pesticide use is unknown. The climate conditions in South Australia and Queensland are different. Queensland may have a different level of pest infestations and subsequently varies the usage of pesticides and the crops to protect as well. To sum up, we cannot assume the same level of exposure among these two population in these two different states or even other states in Australia.

Although there was not any creatinine correction done, Heffernan *et al.*, (2016) reported that after 10 years of regulatory restriction of CPF, urinary TCPy level of pooled sample in Brisbane, Queensland were almost the same as what reported in SA as in what reported in year 2003-2006 (Babina, 2007; Babina *et al.*, 2012). It is difficult to understand this scenario as there is not any pesticides usage record tracked in both states. Moreover, as previously mentioned, there has not been any studies conducted before or the earliest stage of the restriction and therefore we have no baseline data for our population to compare to.

FUTURE STUDIES

Biomonitoring is an important tool to assess the exposure to pesticides and other environmental contaminants in the general population. In Australia, especially, biomonitoring studies of chemical exposures (including CPF) in the general population are not conducted systematically. Hence, there are is not much information on what are the chemicals of the population are exposed to. The level of body burden data has so many uses. For example, knowledge of trends of pesticide exposure among the population can assist in shaping a better pesticide policy.

Biomonitoring studies are connected strongly with analytical methodology. For this study, it is recommended that the developed method is complemented with the addition of the isolation process, whether with acid or enzyme. This step is crucial to liberate conjugated TCPy from urine so that total TCPy excreted from urine can be analysed so the estimation of exposure can be done for the said population.

QuEChERS extraction is a greener and cheaper way to do extract multiple pesticides/chemicals at once. In future, other pesticides/metabolites can be tested to be extracted from urine simultaneously with TCPy. Moreover, realistically, the public are exposed to multiple pesticides at once from multiple sources of food and the environment. Farmworkers sometimes use a type of pesticide to enhance the performance of other pesticides (Okeke, 2018). With simultaneous extraction and analysis method, urine sample obtained can be analysed for different pesticides as well.

There is also a need to study the motivation of healthy Australian to participate in a biomonitoring study or any other research. This information can help researchers to understand a healthy individual who may not gain anything from such research as opposed to non-healthy individuals that participate in a clinical trial that benefited them directly.

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APPENDIX 1

PARTICIPANT INFORMATION SHEET, CONSENT FORM AND QUESTIONNAIRES

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APPENDIX 2

TCPy URINARY CONCENTRATIONS

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