Ecological risk assessment of amphibians in the Phongolo River floodplain

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Ecologische risicobeoordeling van amfibieën in de uiterwaarden van de Phongolo-rivier

ABSTRACT

The Phongolo River floodplain in South Africa hosts the highest floodplain biodiversity in the country, while also being highly utilised for commercial and subsistence agriculture. The floodplain falls within the malaria risk region where vector control in the form of indoor residual spraying is still practised with dichlorodiphenyltrichloroethane (DDT). This region operates on a fragile socio-ecological balance and as such has been the subject of socio-ecological assessments in the past. Previous studies identified a gap in available knowledge on the ecological risk to amphibians within the floodplain. Specific focus was drawn to malaria vector control pesticides and the associated toxicity to amphibians. This study used a tiered assessment approach to generate required data and assess the risk to amphibian well-being in the Phongolo River floodplain. Ecosystem services were incorporated into the assessment alongside the risk to amphibian well-being to assess the relationship between these aspects. The first tier to this study involved identifying the data requirements, which included sub-lethal effects data regarding amphibians and malaria vector control pesticides along with a lack of current field monitoring data of these pesticide in amphibians. The second tier involved generating field monitoring data. In this phase of the study it was concluded that close proximity to spraying sources in the Phongolo River floodplain resulted in amphibians from a conservation region in the floodplain actively accumulating DDT at sub-lethal levels. The next tier in the assessment involved sub-lethal toxicity data generation. This was done through laboratory based acute exposures of *Xenopus laevis* to vector control pesticides measuring behaviour, metabolomics and pesticide accumulation as effect outcomes. Behavioural changes were seen in frogs exposed to a mixture of DDT and deltamethrin. Metabolomic changes were also determined, but were mostly attributed to a general stress response as similar metabolic pathways were affected in all exposures compared to control. The next tier of the study involved a simulated field exposure assessment where pesticide residue accumulation, metabolomic response and aquatic invertebrate community effects were measured. Metabolomic responses had low overlap with those found in laboratory exposures which was attributed to the addition of food sources in the simulated field environment. The mixture exposure of DDT and deltamethrin resulted in massive loss of invertebrate diversity. The final risk assessment incorporated data generated in this study to determine risk levels and current risk impacts on amphibians using a relative risk model. Using source–habitat effect interactions the relative risk to different habitats in the floodplain were determined.

Overall amphibian well-being was at moderate risk, driven by the likelihood of chronic or sub-lethal effects from pesticides and high amphibian biodiversity in the region. Based on both ecosystem service and amphibian well-being risk outcomes, the aquatic habitats in the floodplain (river, temporary pans, and permanent pans) were identified as priority habitats for conservation management and development of protection goals in order to benefit total socio-ecological functioning and maximise amphibian wellbeing management in the process. The outcome of this study supports the use of amphibian health and well-being as a monitoring tool for the Phongolo River floodplain. Long term pesticide monitoring alongside amphibian well-being and biodiversity monitoring will serve as sensitive indicators of ecological change and allow intervention prior to ecosystem service impacts being observed.

Keywords: amphibian ecotoxicology, DDT, deltamethrin, ecosystem services, malaria vector control, metabolomics, pesticides, regional risk assessment, sub-lethal effects

SAMENVATTING

Het overstromingsgebied van de Phongolo-rivier in Zuid-Afrika herbergt de grootste biodiversiteit in dit type ecosysteem van heel het land, terwijl het ook intensief wordt gebruikt voor commerciële- en zelfvoorzieningslandbouw. De uiterwaarden vallen in het malariarisicogebied waar vectoren nog steeds worden bestreden door binnenshuis residueel te sproeien met dichloordifenyltrichloorethaan (DDT). In deze regio heerst een kwetsbaar sociaal-ecologisch evenwicht en heel de regio is als zodanig in het verleden onderworpen aan sociaal-ecologische evaluaties. Eerdere studies brachten een leemte aan het licht in de beschikbare kennis over de ecologische risico's voor amfibieën in de uiterwaarden. Specifieke aandacht ging uit naar bestrijdingsmiddelen tegen malariavectoren en de daarmee samenhangende toxiciteit voor amfibieën. In de huidige studie is gebruik gemaakt van een gefaseerde aanpak om de benodigde gegevens te verzamelen en het risico voor de amfibie-biodiversiteit in de uiterwaarden van de Phongolo-rivier in kaart te brengen. Naast het risico voor de biodiversiteit van amfibieën, werden ook ecosysteemdiensten in de beoordeling betrokken. De eerste fase van deze studie bestond uit het identificeren van de vereiste gegevens, waaronder gegevens over subletale effecten van bestrijdingsmiddelen tegen malariavectoren op amfibieën, en een gebrek aan actuele veldmonitoringgegevens over deze bestrijdingsmiddelen bij amfibieën. Het tweede niveau betrof het genereren van veldmonitoringgegevens. In deze fase van de studie werd geconcludeerd dat de nabijheid van pesticidebronnen in de uiterwaard van de Phongolo ertoe leidde dat amfibieën uit een beschermingsgebied in de uiterwaard actief DDT accumuleerden in subletale hoeveelheden. Het volgende niveau in de beoordeling omvatte het genereren van gegevens over subletale toxiciteit. Dit gebeurde door acute blootstelling van *Xenopus laevis* aan anti-vectoren bestrijdingsmiddelen in het laboratorium, waarbij gedrag, metabolomica en accumulatie van bestrijdingsmiddelen als effectresultaten werden gemeten. Gedragsveranderingen werden waargenomen bij kikkers die waren blootgesteld aan een mengsel van DDT en deltamethrine. Er werden ook metabolomische veranderingen vastgesteld, maar deze werden meestal toegeschreven aan een algemene stressrespons, aangezien vergelijkbare metabolische routes werden beïnvloed bij alle blootstellingen in vergelijking met de controle. De volgende fase van de studie omvatte een gesimuleerde beoordeling van de blootstelling in het veld, waarbij de accumulatie van residuen van bestrijdingsmiddelen, de metabolomische respons en de effecten op de gemeenschap van ongewervelde waterdieren werden gemeten. De metabolomische reacties vertoonden weinig overeenkomst met die welke bij de blootstelling in het laboratorium werden gevonden, hetgeen werd toegeschreven aan de toevoeging van voeding in de gesimuleerde veldomgeving. De blootstelling aan een mengsel van DDT en deltamethrine resulteerde in een massaal verlies van de diversiteit van ongewervelde dieren. In de uiteindelijke

risicobeoordeling werden de gegevens uit deze studie verwerkt om met behulp van een risico model de huidige risico's voor amfibieën te bepalen. Met behulp van bron-habitat-effect interacties werd het relatieve risico voor verschillende habitats in de uiterwaarden bepaald. In het algemeen was het amfibieënwelzijn matig bedreigd en dit door chronische of subletale effecten van bestrijdingsmiddelen en de grote amfibieënbiodiversiteit. Op basis van de uitkomsten van zowel ecosysteemdiensten als welzijn van amfibieën werden de aquatische habitats in de uiterwaarden (rivier, tijdelijke pannen en permanente pannen) geïdentificeerd als prioritaire habitats voor instandhoudingsbeheer en ontwikkeling van beschermingsdoelen om zo het totale sociaalecologische functioneren te bevorderen en daarbij het amfibieënwelzijnsbeheer te maximaliseren. De uitkomst van deze studie ondersteunt het gebruik van de gezondheid en het welzijn van amfibieën als een monitoringinstrument voor de uiterwaarden van de Phongolo-rivier. Langetermijnmonitoring van pesticiden naast het welzijn van amfibieën en monitoring van de biodiversiteit zullen dienen als gevoelige indicatoren van ecologische verandering en zullen interventie mogelijk maken voordat de effecten van ecosysteemdiensten worden waargenomen.

Trefwoorden: amfibieën ecotoxicologie, DDT, deltamethrine, ecosysteemdiensten, malaria vector bestrijding, metabolomics, pesticiden, regionale risicobeoordeling, subletale effecten

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LIST OF ABBREVIATIONS

A — Aquatic (category)
AHR — Aryl hydrocarbon receptor
AMA — Amphibian metamorphosis assay
ANOVA — Analysis of variance
AOP — Adverse outcome pathway
ATSDR — Agency for Toxic Substances and Disease Registry
BM — Body mass
CAS — Chemical Abstracts Service
Cat — Category
CB— Chlorinated biphenyl
CF — Composition factor
CHIETA — Chemical Industries Education & Training Authority
CHLs — Chlodrane, oxychlordane and nonachlor (collective)
CSA — Cockayne syndrome A (gene)
CX — *Chiromantis xerampelina*
DDD — Dichlorodiphenyldichoroethane
DDE — Dichlorodiphenyldichoroethylene
DDT — Dichlorodiphenyltrichloroethane
DDx —DDT,DDD and DDE (collective)
DEA — Department of Environmental Affairs
DFA — Discriminant function analysis
Drins — Aldrin, Dieldrin and Endrin (collective)
DTM — Deltamethrin
EDC — Endocrine disrupting compound
EI — Electron ionization
FC — Fold change
FDR— False discovery rate
FEOW — Freshwater Ecoregions of the World
FETAX — Frog Embryo Teratogenesis Assay-*Xenopus*
GAS — General adaptation syndrome
GC — Gas Chromatography
GC-(µ)ECD — Gas chromatography coupled with a Ni (micro) electron capture detector
GC/TOF-MS — Gas chromatography coupled with Time-Of-Flight mass spectrometry
GC-MS/MS — Gas chromatography coupled with tandem mass spectrometry
GPC — Gel permeation chromatography
HAI — Health assessment index
HCB — Hexachlorbenzene
HCH — Hexachlorocyclohexane
HO — *Hildebrantia ornata*

HPI — Hypothalamus-pituitary-interrenal

HPTs — Heptachlor; Heptachor epoxide (collective)

HSI — Hepatosomatic index

HT — *Hyperoluis tuberlinguis*

IC50 — 50th percentile inhibition concentration

IRS — indoor residual spraying

IS — Internal standard

ITN — Insecticide treated net

IWRM — Integrated Water Resource Management

KEGG — Kyoto Encyclopedia of Genes and Genomes

KNP — Kruger National Park

LAGDA — Larval amphibian growth, and development assay

LC50 — 50th percentile lethal concentration

LC-MS/MS — Liquid chromatography coupled with tandem mass spectrometry

LOD — Limit of detection

LOEC — Lowest-observed-effects concentration

LOQ – Limit of quantitation

MCIG — Minimum concentration to inhibit growth

MFO — Mixed function oxidase

MUTL — Muir-torre syndrome (gene)

MVC malaria vector control

NABF — National Aquatic Bioassay Facility

NAD — Nicotinamide adenine dinucleotide

ND — Not detected

NF — Nieuwkoop-Faber lifestage

NGR — Ndumo Game Reserve

NMDS —Non-metric multi-dimensional scaling

NMP — National Metabolomics Platform

NMR — Nuclear magnetic resonance

NRF — National Research Foundation

NWU — North-West University

OCP — Organochlorine pesticide

OECD — Economic Co-operation and Development

P/D — Parent/daughter ratio

PA — *Ptychadena anchietae*

PCA — Principle component analysis

PCB — Polychlorinated biphenyl

PE — *Pixycephalus edulis*

PI — Pathway impact

PLS-DA — Partial least squares-discriminant analysis

POP — Persistent organic pollutant

PXR — Pregnane X receptor

RDA — Redundancy analyses
RQO — Resource Quality Objective
RR — Risk region
RRA — Regional risk assessment
RRM — Relative risk model
RT — Retention time
S/N — Signal to noise ratio
SA — Semi-aquatic (category)
SG — *Sclerophrys garmani*;
SPHERE — Systemic Physiological and Ecotoxicological Research
SSD — Species sensitivity distribution
ST — Semi-terrestrial (category)
SUR – Survey
TCA — Tricarboxylic acid cycle
TD — Tree dwelling (category)
TDS — Total dissolved solids
TI — Teratogenic index
T*m*X — Tetrachloro-*m*-xylene
UAntwerpen — University of Antwerp
UNEP — United Nations Environment Programme
UV — Ultraviolet
UVB — Ultraviolet-B
VIP — Variable importance in projection
VLIR — Vlaamse Interuniversitaire Raad
WHO — World health Organisation
WRC — Water Research Commission
WRG — Water Research Group
WWF — World Wildlife Fund
XM — *Xenopus muelleri*.
XPA — Xeroderma pigmentosum group A (gene)
XPG — Xeroderma pigmentosum group G (gene)

CHAPTER 1 INTRODUCTION

1.1 Malaria and vector control in South Africa

It is a well-known fact that malaria is the most prolific killer in the world. Caused by infection with *Plasmodium* sp. parasites, it results in a state of periodic fevers and anaemia linked to the life cycle of the parasites in human red blood cells (Nacher 2005; Manguin et al. 2010). Malaria was responsible for 409 000 casualties in 2019 worldwide, with 94% of malaria cases occurring in Africa (WHO 2019). In South Africa, Three of the nine provinces include areas classified as malaria risk regions (Craig et al. 1999). The risk within these regions have decreased over time due to implemented prevention strategies, but periodic increases (e.g. 2016 and 2017) have also shown that malaria risk is highly dependent on climate variables (Kim et al. 2019) and that the fight against this disease is not yet won. Malaria eradication (more precisely, global reduction by 90%) is currently one of the World health Organisation's targets for 2030 (WHO 2015). The most effective methods of malaria spread prevention have always been related to controlling the population of the vector mosquitoes (*Anopheles* sp.) in order to limit the probability of *Plasmodium* sp. transfer between humans. In the early 1930's the use of insecticides as a method for malaria vector control was first introduced in the country by spraying the insides of houses and spraying water bodies for larval control. The first pesticide used for this project was pyrethrum which was replaced by dichlorodiphenyltrichloroethane (DDT) in 1946 after its effectiveness against mosquitoes was shown, and its infrequent spraying cost was lower than weekly pyrethrum requirements (Coetzee et al. 2013). This had an enormous impact on reducing the malaria incidence in South Africa and appeared to be the best method to combat the disease, even enabling previously deserted regions such as the Phongolo River region to be cultivated for the first time (Coetzee et al. 2013).

The fight against malaria continued, but in the 1980's as the secondary environmental effects of DDT started emerging and new synthetic pyrethroids and carbamates were developed,

these newer pesticides started replacing DDT in the vector control program. South Africa first ceased the use of DDT in their malaria vector control (MVC) program in 1995 (Bouwman et al. 2013a). It was initially replaced by pyrethroids. Unfortunately *Anopheles funestus*, built up a genetic resistance to pyrethroids. Consequently pyrethroid resistance along with several factors, such as increased human movement over the Mozambique and Zimbabwe borders and high rainfall, resulted in a dramatic rise in malaria incidence (Coetzee et al. 2013). The rise in malaria cases prompted the reintroduction of DDT in MVC (Bouwman et al. 2013a). Around the same time the Stockholm convention classified DDT and other organochlorine pesticides as persistent organic pollutants (POPs) of which production and use was to be ceased (Bouwman et al. 2013a). South Africa officially made the case for continued DDT use in vector control for indoor residual spraying (IRS) and resulted in this exemption being allowed for all countries under the Stockholm convention. South Africa since has been using DDT for IRS in combination with other recommended MVC pesticides to prevent the build-up of genetic resistance (Coetzee et al. 2013). Apart from DDT as the only organochlorine pesticide, the recommended MVC pesticides belong to the organophosphate, carbamate and pyrethroid chemical groups (detailed description is given in Chapter 2). Although there has been a global increase in the use of organophosphates in vector control in recent years, South Africa mainly uses DDT and pyrethroids. These two are also the pesticide groups most commonly used in simultaneously for IRS (Tangena et al. 2020). Pesticide resistance management for both DDT and pyrethroids remains an important factor in MVC.

1.1.1 MVC pesticides in the environment

There is a continuing debate across the globe on what is referred to as the DDT paradox by Bouwman et al. (2011). On the one side the use of MVC pesticides (not limited to DDT) is saving potentially millions of lives from malaria. On the other hand the introduction of these pesticides into the environment has acute and chronic negative effects on the exposed environment. It has been reported by several sources that even though IRS is considered the environmentally safer option for vector control, these pesticides still transfer into and

accumulate in the environment posing potential risks to humans and wildlife (Bouwman et al. 2012; Bouwman et al. 2013a; Thompson et al. 2017a,b) with especially aquatic ecosystems being vulnerable (Van Dyk et al., 2010; Wepener et al. 2012; Gerber et al. 2016; Viljoen et al. 2016; Pheiffer et al. 2018; Wolmarans et al. 2018; Buah Kwofie et al. 2018b; Volschenk et al. 2019; Gerber et al. 2021).

1.2 The Phongolo River floodplain

Situated in the northern part of the KwaZulu-Natal Province of South Africa within the malaria risk region, the Phongolo River floodplain stretches from the Pongolapoort dam in a northern direction toward the South Africa - Mozambique border where the Phongolo and Usuthu rivers join and flow into Mozambique and eventually into the Indian Ocean at Maputo bay. The Phongolo River floodplain is considered one of South Africa's most important floodplain systems due to its host of biodiversity and utilisation for subsistence and commercial agriculture (Smit et al. 2016). The biodiversity in the floodplain is of significant importance as Ndumo Game Reserve (NGR) inside the floodplain is classified as a "Ramsar site" under the Ramsar wetland convention (DEAT 1996) due to the high biodiversity hosted by the floodplain pan (pans are endorheic wetlands with a flat basin floor that do not have any outlet for water flows) system inside the reserve. Construction of the Pongolapoort Dam in the 1970s resulted in a unique situation. Built to provide irrigation of commercial cropland upstream of the town of Jozini, the dam disrupts the natural annual flooding patterns of the lower Phongolo River and unique flood dependent pans and wetlands in the region. Originally the ecological needs of the floodplain and economic and agricultural opportunities were assessed and an artificial dam release strategy was devised (Breen et al. 1998). Various management challenges have since led to adjusted flooding regimes driven largely by the agricultural needs of the floodplain community (Lankford et al. 2011), leaving the extent of ecosystem functioning in question. Early studies such as Heeg and Breen (1982) indicated the importance of preserving ecosystem services in the floodplain. Large scale irrigation plans conceptualised with construction of the dam never reached development (Van Vuuren 2009) which unwittingly

helped preserve ecosystem functioning in terms of water use, while also resulting in expansion of subsistence cropping around floodplain pans (Lankford et al. 2011). Jaganyi et al. (2008) showed that the artificial releases did not effectively simulate natural floods as peak flows are lower and more consistent with sudden stops instead of gradual declines. While the floodplain remains productive under these conditions all of the aforementioned factors, along with a population increase in the human floodplain community, have led to a shift in the balance between provision and use of ecosystem services. Add to this the constant introduction of pesticides that could have long term effects on wildlife and habitats, and the potential for loss of ecosystem functioning becomes an undeniable concern.

1.3 Amphibians as indicators

Biodiversity is one of the most important aspects of the Phongolo floodplain for South Africa. While famous for its bird and fish diversity, the floodplain also hosts ≈ 30% of the amphibian species native to Southern Africa with 46 species (based on distributions from Phaka et al. 2017; Du Preez and Carruthers 2017). Amphibians are considered to be amongst the most threatened taxa on earth (Monastersky 2014) while also being one of the most sensitive taxa to pollution effects and habitat degradation (WWF 2018). It is therefore essential that threats influencing amphibian population health and fitness are evaluated (Melvin et al. 2016).

Broadly defined, the anuran (most prominent class of amphibians in Southern Africa) life cycle involves aquatic embryo and larval stages, followed by metamorphosis into an adult stage that can range from fully aquatic to fully terrestrial depending on species characteristics (Du Preez and Carruthers 2017). Exceptions to the standard life cycle include frogs with that make use of terrestrial jelly or foam nests for eggs. In such cases the embryos develop into tadpoles in a terrestrial environment with the nest providing moisture for this (technically) aquatic life stage. Nests are usually made on vegetation or structures that cover a waterbody and developed tadpoles are released into the waterbody. Rain frogs (*Breviceps* sp.) defy this rule completely with subterranean jelly nests in which complete embryo development and

metamorphosis takes place with no free swimming tadpole stage and metamorphs emerge from the nest with limbs already developed (Du Preez and Carruthers 2017). Although there is vast species specific variation in anuran life cycles and ecology, the interaction between aquatic and terrestrial habitats throughout their development place them in a unique position in the food web. Tadpoles can be either herbivores or carnivores depending on the species. Adults are moslty predatory and form part of both terrestrial and aquatic systems (Böll et al. 2013) and often feed on invertebrates from both, while also being consumed by higher trophic level predators from both systems. Amphibians have also been shown to contribute to the regulation of ecosystem services (Hocking and Babbitt 2014; detailed description given in section 1.6). These factors all make amphibians one of the best indicators to assess ecosystem changes (Böll et al. 2013). Ecological risk assessments are an effective method of assessing such ecosystem change. The African Clawed Frog (*Xenopus laevis*) is an African frog with a fully aquatic adult lifestage. This is a globally established standard test species for toxicity testing. They are of particular interest in terms of testing teratogenicity of pollutants in aquatic systems. *Xenopus laevis* was selected as amphibian model species for toxicity data generation in this study. Benefits to selecting this species include their ease of use in aquatic exposure scenarios, high dermal pollutant uptake uniquely representative of amphibians, and well established literature base for use in toxicity testing (full description on *X. laevis* as test species is given in Chapter 2).

1.4 Risk Assessment

Ecological risk assessment generally involves quantifying the potential for, and extent of change (or damage) to individuals, populations, or ecosystems due to the influence of one or multiple stressors impacting the assessed environment. It is a very versatile toolset that can incorporate different methodologies. The regional risk assessment (RRA) concept was originally introduced into ecological risk assessment methodology by Landis and Wiegers (1997). This method focuses on interactions between stressor sources and habitats and their importance to both the ecosystem and regional stakeholders. The risk is then measured as

the impact the stressor has on the specific habitat weighted by the importance ranking of the source-habitat-effect interaction within the regional context (Landis and Wiegers 1997). Refined over time the RRA method has become an essential tool in modern ecological risk assessment. O'Brien and Wepener (2012) specified the steps and strategies required for implementation of this assessment method in a South African context linking local policies on integrated water resources management with steps in the relative risk model (RRM) to enable effective implementation of regional risk assessments within South Africa. This opened up opportunity for the implementation of such assessments on the Phongolo River floodplain.

1.5 Ecosystem services

Ecosystem services are defined as interactions between man and the environment from an anthropocentric perspective. In the development of guidelines on environmental risk assessment of pesticides the European Food Safety Authority (ESFSA 2010) and Maltby et al. (2017) make use of ecosystem service classifications as defined by the millenium ecosystem assessment (MEA 2005). This classification consists of four broad categories namely provisioning services, is used in the current study to define ecosystem services within the Phongolo River floodplain for assessment purposes.

Hocking and Babbitt (2014) assessed the contribution of amphibians to ecosystem services on a global scale and found that they play a role in provisioning services through human consumption of frog meat and medical advancements achieved through the use of amphibians. Amphibians contribute to regulating services through predation on disease vectors (mosquitoes in particular). They also have cultural importance, mainly in South America and East Asia. The most important ecosystem service contribution of amphibians was found to be in terms of supporting services through the alteration of ecosystem functioning. These contributions fall within the ecosystem service categories as set out by MEA (2005). In Chapter 6 the specific ecosystem service contributions identified by Hocking

and Babbitt (2014) are categorised into broader groups as they are used in the current study (*see* Table 6.1).

1.6 Ethical research practise

This study in its entirety, including all animal interventions applied, was reviewed and completed under ethical approval by the AnimCare ethics committee of North-West University (ref: NWU-0264-16-A5).

1.7 Rationale, scope, hypothesis, aims and objectives

Smit et al. (2016) implemented a socio-ecological risk assessment on the Phongolo River floodplain to assess, among others, the impact of DDT use for MVC as a potential stressor to the ecosystem. The study involved using fish as ecosystem health indicators and assessing the socio-economic impacts of the effects on fish and fisheries due to stressors in the floodplain. Alongside fish assessments, the well-being of bird, invertebrate, and amphibian communities were also included in the regional risk assessment model for the floodlpain, which was published by O'Brien et al. (2021). The amphibian well-being model used by Smit et al. (2016) and O'Brien et al. (2021) accounts for the influence of pesticide accumulation, habitat suitability, river flow requirements, parasitemia and amphibian biodiversity as influences determining the risk to amphibians in the floodplain. Smit et al. (2016) identified a lack of data supporting the effects of MVC pesticides on amphibians and how the potential effects relate to measurable accumulation of DDT in amphibians. The need was identified for better quality data in this regard as to lower the uncertainty involved in the risk to amphibians assessed in the floodplain.

Stemming from these knowledge gaps identified by Smit et al. (2016) and the great potential for loss of biodiversity, the current study was conceptualized to investigate these amphibian-

pesticide interactions in more detail. Through the development of the current study a need was also identified to assess the risk to ecosystem services in this region based on the MVC pesticide input. Comparing risks posed to amphibians and risks posed to ecosystem services would also broaden the current extent in which amphibians can be used as indicators of environmental changes.

The general hypothesis tested in this study is that MVC pesticide use in the Phongolo River floodplain contributes as the major risk factor to the local amphibian well-being, and that there is a strong correspondence between the risks to amphibian well-being and risks to ecosystem services in the floodplain.

In order to test this hypothesis the following aims were put forward:

- To assess the specific data needs for the implementation of a risk assessment of amphibians in the Phongolo River floodplain with regard to MVC pesticides.
- To generate the necessary toxicological data for amphibians and MVC pesticides required as identified through the first aim.
- To implement a risk assessment on amphibian well-being within the floodplain with regard to MVC pesticide exposure.
- To assess the risk to ecosystem services in the floodplain and compare how these risks relate to risks to amphibian well-being assessed in the third aim.

As the study originated from a lack of data, a tiered approach was taken to the implementation of this risk assessment and meeting the aims set for this study. A tiered approach involves increasing the expanse and complexity of data generation steps based on the necessary requirements and gaps identified at the current data level. Based on this approach the study developed into five different sub-studies each performed as a separate chapter within this thesis (Figure 1.1). Initially the exact lack of data in the current literature regarding amphibian

interactions with MVC pesticides was identified in Chapter 2 through a literature review. Confirmation of the hazard posed by MVC pesticides to amphibians in the Phongolo floodplain was performed in Chapter 3 through a field assessment of pesticide accumulation and comparative study with another MVC area in South Africa. Based on the results of these two studies the next tier involved a laboratory based exposure assessment in Chapter 4 in order to generate data on the sub-lethal effects of these pesticides on amphibians.

Another tier shift in the investigation was prompted by the Chapter 4 results and in Chapter 5 the environmental relevance of the sub-lethal effects identified were tested. Utilising the outcomes of chapters 2 to 5, a risk assessment was then implemented in Chapter 6 as the final study. Chapter 7 summarises the conclusions of the collective study and relevant recommendations are made for implementation in future research.

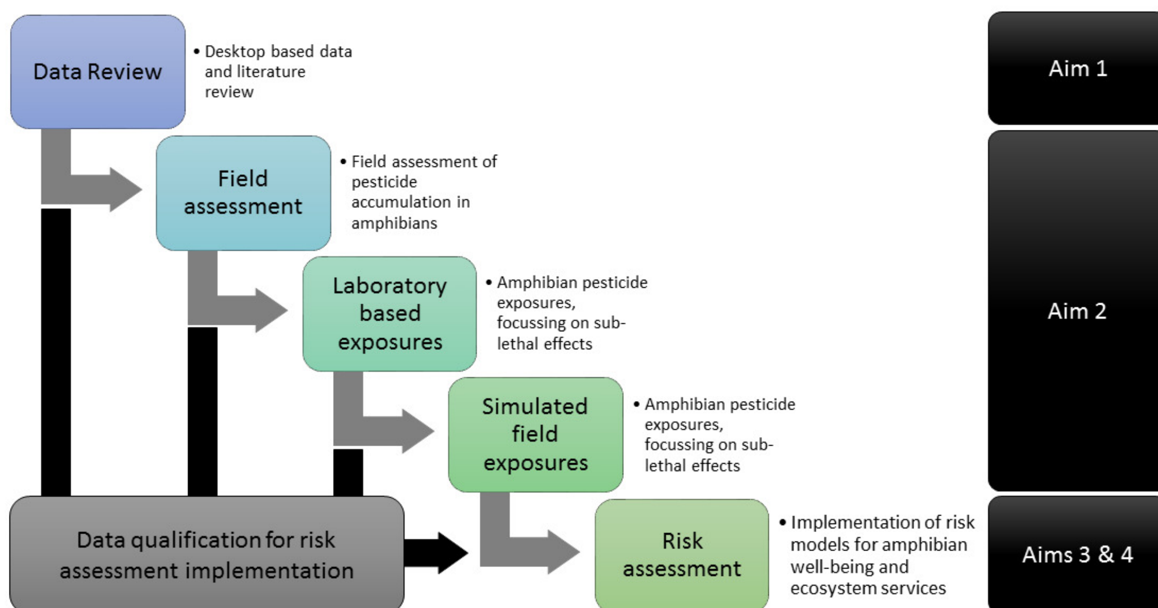

Figure 1.1 **Outline of the tiered study approach followed. After completion of each tier, qualification of data for sufficient implementation of the risk assessment model was assessed. Based on remaining data needs investigation into the subsequent next tier was initiated. Through**

implementation of tier 1 (data review) the first aim of this study is addressed. Through implementation of tiers 2-4 (field assessment, laboratory based exposures, and simulated field exposures) the second aim of this study is addressed. Implementation of the risk assessment addresses the third and fourth aim of this study

CHAPTER 2 CURRENT STATUS AND FUTURE PROGNOSIS OF MALARIA VECTOR-CONTROL PESTICIDE ECOTOXICOLOGY AND *XENOPUS* SP.

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2.1 Introduction

The global fight against malaria, nature's most prolific killer, is largely structured around vector control (i.e. controlling mosquito populations). This entails the use of various methods including biological control through the use of larvivorous fish, the efficacy of which has not yet been validated (Walshe et al. 2017). But, for the most part, vector control globally relies on chemical control through the use of various insecticides. These MVCPs include persistent and non-persistent compounds from various pesticide classes. Pesticides are used individually or in combination and through different application methods. The most common and effective chemical methods of intervention are indoor residual spraying (IRS) and insecticide treated nets (ITNs) (Glunt et al. 2013). The World Health Organisation (WHO) Global Malaria Programme lists 12 recommended insecticides for vector control (Table 2.1). These insecticides belong to four chemical groups: organochlorines, pyrethroids, organophosphates, and carbamates (WHO 2006). The use of these pesticides also varies between countries based on policy. Recently there has been a global decline in the use of pesticides for IRS, partly as efforts to reduce selection resistance in mosquitoes (Alonso and Noor 2017). Efforts to find alternative insecticides started in the late 1960's to early 1970's. Driven mainly by resistance in mosquitoes and subsequently by the secondary environmental effects of some pesticides such as DDT.

The first official effort by the WHO to explore alternative insecticides was at the International Conference on Alternative Insecticides for Vector Control in 1971 (WHO 1971). Here it was stated that ideal insecticides should be of low toxicity to mammals, and high toxicity to insects, however not much focus was drawn to the effects on lower vertebrates. Acetylcholinesterase acting insecticides such as carbamates and organophosphates, received major attention as the first alternatives, along with pyrethroids. The development of new synthetic pyrethroids introduced this group as a promising alternative. The pyrethroids currently recommended by the WHO for malaria vector control are considered the more advanced synthetic pyrethroids able to show better photo-stability and potency towards mosquitoes than the natural pyrethroids (pyrethrin I and II; CAS: 121-21-1 and CAS: 121-29-9). The chirality of pyrethroids plays a crucial role in their activity and stability. The ability to separate single isomers allowed for more targeted efficacy such as with bioresmethrin (CAS: 28434-01-7), the 1R-*trans* isomer of resmethrin (CAS: 10453-86-8), showing very low toxicity to mammals, yet equal efficacy against insects to the parent resmethrin (Knaak et al. 2012). The addition of the α-cyano group in the development of cypermethrin (CAS: 67375-30-8) and deltamethrin (CAS: 52918-63-5) greatly increased photo-stability of these insecticides (Knaak et al. 2012). Regardless of efforts made to improve on vector control insecticides since the1970's, a "golden bullet" that is highly toxic to mosquitoes, is persistent in its effect on insects, has no resistance build-up in mosquito populations, and with zero to minimal non-target effects, has not been discovered. The nature of pesticides and some application processes used, such as spraying a water-based solution, causes relatively easy transfer to surrounding environments, especially aquatic environments that tend to act as sinks for accumulation of chemical pollution (Van Der Oost et al. 2003). Lambert (2001a) reported on amphibian deaths in Africa with regard to pesticide usage. Although pesticides may not be the only factors involved, some of the reported deaths occurred even after lower application rates than those recommended for MVC by the WHO (Table 2.1).

Table 2.1 **List of insecticides recommended by the World Health Organisation (WHO) for use for indoor residual spraying against malaria vectors. (Adapted from WHO original list: http://www.who.int/whopes/Insecticides_IRS_malaria_09.pdf)**

Insecticide	CAS#	Formulations	Chemical class	Dosage (g AI/m^2)	Mode of action	Effective duration (months)
α-Cypermethrin	67375-30-8	wettable powder / suspension concentrate	Pyrethroid	0.02–0.03	contact	4–6
λ-Cyhalothrin	91465-08-6	wettable powder / capsule suspension	Pyrethroid	0.02–0.03	contact	3–6
Bendiocarb	22781-23-3	wettable powder	Carbamate	0.1–0.4	contact / airborne	2–6
Bifenthrin	82657-04-3	wettable powder	Pyrethroid	0.025–0.05	contact	3–6
Cyfluthrin	68359-37-5	wettable powder	Pyrethroid	0.02–0.05	contact	3–6
DDT	8017-34-3	wettable powder	Organochlorine	1–2	contact	>6
Deltamethrin	52918-63-5	wettable powder / water dispersible granule	Pyrethroid	0.02–0.025	contact	3–6
Etofenprox	80844-07-1	wettable powder	Pyrethroid	0.1–0.3	contact	3–6
Fenitrothion	122-14-5	wettable powder	Organophosphate	2	contact / airborne	3–6
Malathion	121-75-5	wettable powder	Organophosphate	2	contact	2–3
Pirimiphos-methyl	29232-93-7	wettable powder / emulsifiable concentrate	Organophosphate	1–2	contact / airborne	2–3
Propoxur	114-26-1	wettable powder	Carbamate	1–2	contact / airborne	3–6

The principle of introducing neurotoxic chemical agents to any ecosystem (aquatic or terrestrial) on a regular basis inevitably results in a potential risk to non-target organisms in that ecosystem. To assess these potential effects, model organisms are often used. This review discusses anurans from the genus *Xenopus*, with particular focus on *Xenopus laevis* (African clawed frog) and its use as a model organism in research regarding MVCPs around the world.

2.1.1 History of *Xenopus* use in research

Xenopus laevis, the frog species most commonly used in research, is endemic to southern Africa. Starting in the late 1930s, *X. laevis* was distributed to laboratories worldwide, since they served as the most reliable pregnancy test until the 1960s (Gurdon and Hopwood 2000). With laboratory housing conditions becoming refined, and *X. laevis* being distributed to laboratories around the world, it quickly became a model for scientific teaching and research. Its use in pregnancy testing relied on ovulation being induced in the female frogs, which paved the way for harvesting and artificial insemination of the ova for regulated breeding. Nieuwkoop and Faber (1994) studied the development of *X. laevis* in great detail. They produced a normal development table for 66 stages of *X. laevis* development from fertilization to adulthood, first published in 1956, with a final refined version in 1994. This opened the door for the use of *X. laevis* as a model for frog development studies.

The immune system of *Xenopus* changes drastically as it develops (Robert and Ohta 2009), which may cause sensitivity to the teratogenic effects of compounds they are exposed to, or even increase susceptibility to secondary infection (Mann et al. 2009). *Xenopus laevis* tadpoles are filter feeders, which increases their uptake of chemicals in the water column. Dumont et al. (1983) used the Nieuwkoop-Faber developmental staging model and developed a standardised test called the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) (updated document: ASTM 2012), which allows for the measurement of developmental toxicity at different degrees through mortality, morphological changes, and growth rate. All the measured

morphological changes, and the severity thereof, were categorized by Hu et al. (2015). Most of the available data produced on amphibian ecotoxicology regarding this species is based on FETAX.

The validity of *X. laevis* as a model frog can be questioned, besides its use in FETAX. Two arguments against its use as a model are, firstly, that it is a fully aquatic species and therefore not necessarily representative of anurans as a whole. Secondly, adult *X. laevis* are known as being hardy and can tolerate a wider range of physicochemical water parameters and, in some cases, toxicants as well, compared to other anurans. The basis of FETAX, however, is the sensitivity of the embryos and tadpoles. This indicates that the life-stage examined should be considered carefully when *X. laevis* is used as test model. The context in which a study is done can also validate the use of the species, as members of the *Xenopus* genus can be seen as good representatives of freshwater organisms in general. This is based on the mid-level trophic position they hold in aquatic ecosystems (Lindholm et al. 2007; Wolmarans et al. 2015; Dalu et al. 2016). Their permeable skin can lead to increased dermal uptake, which also makes them good models for bioaccumulation studies. Increased uptake plays a large role when investigating toxicants with high affinity to sediments. These frogs tend to dwell in the sediment with their highly permeable belly skin exposed for extended periods. They also eat by stuffing food into their mouths and ingesting it by means of a hyobranchial pump (Avila and Frye 1978), increasing the likelihood of sediment ingestion and subsequent chemical exposure. *Xenopus* laevis tadpoles are filter feeders, which also increases sediment ingestion throughout their development. Dalu et al. (2016) showed, through means of stable isotope ratio analysis that sediments along with coarse particulate organic matter, contribute quite substantially to the diet of *X. laevis* tadpoles in ephemeral ponds, with sediment ranging between 1 and 33% and coarse particulate organic matter up to 40.1% of their diet.

The relative ease of housing, artificially harvesting and fertilizing eggs, and reproducing these organisms in a laboratory setting, as described by ASTM (2012), has also played a role in the continued use of this species. The introduction of *X. tropicalis* (*Silurana tropicalis* in some

literature) as a replacement for *X. laevis* in some research studies in the late 1990s came about largely due the tetraploidy of *X. laevis*, which hindered research in genetic and gene expression fields (Burggren and Warburton 2007). *Xenopus tropicalis* is a diploid and has a faster development timeframe (≈ six months to adulthood vs ≈ 12 months for *X. laevis*), and can also be used effectively with FETAX (Hu et al. 2015). Regardless of the use of *Xenopus* sp. in other types of studies, the FETAX model is still considered one of the primary assays to be used for testing teratogenic effects of compounds on lower vertebrate animals. Bearing this information in mind, it is interesting to note that the number of publications produced using *Xenopus* spp. as a model for pesticide research is quite low (Table 2.2). Although Boolean search systems (search system use is explained under availability of literature) for locating electronically-available publications in online databases do not necessarily provide an accurate number of publications available on a subject, it gives a good indication of whether the topic is popularly explored by scientists around the world.

2.1.2 Scope of the review

In this review we summarize the literature available on *Xenopus* sp. in relation to the 12 WHO-recommended MVCPs in terms of acute, sub-lethal, and chronic toxicity. As supplementary information (Figure 2.1, Figure 2.2), these data were evaluated using meta-analyses for theoretical relative toxicity of the different pesticides to *Xenopus* sp. in order to pinpoint which pesticides might require more focus for future research. This review serves to bring to attention the lack of available literature in this field and the inconsistency of results between some of the existing records. We also identify gaps in the current knowledge, in order to promote more focussed future research in this field. Zippel and Mendelson (2008) proposed certain challenges to the global amphibian crisis, one of which was directly aimed at environmental contamination in generating data on sub-lethal and chronic effects. Now, a decade later, we hope that this review can stress the lack of progress made in this regard in the context of the pesticides in question.

2.1.3 Data inclusion methods and criteria

Search queries were performed on Scopus, Web of Science, and other online databases using selected keywords (Scopus search outcomes given in Table 2.2) pertaining to MCVPs and *Xenopus*. The US-EPA ECOTOX database was also queried, with Pauli et al. (2000) as secondary sources. Some criteria were necessary in the filtering of data used for this review. To be acceptable for inclusion, a study had to report on *Xenopus* sp. in relation to one or more of the 12 WHO-recommended malaria vector control pesticides listed in Table 2.1. Studies reporting toxicological effects without any corresponding reference as to exposure amount or biological load were excluded. Laboratory and field-generated data were included. As dichlorodiphenyltrichloroethane (DDT; CAS: 8017-34-3), one of the pesticides included, is considered a persistent organic pollutant (POP), studies reporting only bioaccumulation data were also included. The main breakdown products of DDT, Dichlorodiphenyldichloroethane (DDD; CAS: 72-54-8) and Dichlorodiphenyldichloroethylene (DDE; CAS:72-55-9), can be even more persistent in the environment and are therefore indirectly introduced via DDT introduction. Studies reporting sub-lethal effects of these compounds were also included in the study. The majority of studies within search parameters that were excluded from this review, involved transgenic *Xenopus* oocytes used merely as a medium in which to analyse the reaction of transplanted genes to pesticides. If such studies did not report effects relatable to *Xenopus* sp. itself, they were excluded from this review.

Table 2.2 Scopus database search outputs using the search field "Article, Abstract and Keywords" for various search phrases pertaining to *Xenopus* sp. and malaria vector control pesticides

Search phrase	Year	Records found	Records within review criteria
***Xenopus* AND cypermethrin**	1982 - 2018	22	8
***Xenopus* AND cyhalothrin**	2006 - 2012	5	1
***Xenopus* AND bendiocarb**	2003	1	0
***Xenopus* AND bifentrhin**	2006 - 2017	3	0
***Xenopus* AND cyfluthrin**	1986 - 2017	7	2
***Xenopus* AND DDT**	1969 - 2017	34	16
***Xenopus* and deltamethrin**	1979 - 2017	47	8
***Xenopus* and etofenprox**	2010	1	0
***Xenopus* and fenitrothion**	1981	1	1
***Xenopus* AND malathion**	1989 - 2016	11 (+ 1 duplicate)	10
***Xenopus* AND propoxur**	1994 & 2003	2	0
***Xenopus* AND DDT AND bioaccumulation**	1995 & 2006	2	2
***Xenopus* AND deltamethrin AND bioaccumulation**	2007	1	1
***Xenopus* AND DDT AND field**	2004 & 2009	2	1
***Xenopus* AND deltamethrin AND field**	2014	1	0
***Xenopus* AND malathion AND field**	1994	1	0
***Xenopus* AND propoxur AND field**	1994	1	0

2.1.4 Availability of literature

Amphibian ecotoxicology is a research topic that has increased in popularity over the last decade, a trend also seen in literature included in this review. A Scopus database query performed in August 2018 using the search phrase "anura AND pesticide" in the field "Abstract, title and keywords" returned 395 entries. When the more refined search term "*Xenopus* AND insecticide" was used 267 entries were given. Replacing "insecticide" with the names of the 12 WHO-recommended MVCPs, the highest output was for "*Xenopus* AND deltamethrin" with 47 entries. Whilst this number may seem high, only eight of these publications fitted the criteria for inclusion in this review (See search results provided in Table 2.2). Through individual pesticide and *Xenopus* combination searches, 135 entries were given in total, of which only 46 entries fitted the criteria for inclusion in this review.

Globally, amphibians are widely distributed, with their highest species density being found in tropical and sub-tropical areas (FEOW 2008). These are also the regions with temperatures suitable for transmission of *Plasmodium* sp. throughout the world (MAP 2018), yet there seems to be a lack of literature on amphibians — especially from tropical and sub-tropical regions — and on ecotoxicology with regard to MVCPs. With overlap in pesticide use between MVC, vector control for other tropical diseases and agriculture, the need for data from species relevant to these tropical and subtropical systems becomes clear. In the case of Africa, *Xenopus* sp. is a good starting point for collecting relevant anuran data.

2.2 Summary and discussion of reviewed literature

2.2.1 FETAX studies

With FETAX long being the most commonly used teratogenic toxicity model, it would be expected that most available literature on *Xenopus* and MVCP toxicity would originate from FETAX studies. Another advantage of this type of data is comparability, as FETAX allows for a standardized testing method with standardized endpoints. This is not really the case with MVCPs, though the literature available is much scarcer than expected, lowering the confidence of comparisons between data. FETAX data relating to MVCPs available in literature are summarized in Table 2.3. Data relating to only three of the 12 WHO-recommended MVCPs could be found, namely DDT, deltamethrin, and malathion (CAS: 121-75-5).

Table 2.3 Summary of results from studies included in this review which performed teratogenic toxicity exposures on *Xenopus laevis* embryos with regard to any of the 12 WHO-recommended MVCPs using the FETAX protocol. NR =not reported

Pesticide	Exposure range (mg/L)	LC50 (95%CI; mg/L)	EC50 (mg/L)	TI	MCIG	LOEC	Notes	Source
Deltamethrin	NR	0.19 (0.16–0.24)	0.006	31	NR	NR		Channing 1998
Malathion	0.1–100	10.9 (10.6–11.3)	P =0.33 G =0.79 N =2.16	P =33.2 N =5.1 G =13.8	NR	NR	P= effect of abnormal pigmentation observed; G= effect of abnormal gut observed; N= abnormal notochord	Snawder & Chambers 1989
Malathion	0.05–6.0	NR	2.39	NR	NR	NR	Mortality at 6 mg/L =12%	Bonfanti et al. 2004
***p,p*-DDT**	0.39–99.9	35.8 (31.3–41.12)	14.7 (10.7–20.8)	2.4	1.5	6.24	LOEC = malformation	Saka 2004
***p,p*-DDD**	0.486–31.2	14.11 (12.0–16.89)	4.7 (3.3–6.9)	3	1.95	1.95	LOEC = malformation	Saka 2004
***p,p*-DDE**	$1.24x10^{-4}$–124.9	>124.98	>124.98	NR	1.25–12.5	NR		Saka 2004

2.2.1.1 Organochlorines

Saka (2004) analysed teratogenic effects through FETAX for three major DDT metabolites. For the *para-para-* isomers of DDT, DDD, and DDE assayed, the results showed sensitivity of *X. laevis* both in terms of mortality and malformations in the order of *p,p*-DDD > *p,p*-DDT > *p,p*-DDE, which happens to be the inverse of their expected half-lives in the environment. The LC50 of *p,p*-DDT was 35.8 mg/L with the EC50 at 14.71 mg/L. The LOEC for malformations was 6.24 mg/L, but the minimum concentration to inhibit growth (MCIG) was 1.5 mg/L. This places the MCIG/LC50 ratio at 0.042. The teratogenic index (TI = LC50/EC50) value for *p,p*-DDT was 2.4. As a standard in FETAX, any compound with a MCIG/LC50 ratio lower than 0.3 and a TI above 1.5 is considered teratogenic.

2.2.1.2 Pyrethroids

Teratogenicity of deltamethrin was analysed by Channing (1998), who reported an LC50 and EC50 of 0.19 mg/L and 0.006 mg/L, respectively, with a resulting TI of 31. Even though Channing (1998) did not report the MCIG or LOEC a TI value 20 times higher than the 1.5 cut-off means that deltamethrin is certainly regarded as teratogenic.

2.2.1.3 Organophosphates

Malathion was the only compound for which FETAX results were reported from multiple sources. Snawder and Chambers (1989) showed malathion to have an LC50 of 10.9 mg/L. They reported the EC50 in terms of specific malformations, the lowest of which was an abnormal pigmentation EC50 of 0.33 mg/L, and the highest an abnormal notochord EC50 of 2.16 mg/L. Bonfanti et al. (2004) did not expose tadpoles to a high enough range of malathion to calculate an LC50, with their highest concentration of 6 mg/L showing mortality of only 12%. They did, however, report an EC50 of 2.39 mg/L for total malformations. This value is similar to the notochord abnormality EC50 of Snawder and Chambers (1989), as Bonfanti et al. (2004) reported the malformations observed to consist mostly of abnormal tail flexure, which is relatable to abnormalities of the notochord. Neither Channing (1998) nor Snawder and

Chambers (1989) reported a lowest-observed-effects concentration (LOEC) or minimum concentration to inhibit growth (MCIG) to compare with results from Saka (2004).

Technical DDT consists of about 85% *p,p*-DDT and about 15% *o,p*-DDT (ATSDR, 2002) with variable traces of other forms such as *p,p*- and *o,p*- DDD, and sometimes *o,o*-DDT. The majority of risk from direct exposure is therefore related to *p,p*-DDT. Hence, in analysing initial toxicity from MVC application, acute toxicity and teratogenic risk to *Xenopus* embryos, when exposed to the equal amounts of each MCVP, would be greatest from deltamethrin n, followed by malathion, and lastly DDT. However, these compounds are not necessarily used in equal amounts during MVC. With DDT being banned under the Stockholm convention (UNEP 2009) from other forms of use in most countries, it also carries the lowest risk of being used for other purposes in the environment. Malathion and deltamethrin are, however, actively used in agriculture, in turn increasing risk to non-target organisms, including *X. laevis*, in aquatic environments. Deltamethrin further has the highest potential to cause developmental malformations at concentrations that would not cause significant mortality, based on its high TI value.

Several other studies included in this review followed the FETAX protocol in terms of exposure, but reported on different endpoints to that of the standardized FETAX format, as in the previously-described studies. These studies were therefore included in the acute toxicity or sub-lethal and chronic toxicity data collections (Table 2.4, Table 2.5), based on exposure duration and endpoints measured.

2.2.2 Acute toxicity

Given greater variability in methods, age of animals and report structure outside the FETAX parameters, there were a few more acute toxicity studies that met the requirements for inclusion in this review. Acute toxicity records could be found only for deltamethrin, cyhalothrin (CAS: 91465-08-6), cypermethrin, fenitrothion (CAS: 122-14-5), and malathion (Table 2.4).

Table 2.4 **Summary of results from studies that performed non-FETAX acute toxicity tests on *Xenopus laevis* with regard to any of the 12 WHO-recommended MVCPs. Route of exposure for all studies in this table was immersion. NA = not applicable**

Pesticide	Life-stage	Exposure range (mg/L)	Temperature (°C)	LC50 24 h	LC50 96 h	LC50 168 h	Notes	Source
Fenitrothion	Embryo	0.1-10	18 25 30	18-25°C > 10 30°C = 0.33	NA	NA	EC50 at 18°C= 4.2; 25°C= 3.7; 30°C= 0.17 (abnormalities leading to post-hatching death)	Elliot-Feeley and Armstrong 1982
Deltamethrin	Larvae (NF 46)	1.1 x 10^{-3}–38.4 x 10^{-3}	23	NA	>38.4 x10^{-3} (21.7% mortality)	6.26 x10^{-3}	AI based concentration in commercial formulation	Aydin-Sinan et al. 2012
λ-Cyhalothrin	Larvae (NF 46)	1.1 x10^{-3}–10 x10^{-3}	23	NA	>10 x10^{-3} (46.7% mortality)	3.94 x10^{-3}	AI based concentration in commercial formulation	Aydin-Sinan et al. 2012
Malathion	Embryo	0.5–8.0	22–24	NA	5.4 (4.9–6.0)	NA		Yu et al. 2013
***α*-Cypermethrin**	Embryo	10 x10^{-3}–0.16	22–24	NA	30.6 x10^{-3} (27.7 x10^{-3}–34.8 x10^{-3})	NA		Yu et al. 2013
Malathion	Larvae (NF 46)	1.3–10	22–24	NA	6.76 (6.11-7.52)	NA		Yu et al. 2013
***α*-Cypermethrin**	Larvae (NF 46)	1.25 x10^{-3}–20 x10^{-3}	22–24	NA	6.9x10^{-3} (5.7 x10^{-3}–8.5 x10^{-3})	NA		Yu et al. 2013
***α*-Cypermethrin**	Larvae (NF47/48)	0.5–5	22–23	NA	NA	NA	Combined UVB exposure increased mortality, but was similar to UVB alone	Yu et al. 2015b
Malathion	Larvae (NF47/48)	0.5 x10^{-3}–5 x10^{-3}	22–23	NA	NA	NA	Combined UVB exposure had no mortality interaction	Yu et al. 2015b

2.2.2.1 Pyrethroids

Aydin-Sinan et al. (2012) used a modified FETAX protocol to expose *X. laevis* Nieuwkoop-Faber (NF) stage 46 tadpoles to deltamethrin in a commercial formulation. The concentration range of deltamethrin (1.1 – 38.4 µg AI/L) was too low to induce 50% mortality at 96 h. Maximum concentration induced 21.7% mortality, which is within reasonable expectations compared to the 190 µg/L LC50 (also 96 h) reported by Channing (1998) on embryos using the FETAX method. A 168 h LC50 reported by Aydin-Sinan et al. (2012) 6.26 µg AI/L.

Aydin-Sinan et al. (2012) also exposed *X. laevis* to *λ*-cyhalothrin in commercial formulation which showed greater toxicity than deltamethrin. The 96 h exposure also did not induce 50% mortality, but at 10 µg AI/L as the maximum concentration, 46.7% of the test population died. The 168 h LC50 was reported as 3.94 µg AI/L.

Yu et al. (2013) analysed the toxicity of *α*-cypermethrin to *X. laevis*. Their study showed *X. laevis* to be one of the most sensitive anurans to *α*–cypermethrin based on literature comparisons, and it was suggested as a useful species for studying the effects of pyrethroids. Very little literature on other anurans was available for comparison at the time of publication, which should be taken into account. The LC50 for *X. laevis* to *α*-cypermethrin was recorded as 30.6 (CI 27.1-34.7) µg/L and 6.9 (CI 5.7-8.5) µg/L for embryos and free swimming larvae, respectively, making *α*–cypermethrin more toxic to free-swimming larvae. This result brings into question the use of a single method such as FETAX as a definitive sensitivity test for these compounds to anurans. Incorporation of other life-stages seems necessary for a holistic approach to assess the effect these chemicals have on anuran populations and community structure. Publications by Yu et al. (2013, 2015b) were the only records of *α*-cypermethrin acute toxicity testing on *Xenopus* available for the present review. Yu et al. (2015b) investigated combination effects of simultaneous exposure to *α*-cypermethrin and ultraviolet-B radiation (UVB). The addition of UVB (intensity of 22-28 uW/cm2) showed increased mortality, but this was mostly attributed to the toxic effects of UVB itself and no discernible

relationship between pesticide-caused mortality and UVB-caused mortality could be distinguished. The results did however indicate a synergistic effect between the two in terms of sub-lethal effects such as growth and malformations.

2.2.2.2 Organophosphates

The only study on acute toxicity listed in Pauli et al. (2000) involving *Xenopus* and an MVCP was Elliot-Feeley and Armstrong (1982) involving *X. laevis* and fenitrothion. This was also the only existing acute toxicity record of fenitrothion on *Xenopus* we could find. The authors exposed *X. laevis* embryos at gastrulation phase to a range of concentrations (0.1-10 mg/L) of fenitrothion for 24h at various temperatures. The results showed an LC50 above 10 mg/L at 18°C (8% mortality at 10 mg/L) and 25°C, and an LC50 of 0.33 mg/L at 30°C. At 18°C, when exposed to a saturated solution (estimated at 30 mg/L), mortality increased to 89%, which places the theoretical LC50 at 18°C somewhere between 10 and 30 mg/L. The surviving embryos were raised to NF stage 37 and morphological changes recorded. The authors classed tadpoles based on the severity of morphological change, which showed increased severity corresponding to increased concentration. Significant differences in morphology were observed at 1 mg/L (at 18°C), while increased mortality from the control was measured at only 10 mg/L. Sensitivity tests were also conducted with 10 mg/L exposures at 25°C which indicated the highest mortality when exposed during stage 11-15, but with the highest incidence of morphological changes when exposed at stage 8-11. The sensitivity differences in terms of mortality at different developmental stages once again (as with *α*-cypermethrin) seems to indicate that a single life-stage exposure is inadequate to determine realistic toxicity information when working with anurans.

Yu et al. (2013) also investigated the toxic effects of several pesticides, including malathion, following the FETAX method, except for a few modifications such as not de-jellying the eggs. This provided a more realistic exposure scenario, but excluded this study from outright comparison to other FETAX studies. Other parameters they adjusted were the mixing of egg

clutches, larger test volumes, and tadpoles were considered dead when they did not respond to prodding rather than when they had no heartbeat (as described in the latest version of FETAX; ASTM 2012). The results from Yu et al. (2013) showed malathion as having an LC50 of 5.4 (95%CI 4.9-6.0) mg/L and 6.7 (95%CI 6.1-7.5) mg/L for stage-8-11 and stage-46 larvae, respectively. This is lower than the values measured by Snawder and Chambers (1989) and Bonfanti et al. (2004), which the authors ascribed to their alterations on the FETAX protocol. In Yu et al. (2013), malathion showed less acute toxicity (endpoint: mortality) to free-swimming larvae (NF stage 46) than to embryos (NF stages 8-11) with similar sensitivity indicated by growth and malformation endpoints. The authors also compared the sensitivity of *X. laevis* larvae to values from the literature on other amphibian species with *X. laevis* showing median sensitivity to malathion. Yu et al. (2015b) also investigated additive effects of UVB radiation to malathion toxicity, but with no discernible interaction in terms of mortality. However at lower concentrations the addition of UVB significantly increased the incidence of malformations such as abnormal tail tip and gut observed in conjunction with malathion exposure. Edema incidence, caused by UVB alone, was reduced in combined exposure with higher concentrations of malathion.

2.2.2.3 Relative toxicity of MVCPs towards *Xenopus*

In order to evaluate the sensitivity of *Xenopus* sp. (data only available for *X. laevis* as representative of *Xenopus* sp.) to MVCPs, we analysed the acute toxicity data above to rank the 12 WHO MVCPs based on their relative potency towards *Xenopus* sp. We utilise the term potency in a generic manner to indicate comparative toxicity towards *Xenopus* sp. rather than terms such as hazard or risk since in the context of risk assessment these terms would imply some form of known relationship between exposure and effect. By plotting the available LC50 values for each pesticide vs the recommended manufacturer's application rate (m^2) we derived course relative MVCP potency categories (Figure 2.1). High potency towards *Xenopus* sp. relates to a high toxicity (i.e. low LC50 value) and a high application rate, and conversely the lowest application rate and highest LC50 value would result in the lowest potency. The

boundary between high and low potency was derived by the combination of highest application rate between all compounds and highest LC50 between all compounds as the best case for toxicity and worst case for application. This combination then provides a separation point for compounds having worse or better combination outcomes than the combination of worst and best case scenarios. Any points below this line are considered moderate to high risk, while any points above are considered low to very low risk. Zero risk cannot be used as a predicted value if application is a non-zero value. Based on these combinations, relative potency values were calculated using the following formula: Log((average dose x 1/mean LC50)+1) (Figure 2.2).

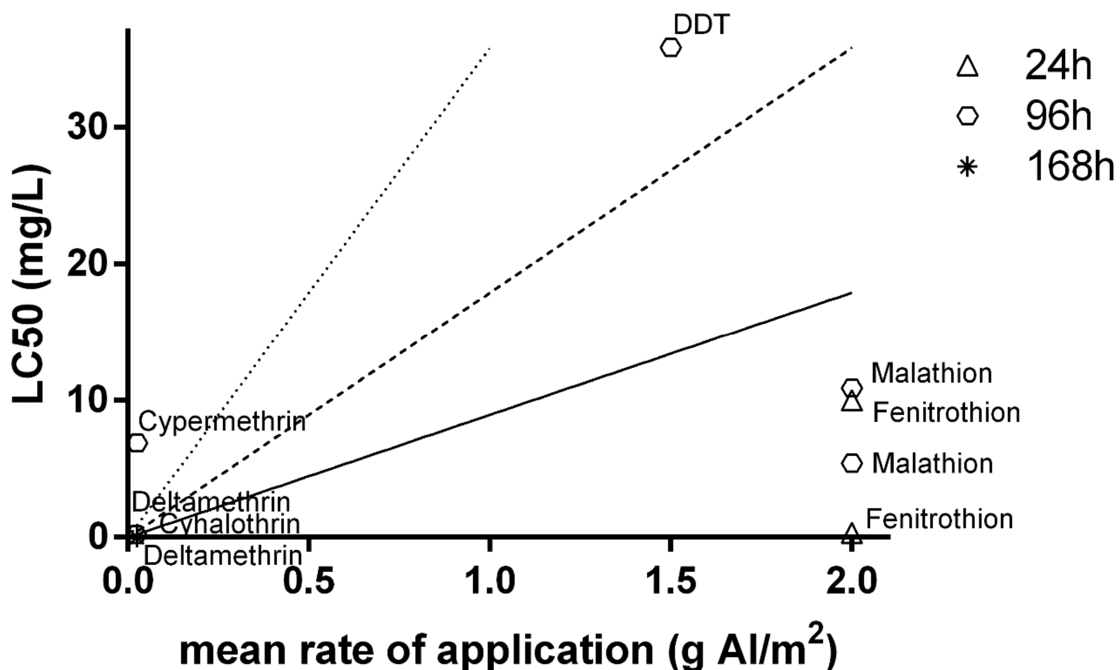

Figure 2.1 **Scatterplot of acute toxicity to *Xenopus laevis* vs recommended application rates for malaria vector control pesticides with relative potency categories indicated. Top quadrant shows lowest potency, bottom quadrant shows greatest potency relative to the other data on the graph. ··· = very low to low potency, --- = low to moderate potency, and — = moderate to high potency**

The use of cyhalothrin for MVC theoretically contributes the greatest acute toxicity potency to *Xenopus* sp. in a natural setting during MVC application out of all the MVCPs for which toxicity data is available. Fenotrothin, deltamethrin and malathion also have high relative potency while DDT has a moderate relative potency. *α*-Cypermethrin poses the lowest risk for acute toxicity to *Xenopus* sp. out of the possible MVCPs. The lack of acute toxicity data for bendiocarb (CAS: 22781-23-3), bifentrhin (CAS: 82657-04-3), cyfluthrin (CAS: 68359-37-5), etofenprox (CAS: 80844-07-1), pirimiphos-methyl (CAS: 29232-93-7) and propoxur (CAS: 114-26-1) means that 50% of the WHO MVCPs could not be evaluated in this manner.

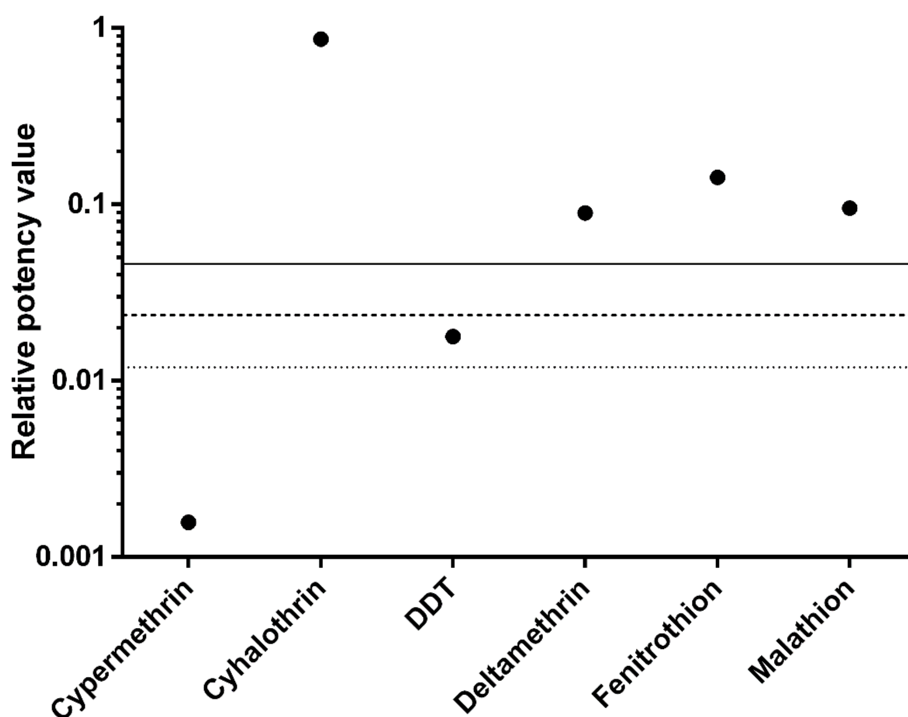

Figure 2.2 **Relative potency values for malaria vector control pesticides for causing acute toxic effects to *Xenopus laevis* calculated as Log((mean application rate x 1/mean LC50)+1) with relative potency categories indicated as in Figure 2.1. ··· = very low to low potency, --- = low to moderate potency, and — = moderate to high potency**

It should be stressed that there are specific limitations to the use of the relative potency values calculated in this manner. Firstly factors such as frequency of use, distance from spraying, temperature, wind, soil composition, runoff from precipitation, photolysis, and other forms of degradation will all affect the extent to which these animals are essentially exposed in the wild. These factors are not included as most of them are going to be scenario specific and using theoretical values can provide even more misleading results. The calculations performed in this review merely compare the potential to be introduced in a broad sense vs the potential to have a toxic effect on these animals. This provides comparative potency between the pesticides discussed in this review only. The lack of toxicity data on many of the discussed pesticides also limits the interpretation of these results in the wider context of all 12 WHO recommended MVCPs. In fact these limitations on the meta-analysis already provides insight into the issues regarding lack of data in this field. That being said, the comparisons provided between these pesticides from the relative potency values provides rationale as to prioritising pesticides for future research.

Pyrethroids are the compound group that contribute the greatest relative potency to *Xenopus* sp. in terms of having the potential to cause frog mortality in quantities used for MVC in Africa (Figure 2.2). This is due to the high sensitivity *Xenopus* has towards these compounds compared to other MVCPs. Even with DDT (organochlorine) application rate 26 times higher than the mean application rate of the pyrethroids, the mean relative potency of the three pyrethroids analysed is still higher than that of DDT. Based on application rates and sensitivity we can discern that *λ*-cyhalothrin holds the greatest potential for acute toxicity to *Xenopus* sp. followed by deltamethrin. However, all the toxicity tests included were not conducted under the exact same conditions, lowering the confidence of such a conclusion.

The use of multiple life-stages in anuran acute toxicity testing plays an important role in amphibian ecotoxicology as the most sensitive life-stage was not consistent throughout reviewed studies where both embryo and larval stages were used. The most sensitive life-stage appears to be compound-specific and may be related to the specific mechanism of

action of each pesticide. Another issue already touched on with the available toxicity data is the lack of structure. Currently three internationally standardised tests for the *Xenopus* model are available. The Organisation for Economic Co-operation and Development (OECD) chemical safety testing protocol No. 231: Amphibian metamorphosis assay (AMA), No. 241: Larval amphibian growth, and development assay (LAGDA), and FETAX. The AMA is to some extent a 21-day variation of FETAX and focuses only on the thyroid axis effects compounds can have that affect the metamorphoses of frogs. It can be applied to different anurans, but was also specifically designed around *X. laevis*, like FETAX. The LAGDA stemmed from the AMA and is considered a more comprehensive toxicity analysis enabling measurement of endocrine disruption through various pathways. The issue with using either of these assays for other species comes down to the developmental staging of the frogs. General frog development stages, as defined by Gosner (1960), has less detail than NF staging (Nieuwkoop and Faber 1991) for *X. laevis*, which is what makes the generalisation possible, but limits quantification during assays, and comparability to NF staged studies. The aquatic-terrestrial range of amphibians also provides complications as not all amphibians can be immersed continuously in exposure media, some species will die if they have prolonged contact with water due to unique osmoregulation adaptations. This is the same uniqueness that makes frogs so habitat-specific. Studying frog ecotoxicology in a laboratory is one thing, but relating those results back to real world exposure scenarios raises complications in itself between species and the respective habitats they inhabit.

The lack of acute toxicity data for WHO-recommended pesticides on a standard model organism is alarming. As *Xenopus* is not the only standard testing frog species in the world, it is fitting also to compare literature on other amphibian species. Species sensitivity distributions (SSDs) were compiled from the LC50 values discussed in this review and US-EPA ECOTOX database results on amphibians in relation to the 12 WHO MVCPs in question. Figure 2.3 shows an SSD of amphibians to malathion. LC50 data for studies ranging between 48 h and 96 h was included in the analysis. Data fitting these criteria were found for nine amphibian

species. *Xenopus laevis* is shown to be the second least susceptible species in this regard. SSDs were also calculated for Deltamethrin, DDT and cypermethrin in the same manner (data not shown), however there were less than seven data points available for each of these pesticides, increasing the error margin severely. Both DDT and deltamethrin showed *X. laevis* to be the least susceptible species, but for cypermethrin *X. laevis* was the second most susceptible species, with only the Asian common toad *Duttaphrynus melanostictus* having a lower LC50. Interestingly, in terms of cypermethrin, *X. laevis* is thus a relatively sensitive anuran, but this pesticide was shown to have the lowest relative potency value to *X. laevis*. Conversely, malathion and deltamethrin had fairly high potency values compared to other MVCPs, but showed *X. laevis* as tolerant, compared to other frog species. This indicates an inflated potency towards most other frog species for these two compounds. Exposure scenarios for other species would differ based on their preferred habitats, which may lower the realistic risk posed by these compounds. The lack of data on other anuran species is also alarming as it indicates *Xenopus* species aren't the only frogs neglected in this topic of research. Larger and more relevant exposure and sensitivity datasets are required in order to be able to properly investigate risks these pesticides pose to frogs. Realistic (or simulated-realism) exposure-response, and site specific application-risk investigations into all MVCPs, not only for *X. laevis* but also other frog species, seems to be a logical and crucial next step for amphibian ecotoxicology research and conservation in MVC regions. Of all the MVCPs with data available, the compound with the highest relative potency towards *X. laevis* was λ-cyhalothrin. The only other publication on acute toxicity to frogs for this compound in the ECOTOX database is for the Bog Frog *Fejervarya limnocharis*; previously *Rana limnocharis*, which showed a 48 h LC50 of 4 µg/L (Pan and Liang 1993), compared to the 3.97 µg/L LC50 for *X. laevis* only after 3.5 times longer exposure. The lack of comparable toxicity data with regard to MVCPs and frogs in general raises the question of how we can be sure that the current use of pesticides for MVC, excluding agricultural use, is not causing permanent damage to frog populations in malaria risk regions.

It is unlikely that mass mortalities of frog populations due to MVC may have gone unnoticed, but even if exposure does not result in mortality, it is conceivable that sub-lethal effects may occur. In the next section we summarize literature available on sub-lethal and chronic effects of MVCPs on *Xenopus* sp.

2.2.3 Sub-lethal and chronic effects

2.2.3.1 Organochlorines

DDT is a well-known endocrine disrupting compound (EDC) (Hoffmann and Kloas 2016). Palmer and Palmer (1995) investigated the use of plasma vitellogenin as a biomarker for oestrogenic activity in *X. laevis* males exposed to DDT. When exposed to 1 µg/g and 250 µg/g o,p-DDT through injection for 7 days, with blood analysed on day 14, both concentrations showed detectable vitellogenin levels significantly higher than control, and increasing in a dose-dependent manner. Hoffmann and Kloas (2016) studied the EDC effects of DDE on male *X. laevis* through analysing call behaviour. Significant reduction in advertising calls (female-attracting call) were observed at 3.18 ng/L exposure. This reduction, however, was not significant at 318.0 ng/L exposure, which did in turn show a significant increase in the amount of ticking (a type of release call). The manner in which calls were altered by the different concentrations of DDE is, according to the authors, suggestive that both estrogenic and antiandrogenic mode of action can be elicited at different concentrations with lower concentrations showing estrogenic activity and higher concentrations showing antiandrogenic activity.

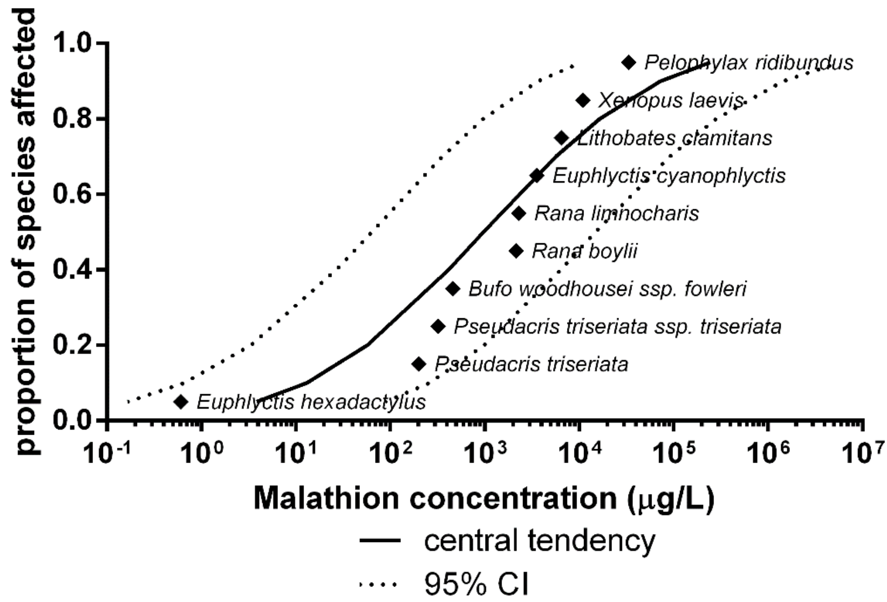

Figure 2.3 **Anuran species sensitivity distribution for malathion showing the proportion of species affected vs. LC50 concentration. Both 48h and 96h LC50 data were included**

Table 2.5 **Studies performed on *Xenopus laevis* with regard to any of the 12 WHO-recommended MVCPs investigating sub-lethal or chronic exposure effects**

Pesticide	Life-stage	Exposure route	Temperature (°C)	Exposure time (days)	Exposure concentration	Notes	Source
***o,p*-DDT**	Adult (males)	injection	21	7	1 & 250 (µg/g)	Injected for 7 days, but analysed at day 14. Vitellogenin was extractable from the plasma of treated frogs but it was not extractable from the plasma of control specimens.	Palmer and Palmer 1995
***p,p*-DDE**	Adult (males)	immersion	21.6	4	3.18×10^{-6} & 3.18×10^{-4} (mg/L)	3.18 ng/L showed estrogenic activity while 318 ng/L showed antiandrogenic activity.	Hoffman and Kloas 2016
***λ*-Cyhalothrin**	Tadpoles (NF 46)	immersion	23	7	5.5×10^{-4}–0.01 (mg AI/L)	Decreased acid phosphatase, aspartate amniotrasferase, glutathione-*S*-transferase and lactose dehydrogenase	Aydin Sinan 2012
Deltamethrin	Tadpoles (NF 46)	immersion	23	7	1.1×10^{-3}–0.0384 (mg AI/L)	Decreased acid phosphatase and aspartate amniotrasferase	Aydin Sinan 2012
***α*-Cypermethrin**	Tadpoles (NF 49)	immersion	20	14	0.001 – 0.025 (mg/L)	0.015 – 0.025 mg/L showed clastogenic activity and increase in number of micronucleated red blood cells.	Rudek and Rożek 1992
***α*-Cypermethrin**	Tadpoles (NF 47)	immersion	22	9	2.4×10^{-4} (mg/L)	Increased heat shock protein 70 and interleukin-1β gene expression	Martini 2010
***α*-Cypermethrin**	Tadpoles (NF 46)	immersion	22-23	4	5.0×10^{-4} (mg/L)	No light (UVB) avoidance behaviour influences were observed	Yu et al. 2014
***α*-Cypermethrin**	Tadpoles (NF 47)	immersion	22-23	4	0.005 (mg/L)	Increased DNA damage. Combined with 63mW/m^2 UVB for last 7 hours showed decrease in most of this damage	Yu et al. 2015a
Malathion	Embryos	immersion	20	4	0.1–100 (mg/L)	Dose dependant reduction in NAD^+	Snawder and Chambers 1989

Table 2.5 (continued)

Pesticide	Life-stage	Exposure route	Temperature (°C)	Exposure time (days)	Exposure concentration	Notes	Source
Malathion	Embryos	immersion	23	4	1, 5 & 10 (mg/L)	Lysyl oxidase inhibition (IC50: 2.3 x 10^{-4} mg/L) and proline hydroxylase inhibition (IC50: 0.2 mg/L) Less collagen fibres observed, but total collagen quantification showed no reduction	Snawder & Chambers 1993
Malathion	Embryos	immersion	23	4	1, 5 &10 (mg/L)	Dose dependent decrease in length. Dose dependent increase in abnormal pigmentation, abnormal gut and notochordal bending. Dose dependent reduction of intestinal loops with a concurrent increase in anterior intestine diameter.	Snawder & Chambers 1990
Malathion	Tadpoles	immersion		30	1.0 x10^{-9}–1.0 (mg/L)	More than 50% mortality at 30 days in 1.0 mg/L. AChE reduced	Webb & Crain 2006
Malathion	embryos	immersion		3	1.0 & 2.5 (mg/L)	Time dependent increase in notochord axis angle and significant reduction in length at both concentrations	Chemotti et al. 2006
Malathion	Tadpoles (NF 46)	immersion	22-23	4	1.0 x10^{-3} & 5.0 x10^{-3} (mg/L)	No light (UVB) avoidance behaviour influences were observed	Yu et al. 2014

2.2.3.2 Pyrethroids

Apart from acute toxicity, Aydin-Sinan et al. (2012) also analysed a set of biomarker responses to analyse the sub-lethal effects of deltamethrin and *λ*-cyhalothrin on *X.laevis*. Both compounds significantly decreased ACP (acid phosphatase) and AST (aspartate aminotransferases) after 24h exposure. Cyhalothrin also significantly decreased CaE (carboxylesterase), and GST (glutathione-*S*-transferase), with LDH (lactate dehydrogenase) being inhibited by the highest concentration (10 µg AI/L). To our knowledge, this publication is the first report on the use of biomarker responses with *Xenopus* sp. in relation to MVCPs.

Rudek and Rożek (1992) exposed *X. laevis* to cypermethrin between 1 and 25 µg/L and found a dose-dependent increase in the occurrence of erythrocytes with micronuclei. The increase was, however, not as severe as in *Rana temporaria*. Martini et al. (2010) exposed *X. laevis* tadpoles to 0.24 µg/L *α*-cypermethrin in order to measure immune responses. Cypermethrin was found to increase the messenger-ribonucleic acid (m-RNA) expression of heat shock protein 70 and interleukin-1β. Both of these play important roles in immune response and alterations to their normal expression could result in a compromised response to influences such as disease. In Yu et al. (2014) the behaviour of tadpoles exposed to pesticides were measured in terms of UVB radiation avoidance. A 0.5 µg/L cypermethrin exposure, however, showed no difference in time spent in or outside of the UVB-exposed area compared to control. A 1 µg/L cypermethrin exposure was excluded from the results as axial malformations interfered with swimming behaviour and subsequently light avoidance behaviour. Yu et al. (2015a) exposed *X. laevis* embryos to cypermethrin and UVB simultaneously looking at sub-lethal effects in the form of Deoxyribonucleic acid (DNA) damage. Cyclobutane pyrimidine dimer accumulation, indicating DNA-adducts, increased significantly compared to both control and UVB-only exposure, at 5 µg/L cypermethrin exposure for 96 h with 63.0 mW/m^2 UVB introduced for the last seven hours of exposure. The m-RNA expression of genes indicative of DNA damage showed cypermethrin alone down-regulated the xeroderma pigmentosum group A (XPA) gene, and up-regulated the xeroderma pigmentosum group G (XPG) gene,

and cockayne syndrome A (CSA) gene playing a role in transcription coupled DNA excision repair, and cypermethrin also upregulated expression of the muir-torre syndrome (MUTL) gene which is involved in mismatch repair. In combination with UV, however, most of these effects were nullified, with only XPG up-regulation being enhanced even further. These results indicate that sub-lethal concentrations of cypermethrin can have stress response effects in *X. laevis* and that some of these effects may be enhanced in a natural environment with combined exposure to UVB radiation.

2.2.3.3 Organophosphates

In addition to mortality and developmental malformation, Snawder and Chambers (1989) also investigated the effects of malathion on nicotinamide adenine dinucleotide (NAD^+) in the exposed *X. laevis* embryos. They found significant reduction of NAD^+ showing a gradual almost linear decline on a log (dose) vs response curve. This dose-related reduction in NAD^+ is deemed independent from malformations observed, as other compounds tested showed variation in malformations observed with similar reduction in NAD^+. Following these results, Snawder and Chambers published two more studies (Snawder and Chambers 1990, 1993) in which they also exposed *X. laevis* embryos to malathion using the FETAX protocol. In Snawder and Chambers (1990) the effect of tryptophan combined with malathion on the NAD^+ levels was tested and, based on malformations observed, it was concluded that tryptophan could effectively combat the NAD^+ reduction effects of malathion, but with no resulting change in the malformations observed from malathion exposure. The authors also showed that 4-day old embryos exposed for one day showed similar malformations to those exposed for four days from zero days old, making the fourth day of development the most critical point in development in terms of malformation. Snawder and Chambers (1993) focused on the effects of malathion on collagen and subsequent notochord bending. Ultrastructural examination showed fewer collagen fibres in the notochords of exposed embryos, but quantification of collagen in homogenised embryos did not differ significantly from controls. Malathion was, however, shown to inhibit lysyl oxidase and collagen proline hydroxylase activity in *X. laevis*

embryos in a dose-dependent manner, with IC50s of 0.7 nM and 0.58 uM, respectively. The inhibition prevents triple helix collagen from forming and in turn reduces the extracellular collagen fibres, even though total collagen content assays show no reduction.

Webb and Crain (2006) exposed *X. laevis* tadpoles to different concentrations of malathion over 30 days, with 1.0 mg/L showing a significant mortality rate compared to the control, and with more than 50% mortality at the end of the experiment. Webb and Crain (2006) exposed three-week-old tadpoles, without proper mention of the exact developmental stages used, rendering comparison difficult. Chemotti et al. (2006) investigated the teratogenic effects of malathion on *X. laevis* embryos using a modified 72 h FETAX method deigned for practical teaching applications. The tadpoles showed significant time-dependent increase in the notochord axis angle over 72 h at 1.0 and 2.5 mg/L malathion exposure, together with a significant reduction in length at 72 h exposure for both concentrations, compared to controls. Yu et al. (2014) also tested UVB avoidance behaviour in relation to malathion exposure in *X. laevis* tadpoles, but neither 1 nor 5 µg/L exposures showed significant differences in behaviour.

2.2.4 *In vitro* studies

Due to *X. laevis*' extensive use in teaching and as a physiology model, many physiological tests have been performed on tissue preparations and cell cultures of these animals, and of their neuromuscular system in particular, in order to better understand the mechanistic action of neurotoxic compounds. Such studies provide useful information with regard to the mechanisms of action of pesticides. These studies are listed in Table 2.5 2.6.

2.2.4.1 Organochlorines

Van Den Bercken et al. (1973b) investigated the action potential of muscle nerve fibres of *X. laevis* exposed to 25mg/kg *p,p*-DDT. The poisoning symptoms were described as restlessness and hyper-excitability, which increased in intensity until convulsions occurred, coupled with excessive skin mucus secretion. After euthanasia the action potential was

measured on excised muscles. The treated animals showed an increase in the amplitude of negative after-potential. The duration of action potential was also increased in treated animals, attributed to a slower falling phase. Van Den Bercken et al. (1973a) investigated the effects of DDT on prepared *X. laevis* lateral line organs at different temperatures. DDT exposure (2-4 mg/L) caused trains of impulse spikes instead of the normal single-impulse spontaneous activity. The number of spikes per train increased at lower temperatures, while the frequency of trains increased at high temperatures. Århem and Frankenhaeuser (1974) measured the permeability effects of DDT and its metabolites on *X. laevis* nerve fibres using potential clamping. Effects seen with *Lithobates pipiens* (previously *Rana pipiens*) from DDT on the permeability of Na^+ could not be replicated with *X. laevis*. Dichlorodiphenylacetic acid (DDA; CAS: 83-05-6), a minor metabolite of DDT was found to increase the permeability of K^+ through the nerve fibres at 4 mg/L. In a study by Århem et al. (1974) the acute effects of the same DDT metabolites were investigated on myelinated nerve fibres of *X. laevis*. This publication seems to be, to some extent, a detailed summary of significant results from Åhrem and Frankenhaeuser (1974) and merely confirms that the differences between *X. laevis* and *Lithobates pipiens*, in terms of DDT exposure mentioned in Åhrem and Frankenhaeuser (1974), are in fact due to differences in species response, along with a more detailed description of how DDA affects the K^+ membrane potential through interfering with inactivation of the potassium-gated channel. Repetitive firing in the lateral line organ of *X. laevis* caused by DDT exposure was also demonstrated by Akkermans et al. (1975). Vijverberg et al. (1982b) found the mechanism of action of DDT to be related to opening of the sodium channels in *Xenopus* myelinated nerve fibres, with DDT having no major effect on depolarisation of sodium channel, but causing sodium tailing after repolarization. Lutz and Kloas (1999) measured the oestrogen binding activity in primary liver cell cytosol from *X. laevis*. Between seven endogenous steroids and seven exogenous ligands known for oestrogen receptor binding analysed, *p,p*-DDT had the lowest affinity for oestrogen receptor binding and was the only exogenous ligand with lower binding affinity than the endogenous steroids measured.

Table 2.6 ***In vitro* studies performed on *Xenopus laevis* with regard to any of the 12 WHO-recommended MVCPs**

Pesticide	Tissue exposed	Temperature (°C)	Exposure concentration	Notes	Source
***p,p*-DDT**	Muscle nerve fibres	21–25	25 (mg/kg)	Adult frogs dosed *in vivo* and nerve fibres analysed after death. Increase in amplitude of negative after-potential and slowing down of the falling phase of action potential without affecting resting potential; no repetitive activity.	Van den Bercken et al. 1973b
DDT	Lateral line organ	4–35	2–4 (mg/L)	Caused impulse spike trains number of spikes per train correlated negatively with temperature and frequency of trains correlated positively with temperature.	Van den Bercken et al. 1973a
DDA	Nerve fibres	12	4 (mg/L)	DDT was shown to affect Na^+ permeability in nerve fibres of *Rana pipiens*, but similar results could not be duplicated with *Xenopus laevis* nerve fibres. DDA caused increased permeability of of K^+ at 4 mg/L.	Åhrem and Frankenhaeuser 1974
DDA	Nerve fibres	12	4 (mg/L)	DDA was shown to affect the K^+ membrane potential through interfering with inactivation of the potassium gated channel.	Åhrem et al. 1974
DDT	Lateral line organ	19–21	2–5 (mg/L)	Repetitive activity recorded after 2-5 mg/L exposure.	Akkermans et al. 1975
DDT	Myelinated nerve fibres	15	3.5–14 (mg/L)	Modified sodium channel gating selectivity reduced the rate of closing of the activation gate in myelinated nerve fibre. Similar results to non-cyano pyrethroids.	Vijverberg et al. 1982b
***p,p*-DDT**	Primary liver cells	4	$3.5*10^{-4}$–354.5 (mg/L)	Low oestrogen binding affinity, only compound tested with lower binding affinity than endogenous steroids tested.	Lutz and Kloas 1999
***α*-Cypermethrin**	Lateral line organ	8–22	2.1 (mg/L)	Repetitive activity was only induced in *in vivo* exposed lateral line organs. Impulse train lengths reduced as temperature increased.	Vijverberg et al. 1982a
***α*-Cypermethrin**	Myelinated nerve fibres	0–25	0.42–8.3 (mg/L)	Significantly reduced the rate of relaxation of sodium channel activation gate.	Vijverberg et al. 1983
***α*-Cypermethrin**	Muscle-nerve preparation	18	0.42–41.6 (mg/L)	No repetitive activity with respect to end plate potentials were observed, however, up to 60 action potentials occurred.	Ruigt and Van den Bercken 1986

Table 2.6 (continued)

Pesticide	Tissue exposed	Temperature (°C)	Exposure concentration	Notes	Source
Deltamethrin	Myelinated nerve fibres	19–21	0.5 (mg/L)	Single fibre experiments were performed at 15°C, Deltamethrin induced frequency dependent reduction in action potential at 10^{-6} M.	Vijverberg and Van den Bercken 1979
Deltamethrin	Lateral line organ	8–22	0.5–2.5 (mg/L)	Deltamethrin induced repetitive activity in lateral line organs at all concentrations through *in vitro* exposure. Impulse train lengths reduced as temperature increased.	Vijverberg et al. 1982a
Deltamethrin	Myelinated nerve fibres	0–25	0.5–10.1 (mg/L)	Significantly reduced the rate of relaxation of sodium channel activation gate. Showed slowest decay of tail currents out of 11 pyrethroids analysed	Vijverberg et al. 1983
Deltamethrin	Muscle-nerve preparation	18	0.5–50.5 (mg/L)	No repetitive end plate potentials for R-cis, S-cis and trans and R-trans and R-cis were able to produce up to 60 action potentials.	Ruigt and Van den Bercken 1986
des-cyano-Deltamethrin	Muscle-nerve preparation	18	15.2 (mg/L)	15-30 end plate potentials and 2-18 action potentials.	Ruigt and Van den Bercken 1986
Malathion	Blood plasma	4	2.6 (mg/L)	Zero to weak binding interference shown by malathion between thyroid hormone receptor binding action of endogenous hormones.	Yamauchi et al. 2002
Malathion	Oocytes	NR	0.1 (mg/L)	Reduced oocyte maturation time significantly. Effect was pronounced in 1:1 malathion:atrazine mixture exposures at both 0.05 and 0.1 mg/L.	Ji et al. 2016

2.2.4.2 Pyrethroids

Vijverberg et al. (1982a) studied the effect of pyrethroids on the lateral line organs of *X. laevis*. Cypermethrin failed to induce repetitive activity within the first 5h in *in vitro* exposed studies. In *in vivo* exposed lateral line organs, however, long lasting trains of nerve impulses were induced after 4h when exposed to $5x10^{-6}$ M cypermethrin. In a study by Vijverberg et al. (1983) on myelinated nerve fibres Na^{+} tail currents were caused by both cypermethrin and deltamethrin with deltamethrin showing the slowest decay of tail currents out of 11 pyrethroids tested. The rate of decay accounted for the differences in repetitive activity induced by the various pyrethroids. Ruigt and Van den Bercken (1986) tested the effects of pyrethroids on end-plate potentials and muscle action potentials in excised pectoralis nerve-muscle preparations of *X. laevis*. At 100 uM cypermethrin failed to induce repetitive end plate potentials. Cypermethrin was, however, very effective at producing repetitive action in muscle fibre.

In a study on the effects of deltamethrin (referred to as decamethrin in that study), Vijverberg and Van den Bercken (1979) noted that cypermethrin causes frequency-dependent reduction of action potential in *X laevis*. Deltamethrin was shown to induce this same depression at 10^{-6} M. Vijverberg et al. (1982a) also analysed the effect of deltamethrin on the lateral line organ. Long-lasting trains of nerve impulses were observed in *in vitro* experiments. The number of impulses per train was significantly higher in α-cyano containing pyrethroids (deltamethrin, cypermethrin etc.) than in non cyano containing pyrethroids (permethrin etc.). All pyrethroids analysed showed a temperature-dependent reduction in the length of impulse trains induced. Deltamethrin exposure also did not induce repetitive activity in excised sciatic nerves at 10^{-5} M concentrations after 24h. Ruigt and Van den Bercken (1986) also found no repetitive end plate potentials for deltamethrin exposure. End plate potentials were, however, induced by des-cyano-deltamethrin (Deltamethrin without its *α*-cyano group). Muscle action potentials were induced by both forms.

In general, mechanisms of action derived from the above studies showed activity between DDT and pyrethroids were shown to be very similar, with similar sodium channel activity suggested. Slight differences between different compounds can mostly be attributed to relaxation times after action (Vijverberg et al. 1982b).

2.2.4.3 Organophosphates

Yamauchi et al. (2002) studied the effects of known EDCs on the binding of *X. laevis* plasma thyroid hormone-binding proteins. Malathion was analysed as one of the known EDCs, and showed weak inhibition of binding between triiodothyronine and *X.laevis* transthyretin, with no observable binding effects on thyroid hormone receptor-β. Ji et al. (2016) investigated the effects of malathion on *Xenopus* oocyte development and found that 100 µg/L significantly reduced the time to maturation. This effect was greatly increased by both 50 µg/L and 100 µg/L 1:1 mixtures between malathion and atrazine. The mixture of the two pesticides also increased the mortality rate significantly in oocytes that were fertilized post-exposure. However, de-jellying of eggs was observed to influence post-fertilisation death rate which may have pronounced such effects.

2.2.5 Bioaccumulation studies

2.2.5.1 Laboratory studies

2.2.5.1.1 Pyrethroids

For the purposes of in vitro testing with transgenic *Xenopus* oocytes, Harril et al. (2005), investigated the link between dose and accumulation in non-transfected oocytes (normal *Xenopus* oocytes) to determine the active concentration of deltamethrin in *in vitro* studies (Table 2.7). Their study showed deltamethrin having the ability to accumulate actively in cell tissue in both a time-dependent and dose-dependent manner. In exposed oocytes deltamethrin reached an accumulated concentration equivalent to exposure dose at 55.5 min. At 180 min the accumulated dose reached approximately double the media dose. The results from Harril et al. (2005) were further investigated by Watkins et al. (2007) who also showed a

dose-dependent accumulation of deltamethrin in *X. laevis* oocytes after 1h of exposure. Interestingly, voltage-sensitive sodium channel expressing (Na_v 1.2 + β_1)-transfected oocytes showed lower accumulation than non-transfected or sham-transfected oocytes. These sham-transfected oocytes underwent the transfection process as blanks being transfected only with water and thus having no genetic alteration. This was done in order to determine if the transfection method affected the oocyte response.

Oocytes, however, do not have the same enzymatic defences against xenobiotics as fully developed frogs do, thus this type of accumulation may not be seen in developed embryos. Although, the ability of deltamethrin to accumulate in *Xenopus* oocytes proposes a possible risk to freshly laid frog eggs in the wild experiencing exposure events. It is also unknown how the stability of deltamethrin could be altered when accumulated in tissue, perhaps extending the chemical's half-life in the process. Results from Chapter 3 assessing accumulation in adult *Xenopus muelleri* (n=12) from a MVC sprayed area in South Africa collected in May 2016 (outside the MVC spraying season) showed no detectable accumulation of any MVCP (LOQ=0.01 ppm) other than DDT in adult frogs, indicating that deltamethrin or other pyrethroid accumulation in wild *Xenopus*, if at all possible, is most likely temporary, with a quick cessation period, which reduces the risk of chronic exposure effects emerging.

Table 2.7 **Laboratory-based bioaccumulation studies involving *Xenopus laevis* with regard to any of the 12 WHO-recommended MVCPs**

Pesticide	Life-stage	Exposure concentration (mg/L)	Exposure time (min)	Bioaccumulation concentration (µg/g)	Notes	Source
Deltamethrin	Unfertilised oocytes	0.5	180	1.08	Tissue levels increased with time reaching similar level to media dose at 55.5 min. Concentration dependent increase in accumulation was also shown.	Harril et al. 2005
Deltamethrin	Unfertilised oocytes	0.05;0.5	60	0.98;0.62		Watkins et al. 2007

2.2.5.2 Field studies

2.2.5.2.1 Organochlorines

Only DDT is expected to show bioaccumulation in a field setting, as it is the only MVCP considered to be a persistent organic pollutant (POP). Three studies could be found on DDT accumulation in *Xenopus* sp. in the field (Table 2.8). Hothem et al. (2006) investigated the effects of pollutants on water birds from the Mojave Desert in North America (no active DDT use mentioned), incorporating the accumulation of pollutants in *X. laevis* into the study as a potential food source for birds. None of the frogs analysed had accumulated DDT or metabolite concentrations above limit of detection (0.01 µg/g), except one individual which had 0.054 µg/g wm *p,p*-DDE. Viljoen et al. (2016) measured DDT in *X. laevis* and *X. muelleri* fat bodies in an area sprayed with DDT for MVC through means of IRS in the Limpopo Province of South Africa. All DDT metabolites detected showed higher accumulation at sprayed sites compared to reference sites. No parent DDT (neither *p,p*-DDT nor *o,p*-DDT) was however detected in the frog fat bodies. The authors measured various morphometric factors which indicated observed testicular asymmetry, but with no significant statistical differences. The ∑DDT levels measured in *Xenopus* sp. in this study ranged from 41 to 391 ng/g wet mass. The authors compared these values to literature on other frog species and values for this study are at the lower end, but within comparable range to concentrations measured in fat bodies of *Rhinella marina* in Mexico (Gonzalez-Mille et al. 2013) and of *Rana clamitans* in Canada (Harris et al. 1998). Wolmarans et al. (2018) measured DDT accumulation in whole body *X. muelleri* from another MVC-sprayed region in South Africa across four surveys over a two year period. The survey with the highest ∑DDT accumulation in this study showed a mean of 2,062 ng/g lipid. The study showed no discernible correlations between spraying periods and DDT accumulation. However, unlike the results of Viljoen et al. (2016), parent DDT was detected in two of the four surveys, with one survey showing a ratio of DDT:∑DDD/E (sum of metabolites) greater than one, indicative of exposure to new DDT. In Wolmarans et al. (2018) biomarker responses were also measured in liver and muscle tissue. The authors showed a

significant relationship between some of the measured biomarkers and both *p,p*-DDT and *p,p*-DDE, indicating that the accumulation of these two forms of DDT could cause changes in the energy dynamics of *X. muelleri*. The *p,p*-DDE accumulation showed a reduction in acetylcholinesterase and malondialdehyde in the liver and energy availability in the form of carbohydrates and proteins in muscle tissue. The *p,p*-DDT accumulation indicated an increase in lipid energy availability and cellular energy consumption in muscle tissue.

Lambert et al. (1997, 2001a) investigated frog deaths related to pesticide use and bioaccumulation in sub-Saharan Africa extensively, but made no mention of *Xenopus* with regard to accumulation, or deaths due to DDT or any of the other MVCPs. In this sense, the ecological resilience of *Xenopus* sp. is apparent, as population declines are rarely seen in areas where agricultural pesticides are regularly used. In this regard we hypothesise that some form of generational adaptation may result in the development of resistance to pesticides by wild populations. This is a factor that further brings into doubt the relevance of laboratory based toxicity testing without some form of ecological context.

2.2.5.3 Environmental concentrations

To put into perspective the data discussed on *Xenopus*' sensitivity towards, and accumulation of MVCPs, comparisons need to be made to environmental levels globally, with a focus on Africa as being the natural distribution of *Xenopus*.

Table 2.8 Field-based bioaccumulation studies regarding *Xenopus* sp. and DDT

Pesticide	Species	Life-stage	Tissue analysed	Measured concentration (ng/g wet mass)	Location (country)	notes	source
***p,p*-DDE**	*Xenopus laevis*		Whole body	54.0	Edwards Air Force Base, Mojave Desert (USA)	Only one individual with detectable values.	Hothem et al. 2006
DDT (as ∑DDTs)	*Xenopus laevis*	Adult	Fat bodies	NS: 19.1–277.1 S: 5.5–914.4	Luvuvhu River, Limpopo province (South Africa)	NS = non-sprayed area; S = sprayed area. No *p,p*-DDT detected.	Viljoen et al. 2016
DDT (as ∑DDTs)	*Xenopus muelleri*	Adult	Fat bodies	NS: <LOQ–167.1 S: 154.8–658.1	Luvuvhu River, Limpopo province (South Africa)	NS = non-sprayed area; S = sprayed area. No *p,p*-DDT detected.	Viljoen et al. 2016
DDT (as ∑DDTs)	*Xenopus muelleri*	Adult	Whole body	<LOQ–79.59*	Phongolo River floodplain, Northern Kwazulu-Natal (South Africa)	Liver and leg muscle sample removed before analysis.	Wolmarans et al. 2018

2.2.5.3.1 Organochlorines

The highest environmental level of DDT measured in water in Africa was by Sibali et al. (2008) with ∑DDTs of 3.0 mg/L in Hartbeespoort Dam, South Africa (*p,p*-DDT was 1.5mg/L). The highest recording previous to that was by Henry and Kishimba (2003) in Lake Victoria (Kenya) at 1.6 mg/L. Other African studies reported levels well below these levels with most values below 1 µg/L. Van Dyk et al. (2010) measured DDT in in potable water from the Limpopo Province in South Africa (in an MVC area) at 7.6 µg/L of with the maximum *p,p*-DDT at 1 µg/L and maximum o,p-DDT at 0.8 µg/L. This is just below the *o,p*-DDT levels at which significant vittellogenin induction was shown (Palmer and Palmer 1995). However all of these levels are well above the sub-lethal threshold where changes in calling behaviour of *Xenopus laevis* was found (Hoffmann and Kloas 2016). The high levels in natural water bodies reported by Henri and Kishimba (2003) and Sibali et al. (2008) are also still well below acute toxicity levels and lower than the LOEC reported through FETAX studies showing no threat to *Xenopus* in their natural environment in terms of mortality. Most other African studies on DDT focused on sediment. The most recent study in Africa reporting water DDT concentration was Ogbeide et al. (2018) who reported levels <0.001 ng/L ∑DDTs in surface waters from Edo State, Nigeria.

2.2.5.3.2 Pyrethroids

A recent review by Tang et al. (2018) summarizes global environmental levels of pyrethroids who attributed their environmental distribution to agricultural use. Of the pyrethroids mentioned in the water dataset of Tang et al. (2018), cypermethrin is most frequently detected in water samples from around the world with the highest concentration reported in Bangladesh at 80.50 µg/L in lake water. Studies from China, USA and Czech Republic all showed levels close to or above 30 µg/L. These are levels that would lead to acute toxicity in *Xenopus* embryos and larvae according to the data previously discussed under acute toxicity. No African data are available on current pyrethroid levels in water. This makes comparison between *Xenopus*' sensitivity to pyrethroids less relevant as the environmental data are for regions outside of *Xenopus*' natural distribution. Historical levels of pesticides recorded in

South African aquatic ecosystems were summarised by Ansara-Ross et al. (2012). Pyrethroids historically measured in South African waters include cyfluthrin (<0.0006 μg/L) and cypermethrin (up to 40.7 μg/L) in northern KwaZulu-Natal province (an MVC region) (Sereda and Meinhardt, 2003), and deltamethrin (1.43 μg/L) in runoff water (Dabrowski et al. 2002) in the Western Cape (not a MVC region). River water from the same Western Cape area (Louwrens River) where runoff was measured by Dabrowksi et al. (2002), was analysed by Bollmohr et al. (2007) who reported cypermethrin at 0.1 μg/L. Even though these are historical values from more than a decade ago, the maximum concentration of cypermethrin found in a MVC area by Sereda and Meinhardt (2003) was high enough to have theoretically caused acute toxicity to *Xenopus*. This region is host to both *X. laevis* and *X. muelleri*, but the latter is far more commonly found in this region today. Sensitivity differences between these species have never been assessed, but may hypothetically be a contributing factor. In terms of sediment concentrations of pyrethroids, there are two studies from African countries outside of South Africa reporting pyrethroid concentrations in sediments. Daka et al. (2006) sampled sediment from different habitats in an area of the Okavango Delta in Botswana aerially sprayed with 0.26 g/ha deltamethrin for Tsetse fly control. Deltamethrin in pool sediment had the highest levels which reached a maximum five days after spraying at 0.291 μg/g dry mass. This value dropped slightly towards 0.221 μg/g at 17 days after spraying. Olutona et al. (2016) found cyhalothrin at 77.75 μg/g dry mass in sediments from the Aiba stream in Iwo, Nigeria. This is extremely high considering Bollmohr et al. (2007) reported a maximum of 2.78 ng/g dry mass in sediments from the Western Cape in South Africa. Other historical sediment data from South Africa are reported in terms of wet mass concentrations making comparison difficult. Sereda and Meinhardt (2003) measured sediment Deltamethrin levels up to 90 ng/g wet mass and cyfluthrin levels up to 467 ng/g wet mass in the MVC region of northern KwaZulu-Natal.

2.2.5.3.3 Organophosphates

The only historical record of organophosphates in South African waters is malathion found at 20 ng/L in runoff at the Lourens River in the Western Cape Province (Thiere and Schulz 2004).

Although the organic content from these agricultural runoff is higher and would be more likely to transport pesticides, pesticide concentrations are in the same range as levels found in headwaters of the Donjiang River in China (malathion: 14.94-33.11 ng/L) by Chen et al. (2018). This is lower than the 0.7 nM (approximately 231.25 ng/L) IC50 for lysyl oxidase by malathion shown by Snawder and Chambers (1993).

Based on recorded levels of pesticides in African waters, pyrethroids (specifically cypermethrin) seem to pose the greatest risk to *Xenopus* in their natural environment. The data are, however, mostly outdated and unfortunately there is no consistent ongoing monitoring of pesticide levels in African waters. The fact that both environmental levels and the combination of pesticide application use and dose data point to pyrethroids as a possible threat to *Xenopus* well-being in Africa; warrants further investigation into actual environmental pyrethroid and pesticide mixtures levels of exposure to all frogs and not *Xenopus*).

2.3 Future prognosis for amphibian ecotoxicology

Based on the data reviewed, it is clear that gaps exist within amphibian, specifically anuran, ecotoxicology literature. Acute toxicity data for one of the standard test species was available for only half of the WHO-recommended pesticides, and not enough data on different species exists for species sensitivity distribution analyses. In addition, most of the available acute toxicity data can be considered old literature. Ethics in vertebrate toxicological research may well be a contributing reason behind this lack of data. However, considering comments by Lillicrap et al. (2016) on the future of vertebrate ecotoxicology and steps forward, standardised tests such as FETAX, AMA and LAGDA — mentioned specifically as the “go to” current methods for amphibian ecotoxicology — should have minimal issues associated with them in terms of ethical approval as these are globally accepted standardised tests that are also used as alternatives to other animal tests. A recent publication by Brod et al. (2019) does suggest that amphibian research is at a crossroads in terms of animal welfare in research. If questions posed by Brod et al. (2019) on amphibian welfare (such as appropriate euthanasia methods

and measurable biomarkers of welfare applicable to amphibians) aren't sufficiently answered in the near future, along with renewed interest in amphibian research by funders, it may greatly hinder progress made in generating relevant and usable ecotoxicology data on amphibians.

Another possible reason for the lack of data already existing in this field is a lack of interest amongst researchers. Even though the amphibian ecotoxicology research community around the world is quite small, amphibians are one of the fastest-vanishing groups in the world, which should be an incentive for more detailed research. Zippel and Mendelson (2008) wrote a call-to-action on the global amphibian crisis (i.e. the rapid loss of amphibian species globally). The authors state that a valuable lesson from this crisis is the way in which the, then, 20 years of monitoring provided increasing knowledge on amphibian declines, but did little to prevent extinctions globally, and the term "business as usual" is deemed no longer sufficient by itself for resolving environmental issues. In our opinion, a decade later, many of the questions posed have still not been answered adequately. There is a clear need, not only for toxicity or sensitivity data of anurans towards MVCPs, but also linkages between application rates and exposure, and how these relate to accumulation, or effects, as well as linkages between laboratory and real-world scenarios, and monitoring of current environmental exposure levels. The exploration of mixture effects is something that has only started to progress recently in amphibian ecotoxicology, and could prove useful in amphibian health risk assessment studies in future. There is a need for comprehensive datasets on amphibian ecotoxicology, which can be achieved only through inter-laboratory collaboration and data-sharing in this regard.

2.4 Conclusion

The clearest issue with anuran ecotoxicology regarding pesticides is consistency between reports, mostly due to method-related differences. This is partly accounted for by the fairly common use of FETAX. But the second limiting factor, availability of data, then comes into play. Very few records exist regarding the standard test species, *X. laevis*, and, casting an even wider net by including the whole genus *Xenopus*, does not increase the availability by

any significant means. It is of concern, since most of these pesticides are still being used in agriculture and sectors other than MVC around the world, that for the twelve WHO-recommended MVCPs so little data is available. Biomonitoring data of these pesticides in Africa is also outdated.

Based on the available literature, of the four pesticide groups, pyrethroids may hold the greatest acute toxicity potential to *Xenopus*. This does not mean that at current usage *Xenopus* are at risk of acute toxicity, but merely that, if any of the MVCPs were to result in acute toxicity, based on the sensitivity of *Xenopus* towards pyrethroids and current and recommended usage, pyrethroids would be the most likely compound group to show such effects in the field, however, these potentials are based solely on the data available and are not definitive values in a broader context than how they are used in this study. Based on sub-lethal activity, it is clear that very little is known about how these pesticides affect anurans, in particular at sub-lethal concentrations. The field data available speaks to the persistence of DDT in the environment. As DDT is the only one of the MVCPs known to bioaccumulate in frogs, of the twelve MVCPs, it can be considered to carry the greatest chronic risk to all anurans for this reason. The EDC effects of *p,p*-DDE on *Xenopus* calling behaviour raises concern, as *p,p*-DDE is the most persistent metabolite of DDT and shows the greatest bioaccumulation potential. If more sub-lethal effects linking with accumulation, such as those measured by Wolmarans et al. (2018), can be confirmed through laboratory and field testing it will increase the certainty of risk values that can be calculated from field accumulation data for amphibian populations.

2.5 Recommendations

There is a definite need for further monitoring of anurans in Africa with regard to the effects of pesticides, specifically within the genus *Xenopus*. Lambert (2001b) stated the need for using African amphibians, such as *Xenopus* in particular, more extensively as a monitoring tool in Africa. Increased use of *Xenopus* in monitoring is not reflected by literature as of yet. Based

on the aim of this review, the authors encourage new research on anuran ecotoxicology, specifically in terms of insecticides used in vector control and agriculture, in order to fill the gaps in the current knowledge base. In future studies, *Xenopus* sp. could be used as a starting point around which to build a comprehensive toxicological dataset in order better to understand the impact that human activity has on anuran well-being. The use of adverse outcome pathways may serve as a useful tool to understand sub-lethal effects observed, and is yet to be used in anuran studies. However, the basic empirical toxicity of these pesticides towards anurans still requires attention.

CHAPTER 3 BIOACCUMULATION OF DDT AND OTHER ORGANOCHLORINE PESTICIDES IN AMPHIBIANS FROM TWO CONSERVATION AREAS WITHIN MALARIA RISK REGIONS OF SOUTH AFRICA

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3.1 Introduction

Organochlorine pesticides (OCPs) are a group of chlorinated chemicals historically used as insecticides and are classified as persistent organic pollutants (POPs) based on their extended half-lives in the environment. These pesticides were banned or severely restricted by the Stockholm Convention on Persistent Organic Pollutants of 2001 (Ritter et al. 1995; Bouwman et al. 2011). Effectively, use of most OCPs in South Africa were banned by the early 2000s while production ceased between 2004 and 2010. Lindane (γ-HCH), which initially replaced the use of technical grade HCH after the POPs ban was put in place, was only banned for agricultural use in South Africa in 2009 (DEA 2011; DEA 2019). Dichloridiphenyltrichloroethane (DDT) is an exception, where its agricultural use in South Africa was banned in 1983, but DDT use in malaria vector control is still permitted under strict regulation for indoor residual spraying (IRS; Ritter et al. 1995; Bouwman et al. 2011). Despite having been banned in developing countries for decades, high levels of OCPs are still recorded in various African countries in abiotic matrices, aquatic organisms, and various foodstuff as evident in recent reviews by Thompson et al. (2017a), Gwenzi and Chaukura (2018), and Olisah et al. (2020). It is worth noting that illegal continued use of obsolete OCP stocks in South Africa have been speculated by previous studies (Gerber et al. 2016, Gerber et al. 2021), but no definitive evidence has been documented. The constant presence of these

pesticides has led to renewed research interest in the levels of vector control pesticides, such as DDT, and other legacy organochlorine pesticides. Fish are frequently included in OCP accumulation assessments to represent the biotic aspect of aquatic ecosystems as well as having human health implications through consumption (Gerber et al. 2016; Verhaert et al. 2017; Pheiffer et al. 2018; Buah-Kwofie et al. 2019; Volschenk et al. 2019). Birds are of concern with regard to OCPs due to the eggshell thinning effect of DDT bioaccumulation (Bouwman et al. 2013b; Bouwman et al. 2019). Domestic chickens kept around homesteads where vector control spraying occurs are also studied due to their close proximity to the spray source and consumption related human health risks (Bouwman et al. 2015; Thompson et al. 2017b). Other taxa are not studied as extensively, and could therefore unknowingly be under as great or greater threat from the effects of OCPs and other pesticides. Reptiles and amphibians are both insufficiently studied taxa in this regard. Reptiles, specifically crocodiles, have recently become the focus of several studies from conservation areas in South Africa that indicated high OCP concentrations in these predators (Buah-Kwofie et al. 2018a, Gerber et al. 2021). Amphibians are specifically understudied in Africa with regard to pesticides (Wolmarans et al. 2020), but OCP accumulation has been confirmed in amphibians from IRS regions in South Africa (Viljoen et al. 2016; Wolmarans et al. 2018).

Since realisation of the global biodiversity crisis and the specific declines in amphibian populations was brought to public attention in the early 1990s (*see* Blaustein et al. 1994), the class Amphibia has continued to show devastating declines. The 2018 Living Planet Report indicates amphibian and reptile populations as the second group most threatened by pollution after birds. Pollution is still considered one of the major threats to amphibian and reptile biodiversity, albeit to a lesser extent than habitat degradation, exploitation, and invasive species (WWF 2018).

The important role of conservation areas in protection of species from these major threats is undeniable, but whilst physical boundaries and conservation efforts can reduce habitat degradation, exploitation, and invasive species, threats such as chemical pollution can still

affect these areas regardless of physical boundaries. The concept of long range atmospheric transport of pesticides and persistent pollutants, with eventual collection and accumulation in aquatic ecosystems is well documented (Unsworth et al. 1999; Ruggirello et al. 2010; Mackay et al. 2014; Pheiffer et al. 2018). Mast et al. (2012) showed the long range transport of various pesticide groups (including legacy OCPs) into the Yosemite National Park in California, USA, mostly through precipitation in the form of rain and snow. Apart from atmospheric transport, the majority of OCPs have a high affinity to bind to soils and sediments and organic matter due to high octanol-water portioning coefficients. Pesticides bound to sediments are transported through waterways with those sediment particles and have an extended half-life increasing the travel distance and overall persistence of the pesticide, which is unique to each chemical (*see* Beyer et al. 2000). Pollutants such as OCPs can enter conservation regions through both aquatic and atmospheric pathways and chemical pollution is therefore considered a trans-conservation-boundary threat to animal populations, and specifically amphibians.

The malaria risk region in South Africa occupies the north-eastern part of the country. The distribution of malaria falls within the subtropical climate range and subsequently includes biodiversity hotspots within South Africa. For this reason there is a large overlap in conservation regions in the country that play a critical role in biodiversity conservation whilst also falling within the malaria risk region where DDT and other pesticides are in use for IRS purposes (Buah-Kwofie et al. 2018b). Conservation regions in the country are also often surrounded by rural settlements and agricultural land (Pretorius 2009) where historical OCP input could have occurred. In this study we focussed on two important conservation areas, Kruger National Park (KNP) and Ndumo Game Reserve (NGR), both within the IRS region in South Africa. Both KNP and NGR were surveyed and anurans collected for chemical analysis of OCP residues. The primary aim of this study was to assess whether OCP accumulation occurs in frogs from within conservation regions in South Africa. The secondary aim was to compare OCP accumulation patterns in the same species between the two sampling regions

in order to assess possible differences in OCP sources and intensity between the two regions. This study further aimed to assess the differences in OCP patterns between species from the same conservation region in order to assess the role of species specific habits or habitats.

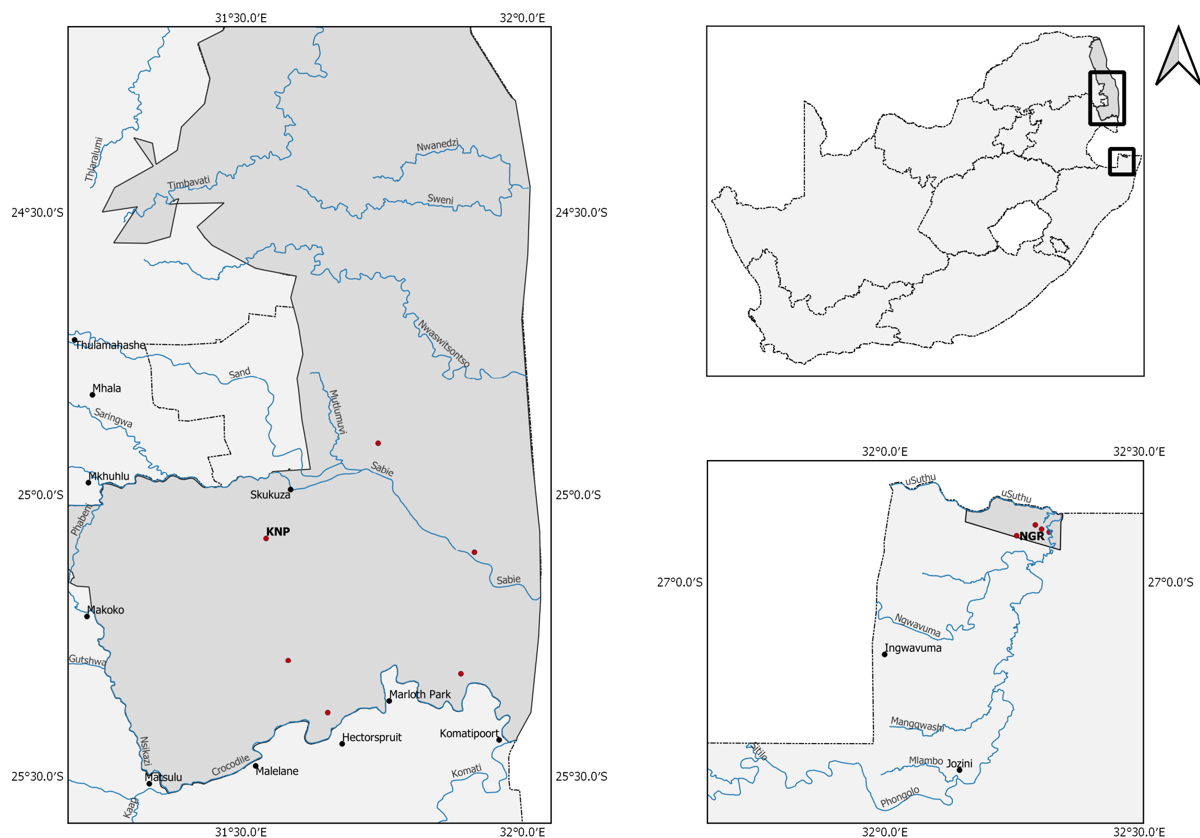

Figure 3.1 **Survey map showing Kruger National Park (KNP) and Ndumo Game Reserve (NGR) indicating sampling locations (red)**

3.2 Materials and methods

3.2.1 Study regions

The KNP (Figure 3.1) is the largest national park in South Africa and is host to 34 frog species (Vlok et al. 2013; Du Preez and Carruthers 2017). The park is situated in the north-eastern

part of South Africa. Mozambique borders the eastern side of the park and Zimbabwe borders the northern side. The park spans over two South African provinces (Limpopo and Mpumalanga) covering 19 485 km^2. During the summer months when malaria risk is highest IRS is actively applied in human settlements surrounding the park, but the use of DDT inside the park is forbidden (SANParks 2006). However, due to the large size of the park, diffuse pollution and long range transport is the main form of organochlorine pesticide input expected at the sample collection sites in this region. The Sabie River and Crocodile River are in close relation to the sampling sites in KNP which increases the possibility of long range aquatic transport of pollutants in the region.

The second conservation area surveyed, NGR (Figure 3.1), is much smaller at 102 km^2 and falls within the Phongolo River floodplain on the eastern side of South Africa with Mozambique bordering the northern side of the park. The Phongolo River floodplain is host to a high diversity of birds, fish and specifically anurans in South Africa, with 45 frog species found in the area (Du Preez and Carruthers 2017; Wolmarans et al. 2018). The reserve is also classified as a Ramsar site under the Ramsar convention on wetlands (DEAT 1996), specifically for the diversity of waterfowl hosted the floodplain pan (wetland) systems inside the reserve. Active IRS occurs inside NGR and in settlements bordering the park (personal communication with NGR staff – Nico Wolmarans). The close proximity of spraying to sampling sites, along with the fact that most wetlands in the area are filled through river overflow or runoff, makes the direct contact or exposure from close-proximity sources (i.e. sprayed homesteads) the expected forms of DDT exposure to amphibians in this region. The Phongolo Catchment is highly utilised for agriculture so legacy OCP exposure via long range aquatic transport is expected as well. There is also long-time practise of livestock dipping (in pesticide) in the land directly bordering the reserve as a form of ectoparasite management, but specific pesticides used could not be confirmed (personal observations and communication with local community members – Nico Wolmarans).

3.2.2 Selection of species

Amphibian species were selected based on their preferred habitats and behaviour around water that would influence the potential OCP exposure pathways. The classification was based on habitat information from Minter et al. (2004), Du Preez and Carruthers (2017), and personal observations in the field. *Pyxicehaplus edulis* (African Bullfrog or Edible Bullfrog), *Hildebrandtia ornata* (Ornate Frog), and *Sclerophrys garmani* (Eastern Olive Toad) are considered semi-terrestrial (ST) as they spend most of their adult lifetime outside water, with only occasional submersion. *Chiromantis xerampelina* (Southern Foam Nest frog) is unique since it is a tree dwelling (TD) frog species that lays its eggs in large foam nests in tree branches overhanging water bodies. These foam nests require a lot of water during production. For this reason, *C. xerampelina* females will soak in water for extended periods during the breeding season in order to hydrate sufficiently. *Ptychadena anchietae* (Common Grass Frog) is considered semi-aquatic (SA) as it stays on river banks and males call from the water edge. If disturbed *P. anchietae* is also more likely to jump towards the water than away from it. *Hyperoluis tuberilinguis* (Tinker Reed Frog) is also considered semi-aquatic as it lives on reeds and has regular contact with water, but males call from outside the water body. Lastly *Xenopus muelleri* (Müller's Clawed Frog) is categorized as fully aquatic (A). Residing in various water bodies and often hiding in the sediment, they have highly permeable skin, which allows for some respiration, extending the time they can spend underwater. Outside of water, they do have the ability to move on wet surfaces and make use of rainy weather to travel. The slightly larger, but also fully aquatic, *X. laevis* has been recorded to travel up to 2.4 km (in six weeks) between water bodies (De Villiers and Measey 2017).

3.2.3 Sample collection and processing

Adult frogs were collected using both active and passive sampling methods. Active methods consisted of frogging at night and catching frogs by hand. Passive methods included drift fence pitfall traps for terrestrial species set up on likely migration pathways to and from water bodies. Aquatic species (*Xenopus muelleri*) were collected with bucket traps baited with commercially

bought chicken liver. Sampling sites are indicated on Figure 3.1. Collection aimed at between 10 and 20 individuals per species sampled evenly between sites based on availability per site, with the exception of *C. xerampelina* in KNP that was collected in higher number for a parallel study. Frogs were therefore treated as one group (per conservation region) representative of the whole region.

Frog samples we collected during November 2011 in KNP (survey 1), November 2012 (survey 2) and April 2013 (survey 3) in NGR. A fourth collection survey took place in May 2016 in NGR for screening of other agricultural pesticides (including other IRS pesticides) in *X. muelleri* only. This region of Southern Africa has a summer rain season spanning from October to March, with October and November considered as onset period and maximal rainfall occurring between December and February (Reason et al. 2005). Surveys conducted in November are therefore considered to be at the start of the rain season, and surveys in April and May at the start of the dry season. The application of IRS occurs in the summer months mostly between November and February. Precise application data for these regions could not be obtained, but based on the combination of the general IRS timeframe and precipitation patterns November surveys are considered to be prior to application and April/May surveys are considered as post application. Application dates could affect DDT exposure from IRS if direct exposure at sprayed homesteads occurs.

Upon collection all frogs were placed in individual plastic containers and for the aquatic and semi-aquatic species water from the sampling site was added. Frogs were then transported to a field station where they were euthanized through double pithing (Amitrano & Tortora, 2012), weighed, and the liver and muscle tissue from the right hind leg were dissected out for separate enzymatic biomarker response analyses not reported in this study (*see* Wolmarans et al. 2018). Chemical euthanasia was not used, to prevent interference with secondary analyses (*see* Wolmarans et al. 2018). The rest of the carcass was wrapped in aluminium foil, labelled, frozen at -20°C until chemical analysis. The 2016 screening samples did not have livers and muscle samples removed.

3.2.4 Chemical analysis

Chemical analysis procedures for both KNP and NGR 2013 samples were performed following the same method described in Wolmarans et al. (2018) and Yohannes et al. (2017). Briefly, frog carcass (lacking liver and muscle from right hind leg) was homogenized and 5-10 g of sample mixed with anhydrous Na_2SO_4, spiked with a surrogate standard PCB 77 and Soxhlet extracted with 150 mL acetone:hexane (1:3 v/v) mixture. A 20% aliquot of the extract was removed and used to determine lipid content gravimetrically. Excess lipid removal from samples was done through gel permeation chromatography (GPC) with the stationary phase consisting of S-X resin beads (Bio-Rad) and mobile phase of 1:1 hexane:dichloromethane mixture. Final clean-up was done using 6 g 5% deactivated Florisil and eluted with 100 mL hexane:dichloromethane (7:3 v/v) solution. The eluate was then evaporated to near dryness, reconstituted in *n*-decane, and spiked with internal standard 2,4,5,6-tetrachloro-*m*-xylene (T*m*X) before instrumental analysis.

Samples were analysed on Shimadzu GC-2014 gas chromatograph coupled with a Ni electron capture detector (GC-ECD). The detector make-up gas flow rate was set at 45 $ml.min^{-1}$. Carrier gas flow rate was set at 1$ml.min^{-1}$ and separation was achieved on an ENV-8MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Splitless injection (1 µl) was used at 250°C inlet temperature. The oven program was initialised at 100°C held for 1 min, ramped at 12°C/min to 180°C, ramped at 4°C/min to 240°C, ramped 10°C/min to 270°C and held for 5 min. The detector temperature was set at 320°C. Five-point calibration curves for a mixture of 22 OCPs (Dr Ehrenstorfer, GmbH) were set up for concentrations ranging between 10 µg/L and 500 µg/L (R^2 ranged between 0.997 and 0.999 for all compounds). The assessed pesticides included the *o,p*- and *p,p*- isomers of DDT, DDD, and DDE (group refered to as DDx); HCB; the *α*-, *β*-, *δ*-, and *γ*- isomers of hexachlorocyclohexane (HCH; grouping referred to as HCHs); Aldrin, dieldrin, endrin (grouping referred to as Drins); the *cis*- and *trans*- isomers of chlordane and nonachlor, oxychlordane (grouping referred to as CHLs); heptachlor; *cis*- and *trans*- isomers of heptachor epoxide (grouping referred to as HPTs). The PCB# 77

recovery rates were > 70% for all samples and concentrations reported were adjusted accordingly. Standard reference material SRM 1947 (Lake Michigan Fish Tissue) analysed using the same method produced recoveries ranging from 75% to 110% with RSD less than 12%. Instrumental limits of quantitation (LOQ) based on 10:1 signal to noise ratio (S/N) were 2.6 to 4 ng/g for HCHs, 0.53 ng/g for HCB, 0.26 to 0.4 ng/g for HPTs, 0.3 for CHLs 0.2 to 0.43 for Drins, and 0.13 to 0.34 ng/g for DDx.

Screening survey samples from NGR (May 2016) were analysed as whole frogs by Primoris analytical laboratories in Belgium using both GC-MS/MS and LC-MS/MS (Primoris Internationally accredited methods: GMSO_01_A, and LMSO_01_A) to screen for the residues of > 500 compounds (listed in supplementary Tables A3 and A4).

3.2.5 Statistical analysis

All concentration data calculated for this study are reported and discussed in terms of wet mass concentrations (Table 3.1). Lipid mass concentrations have been provided in supplementary Table A5 for comparison.

Data followed a non-parametric distribution based on the Shapiro-Wilk normality test. Differences between group (i.e. species per survey) concentration means were analysed using the Kruskal-Wallis analysis coupled with Dunn's post-hoc test. Significance was set at $p < 0.05$. Isomeric ratios of pesticides were also calculated based on wet mass measurements. Values <LOQ were replaced with 0.5 LOQ for all concentration-based analyses.

For compositional analysis, chemical profiles were transformed into relative composition percentages of total OCPs through the equation $CF_{OCP(x)}$ = OCP(x) / total OCPs calculated for each sample where $CF_{OCP(x)}$ is the compositional factor of each OCP measured and the sum of CF values for all measured OCP = 1 for each sample. For this specific analysis, OCPs <LOD were set as zero and a data-filter was applied where samples containing values below LOD for all analysed OCPs were excluded from further analysis to prevent skewing OCP

composition datasets and only include quantifiable contributing compounds in the compositional analysis. Pearson correlation analysis was performed on CF transformed datasets in order to assess similarities in OCP composition between groups, with significance set at $p < 0.05$ and Pearson's $R > 0.5$. The Kurskal-Wallis analyses and correlation analysis were performed using Graphpad Prism 6

To supplement correlation analysis, differentiation between OCP compositions based on CF values were assessed in a discriminant function analysis (DFA). Samples were classified into groups pertaining to both survey and species, and OCP accumulation CF values were used as selection variables. Samples where all OCPs analysed were below LOQ were excluded from this analysis. The DFA was performed using IBM SPSS 24

3.3 Results

3.3.1 Chemical analysis results

Of the 22 analysed OCPs 12 were detected in samples from both regions. Of the six chemical groupings, no Drins or HCB was detected in any of the samples.

Table 3.1 **The organochlorine pesticide (OCP) concentrations measured in different anuran species, given as mean (and range) of detected measurements (ng/g wet mass) per chemical group. Concentrations were measured in whole frog carcasses from Ndumo Game Reserve (NGR) and Kruger National Park (KNP) across four surveys (SUR1-4). DR% = detection rate expressed as percentage of *n*. OCP groups: DDx = total of DDT, DDD, and DDE (all isomers); HCHs = total of all hexachlorocyclohexane isomers; Chls = total Chlordanes; HptChls = total heptachlors. a = in terms of species differences per survey, *C. xerampelina* had significantly higher DDx and ∑OCPs ($p < 0.05$) than all other species within SUR3. The categories (Cat) of association with aquatic systems are represented by semi-terrestrial (ST), tree dwelling (TD), semi-aquatic (SA), and aquatic (A)**

Site	Survey	Species (*n*)	Cat.	∑DDx		∑HCHs		∑Chls		∑HptChls		∑OCPs	
				DR%	Mean (range)	DR%	Mean (range)	DR%	Mean (range)	DR%	Mean (range)	DR%	Mean (range)
KNP	SUR1 (Nov 2011)	*P. edulis* (15)	ST	20	4.45 (0.8 – 11.6)	0	ND	40	11.09 (1 – 42.6)	0	ND	40	13.32 (1.2 – 42.6)
		H. ornata (10)	ST	60	3.98 (1.1 – 12.1)	0	ND	60	10.13 (1.7 – 21.3)	0	ND	70	12.1 (2.4 – 33.5)
		S. garmani (7)	ST	0	ND	0	ND	43	3.94 (1.5 – 8.6)	0	ND	43	3.94 (1.5 – 8.6)
		C. xerampelina (29)	TD	31	3.67 (0.9 – 7.3)	17	3.94 (1 – 8.7)	3	7.17 (single value)	10	3.35 (0.5 – 5.9)	38	6.36 (1 – 12.4)
NGR	SUR2 (Nov 2012)	*C. xerampelina* (4)	TD	100	1.44 (0.5 – 2.7)	100	0.36 (0.2 – 0.6)	0	ND	0	ND	100	1.8 (0.9 – 3.3)
		P. anchietae (6)	SA	33	0.58 (0.4 – 0.7)	50	0.85 (0.2 – 2)	0	ND	0	ND	67	1.18 (0.4 – 3)
		H. tuberilinguis (2)	SA	100	2.16 (2 – 2.3)	0	ND	0	ND	0	ND	100	2.16 (2 – 2.3)
		X. muelleri (6)	A	100	3.2 (0.7 – 6)	67	2.22 (0.5 – 3.7)	0	ND	0	ND	100	4.68 (0.7 – 9.58)

Table 3.1 (continued)

Site	Survey	Species (*n*)	Cat.	∑DDx		∑HCHs		∑Chls		∑HptChls		∑OCPs	
				DR%	Mean (range)	DR%	Mean (range)	DR%	Mean (range)	DR%	Mean (range)	DR%	Mean (range)
NGR	SUR3 (Apr 2013)	*S. garmani* (11)	ST	91	2.23 (1.3 – 7.1)	82	0.65 (0.1 – 1.6)	0	ND	0	ND	100	2.56 (1.3 – 7.2)
		C. xerampelina (10)	TD	100	[a] 9.33 (3.2 – 33.2)	100	0.23 (0.07 – 0.5)	0	ND	0	ND	100	[a] 9.5 (3.4 – 33.3)
		P. anchietae (9)	SA	44	1.39 (0.03 – 3.3)	100	1.6 (0.4 – 3.8)	0	ND	0	ND	100	2.25 (0.4 – 4.4)
		X. muelleri (11)	A	100	1.72 (0.03 – 9.7)	100	2.15 (0.9 – 7)	0	ND	36	0.48 (0.1 – 1.1)	100	4.05 (1 – 14.1)
	SUR4 (May 2016)	*X. muelleri* (12)	A	83	27.3 (13 – 51)	0	ND	0	ND	0	ND	83	27.3 (13 – 51)

3.3.1.1 Spatial differences

No spatial differences were recorded in total OCP concentration between the two species overlapping between conservation areas, *C. xerampelina* and *S. garmani* (Table 3.1, Figure 3.2). Accumulation profiles in all species showed the presence of chlordane in KNP frogs that was below detection limit in NGR frogs and the presence of HCHs in NGR frogs that was not detected in KNP frogs. Exceptions to this include the HCHs not detected from *X. muelleri* (survey four) and from *H. tuberlinguis* (survey two) in NGR and the presence of HCHs in *C. xerampelina* from KNP during survey one. The detection rate for any of the 22 analysed OCPs for the overlapping species from NGR were 100% for both *C. xerampelina* and *S. garmani*, whereas the same species from KNP showed detection rates < 45%. Compositional correlation analysis between overlapping species did not indicate a positive correlation for *C. xerampelina* or *S. garmani* between KNP and NGR, in fact these comparisons showed weak negative correlation (Table 3.2). In terms of contributing OCPs only *p,p*-DDE and *γ*-HCH were shared between *C. xerampelina* from the two regions where *S. garmani* had no common OCPs between the two regions (supplementary Figures A1 and A2).

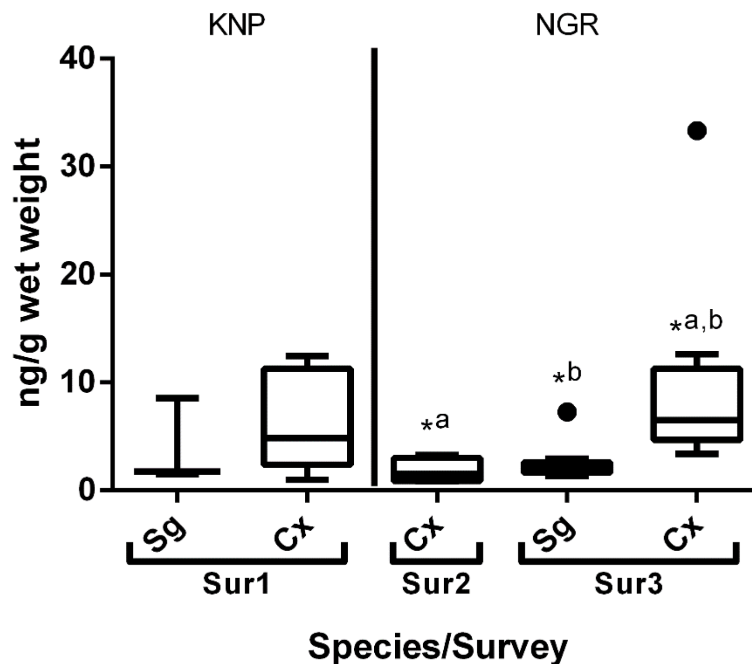

Figure 3.2 **Box plot (Box = 1st and 3rd quartile with line at the mean, whiskers = 5th and 95th percentile) comparison between total OCPs in Kruger National Park (KNP) and Ndumo Game Reserve (NGR) based on bioaccumulation in two anuran species, *C. xerampelina* (Cx) and *S. garmani* (Sg) collected in November 2011 (KNP), November 2012 (NGR), and April 2013 (NGR). Only species that overlap between sites were included. Shared letters (a, b) represent significant difference (α < 0.5) between two groups**

3.3.1.2 Temporal changes

The DDx (sum of all DDT isomers and metabolites) concentration in *X. muelleri* from NGR showed significant temporal changes between seasons with a statistically significant ($p < 0.0001$) increase toward May 2016 compared to previous April surveys (Figure 3.3). Compositional correlations were indicated through moderate to strong positive correlations (All Pearson's $R > 0.55$) between the same species from different surveys within NGR (Table 3.2).

Table 3.2 Correlation table indicating compositional correlations (Pearsons R) for relative contributions of organochlorine pesticides (OCPs) in terms of total OCP accumulation measured in frogs from two conservation areas in South Africa. Survey 1 (SUR1) was conducted in Kruger National Park (KNP). Survey 2, 3, and 4 (SUR2, 3, and 4) were conducted in Ndumo Game Reserve (NGR). SG = *Sclerophrys garmani*; PE = *Pixycephalus edulis*; HO = *Hildebrantia ornata*; CX = *Chiromantis xerampelina*; PA = *Ptychadena anchietae*; HT = *Hyperoluis tuberlinguis*; XM = *Xenopus muelleri*. Grey shading indicates correlations between species within the same survey (indicating compositional similarity between species). Dark blue shading indicates correlations between the same species from NGR over different surveys (indicating temporal compositional similarity). Dark red shading indicates correlations between the same species from different conservation areas (indicating spatial compositional similarity). Significant correlations (Pearson's R values > 0.5 and $p < 0.05$) are indicated with *

	SUR1-SG	SUR1-PE	SUR1-HO	SUR1-CX	SUR2-CX	SUR2-PA	SUR2-XM	SUR2-HT	SUR3-XM	SUR3-PA	SUR3-SG	SUR3-CX
SUR1-PE	***0.97**											
SUR1-HO	***0.83**	***0.95**										
SUR1-CX	-0.04	-0.01	0.03									
SUR2-CX	-0.16	-0.001	0.19	-0.11								
SUR2-PA	-0.18	-0.09	0.02	-0.06	***0.54**							
SUR2-XM	-0.12	0.14	0.43	0.06	***0.62**	0.35						
SUR2-HT	-0.12	-0.07	-0.01	-0.19	0.13	0.45	0.14					
SUR3-XM	-0.15	0.02	0.23	-0.04	***0.77**	***0.88**	***0.68**	0.29				
SUR3-PA	-0.11	-0.1	-0.09	-0.06	0.45	***0.79**	0.1	-0.04	***0.72**			
SUR3-SG	-0.14	0.1	0.37	0.002	***0.77**	***0.69**	***0.87**	0.42	***0.9**	0.36		
SUR3-CX	-0.12	0.09	0.33	-0.02	***0.55**	***0.59**	***0.74**	***0.7**	***0.68**	0.06	***0.9**	
SUR4-XM	-0.08	0.19	0.49	0.12	***0.6**	0.33	***0.96**	0.18	***0.65**	0.01	***0.88**	***0.79**

3.3.1.3 Species differences

The frogs from survey one and two showed no statistically significant difference between species (within each survey) for total OCP concentration. In survey three from NGR *C. xerampelina* showed significantly higher total OCP levels than all three other species, *P. acnhietae*, *S. garmani*, and *X. muelleri* (Table 3.1). Significantly higher DDx concentration in *C. xerampelina* was the main reason for this difference. Heptachlors (consisting of both *cis*- and *trans*-heptachlor epoxide isomers) were detected only in *C. xerampelina* from KNP and *X. muelleri* from survey 3 in NGR (supplementary Figures A1 and A2).

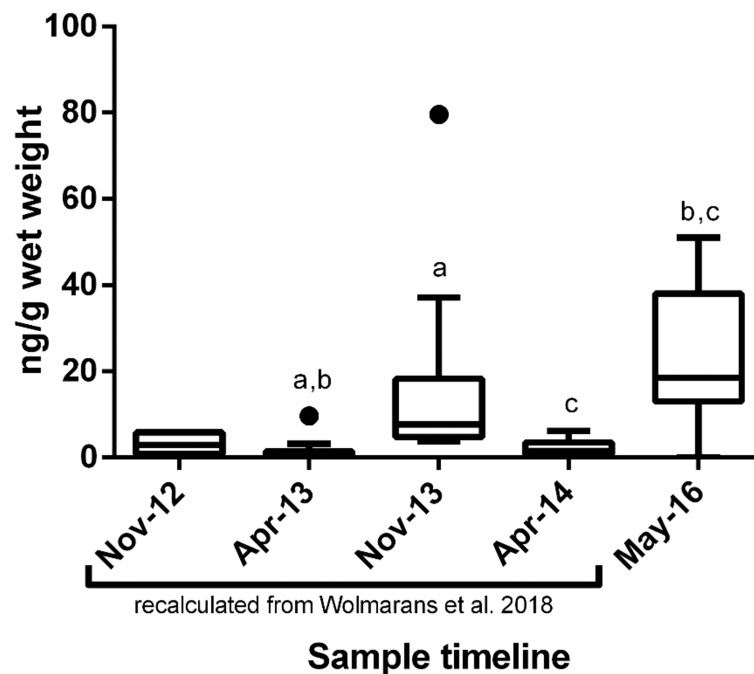

Figure 3.3 **Box plot (Box = 1st and 3rd quartile with line at the mean, whiskers = 5th and 95th percentile) of the temporal variation in DDT bioaccumulation (ng/g wet weight; detected residues only) in *X. laevis* from NGR. Concentrations for November 2012 to April 2014 were recalculated in terms of wet weight from data in Wolmarans et al. (2018). Shared letters (a – c) represent significant difference (α < 0.05) between two groups. Note: May 2016 samples were analysed as whole animals with the inclusion of livers whereas all other samples were analysed without their livers and muscle from one leg**

Compositional correlation analysis between species within survey one showed no correlation (Pearson's $R < 0.01$) between *C. xerampelina* and all other species from that survey (Table 3.2). The *C. xerampelina* samples contained α-HCH, *trans*-heptachlor epoxide, and *o,p*-DDE, as major contributing compounds (> 10% each) that were below detection limits for the other species (supplementary Figure A1). The other three species, *P. edulis*, *S. garmani*, and *H. ornata* (all three in the semi-terrestrial category), had very similar compositions to each other with Pearson's $R \geq 0.83$ between all three species and OCP load consisting only of *trans*-chlordane and *p,p*-DDE. The NGR species' compositional correlations within survey two showed mostly similar OCP compositions between *C. xerampelina* and both *P. anchietae* and *X. muelleri* (Pearson's $R \geq 0.54$; Table 3.2). These correlations are attributed to the major presence of *p,p*-DDE and γ-HCH in each of these species (supplementary Figure A2). *Ptychadena anchietae* did not show significant correlation to *X. muelleri* and *H. tuberlinguis* (Pearson's $R > 0.35$, $p > 0.1$). *Hyperolius tuberlinguis* composition showed even lower correlation to that of both *C. xerampelina* and *X. muelleri* (Pearson's $R = 0.13$ and 0.14 respectively). The survey three species form NGR showed high similarity in OCP composition with *X. muelleri* having a high correlation (Pearson's $R > 0.68$) with all three other species (*C. xerampelina*, *P. anchietae*, and *S. garmani*; Table 3.2). Furthermore, *C. xerampelina* showed strong correlation with *S. garmani*, but *P. anchietae* did not show significant correlation to either *C. xerampelina* or *S. garmani*. Correlations between species in this survey were attributed to *p,p*-DDE, *p,p*-DDT, and γ-HCH as major contributing OCPs (supplementary Figure A2).

3.3.1.4 Discriminant function analysis

The DFA results indicated a partial separation of groups (species per survey) based on OCP contributions across the first two functions with *C. xerampelina* from survey one and *H. tuberlinguis* from survey two showing distinct separation from other groups (supplementary Figure A3). The first five functions significantly explained variance in the OCP contribution data ($\alpha < 0.01$ based on Wilks' Lamda). *trans*-Chlordane was the main OCP correlating with

the first function. Function two and three did not have strong correlations with singular OCPs. Function four showed strong correlation with *p,p*-DDD and *p,p*-DDE and function five showed strong correlation with p,p-DDT and γ-HCH contributions (supplementary Table A1). Overall 61.7% of the sample groups (species per survey) were reclassified correctly based on function one and two with 66.8% of variance explained across these two functions. The first five functions cumulatively explained 96% of the variance in the data. *Sclerophrys garmani* from survey one, *H. tuberlinguis* from survey two, *C. xerampelina* from survey three, and *X. muelleri* from survey four were all correctly reclassified for 100% of samples (supplementary Table A2). This analysis indicated no distinct grouping or separation based on habit/habitat based species classes. Spatial difference was the main factor separated along the first function with the exception of *C. xerampelina* from KNP grouping closer to NGR frogs. Minor species separation is shown along the second function.

3.3.1.5 Isomeric ratios

Isomeric ratios of importance were calculated that have been shown to serve as indicators of exposure age, type, and distance from source in some scenarios (Table 3.3). The ratios between *p,p-*/*o,p*-DDx (DDT + DDD + DDE) were > 2 for all species from both locations except for *C. xerampelina* from KNP which had a ratio of 0.8 slightly favouring the *o,p-* isomers of DDT and its metabolites. The parent/daughter (DDT/DDx) compound ratio between DDT itself and metabolites (DDD + DDE) indicated ratios > 1 for *H. tuberlinguis* in survey 2 and *P. anchietae* in survey 3 both from NGR. All other NGR frogs except *X. muelleri* from survey 4 contained parent *p,p-* and *o,p*-DDT, whereas none of the KNP frogs contained any parent DDT. The *α*-HCH/*γ*-HCH ratios were only applicable in *C. xerampelina* from KNP, as it was the only species from that region with HCH accumulation, but this species had a ratio of 3.3 where all NGR frogs had *α*-HCH/*γ*-HCH ratios < 1. The *trans-*/*cis-* CHLs ratios (chlordanes + heptachlors) were > 1 in all species containing these OCPs, but where markedly higher in KNP (KNP > 16 vs. NGR < 6) frogs due to the dominant presence of *trans*-chlordane.

3.3.2 Secondary pesticide screening

Out of the 538 analysed pesticides (supplementary Tables A3 and A4), only DDx (as *p,p*-DDE) was detected in samples from the May 2016 NGR survey.

Table 3.3 **Isomeric ratios of importance for OCP accumulation in frogs from Kruger National Park (KNP) and Ndumo Game Reserve (NGR) over four surveys based on mean accumulation per species per survey. SUR1 = Survey 1 (KNP), SUR2 = Survey 2 (NGR), SUR3 = Survey 3 (NGR), SUR4 = Survey 4 (NGR). SG = *Sclerophys garmani*, PE = *Pixycephalus edulis* HO = *Hildebrantia ornata*, CX = *Chiromantis xerampelina*, PA = *Ptychadena anchietae*, HT = *Hyperolius tuberlinguis*, XM = *Xenopus muelleri*. N/A = not applicable. a = CHLs in this instance includes both chlordanes and heptachlors**

Species	*p,p*-/*o,p*-DDT	*p,p*-/*o,p*-DDD	*p,p*-/*o,p*-DDE	*p,p*-DDT/(DDD+DDE)	*o,p*-DDT/(DDD+DDE)	α-HCH/γ-HCH	*trans*-/*cis*-CHLs[a]
SUR1-SG	N/A	N/A	N/A	N/A	N/A	N/A	79.4
SUR1-PE	N/A	N/A	89.4	0.011	N/A	N/A	222
SUR1-HO	N/A	N/A	137	0.007	N/A	N/A	174
SUR1-CX	N/A	N/A	0.8	0.018	0.015	3.33	16.7
SUR2-CX	0.5	3.3	32.1	0.22	7.94	0.07	N/A
SUR2-PA	5.18	N/A	8.02	0.57	N/A	0.49	5.82
SUR2-HT	50.7	20.4	15.1	1.42	N/A	N/A	N/A
SUR2-XM	0.13	9.44	112	0.008	3.75	0.47	N/A
SUR3-SG	4.11	2.39	54.1	0.42	2.86	0.05	N/A
SUR3-CX	19.15	8.98	203	0.72	4	0.11	N/A
SUR3-PA	4.73	N/A	13.24	0.77	1.16	0.19	N/A
SUR3-XM	2.52	8	177	0.24	8.88	0.01	4.28
SUR4-XM	N/A	N/A	910	0.001	N/A	N/A	N/A

3.4 Discussion

Gaining an understanding of the threat amphibians face with regard to pesticide exposure in conservation regions, and how this exposure relates to location and species, can produce more reliable risk data for future conservation efforts to build upon. In assessing how these concentrations differ between regions, between species, and between amphibians and other taxa the partitioning of these pesticides in the food web and movement through the environment can be better understood.

Organochlorine pesticide accumulation in KNP from the current study are comparable to that of NGR in terms of total OCP concentrations. DDx has previously been measured in *Xenopus laevis* and *X. mueleri* from Limpopo outside the KNP conservation area by Viljoen et al. (2016), but this study analysed frog lipid bodies resulting in values more directly comparable to lipid concentrations rather than whole body wet mass measurements. Lipid content could, however be influenced by physiological species differences. For instance, species making use of brumation and estivation rely on lipid storage for energy during these dormant periods and increase lipid storage during active periods (Fitzpatrick 1976), thus lipid based concentrations may not reflect the most accurate total bioaccumulation of pesticides for comparison between species. It is worth noting that lipid mass conversions of the current study (supplementary table A5) resulted in all species containing DDx from KNP and NGR having similar (within the same order of magnitude) DDx concentrations to the levels reported by Viljoen et al. (2016). The frogs from Viljoen et al. (2016) included *p,p*-DDE and *p,p*-DDD, but no *p,p*- or *o,p*-DDT itself, even from sprayed areas. This was also the case for frogs from KNP in this study that only included detectable *p,p*-DDE (and *o,p*-DDE only in *C. xerampelina*), whereas frogs from NGR included *p,p*- and *o,p*-DDT, *p,p*-DDD, and *p,p*-DDE.

Compared to results of the studies by Lambert (2001b) on pesticide loads in amphibians from Sub-Saharan Africa measured in sprayed (agricultural) areas during the period when OCPs

were still in active use, the current levels are 1000 times lower than that recorded in *P. anchietae* and *Sclerophrys guttaralis* individuals (maximum levels of 1.5 and 3.9 µg/g ww respectively), indicating a significant reduction in amphibian exposure to DDT since its ban for agricultural and unregulated vector control use. Amphibians form a linkage between the aquatic and terrestrial food webs, but are not considered apex predators in either (Kupfer et al. 2006). This unique position also means they can be exposed to pollutants via both aquatic and terrestrial pathways (Todd et al. 2011). Concentrations in frogs from the current study compared to concentrations in the aquatic apex predator, African Tigerfish (*Hydrocynus vittatus*), from NGR (Volschenk et al. 2019) and KNP (Gerber et al. 2016) indicate this complexity in the trophic transfer of OCPs. The mean concentration of DDx in frogs from the current study were in the same order of magnitude as levels measured in *H. vittatus* (mean values: 2 – 12 ng/g wet mass) for NGR, but one order of magnitude lower than levels in *H. vittatus* (mean values: 12 – 35 ng/g wet mass) for KNP. Total chlordane was one order of magnitude higher in frogs than in *H. vittatus* (mean values: 0.7 – 1.5 ng/g wet mass) from KNP. Total chlordane was below detection limit in NGR frogs, but was present at low levels in *H. vittatus* (mean values: 0.01 – 0.05 ng/g wet mass). Total HCHs were similar between frogs and *H. vitattus* from both KNP (*H. vitattus* mean values: 1.5 – 2.3 ng/g wet mass) and NGR (*H. vitattus* mean values: 0.01 – 0.2 ng/g wet mass).

These comparisons place frogs at similar accumulation levels as aquatic top predators from the same regions even though they hold much lower trophic positions. The reason for the poor differentiation in accumulation at different trophic positions can be attributed in part to different habitats employed by these animals as Tigerfish occur in the mid to top water column, are active swimmers and do not interact with sediments to the extent amphibians do. A secondary, but likely smaller aspect, is the terrestrial exposure pathways that frogs have and *H. vittatus* does not. Although *H. vittatus* has been shown to consume terrestrial birds in some cases (O'Brien et al. 2014), they are still mainly piscivores (Dalu et al. 2012). The increased chlordane levels in amphibians from KNP (compared to *H. vittatus*) could indicate that

chlordane is mostly transferred through the terrestrial habitat (i.e. through terrestrial atmospheric deposits, spray drift, or diet) in this region and not through the aquatic ecosystem. A counter argument to this is the fact that aquatic ecosystems are seen as sinks for OCPs (Arias et al. 2011) and therefore some form or metabolite of chlordane would be expected in the aquatic ecosystem. In this case biotransformation differences between taxa would most likely be responsible for the chlordane concentration differences as frogs and fish do not express the same biotransformation enzymes (*see* Nelson 2009 for CYP450 genomes of different taxa). The accumulation of OCPs in crocodiles from KNP (Gerber et al. 2021) indicated only the presence of nonachlor as a metabolite of chlordane. Crocodiles have higher sediment interaction than *H. vittatus*. This supports the possibility that sediment contact differences would lead to differences in chlordane accumulation patterns between these taxa when coupled with the notion that biotransformation differences between the taxa can cause the differentiation in chlordane accumulation patterns. The Gerber et al. (2021) data do not however exclude the possibility of terrestrial exposure as crocodiles are apex predators in both terrestrial and aquatic ecosystems. The terrestrial exposure pathway possibility for chlordane specifically is somewhat supported by the lower DDx concentrations in frogs compared to *H. vittatus* from KNP. These concentrations follow the expected result for an aquatic exposure route based on trophic differences between the taxa, where DDx in *H. vittatus* is expected (and was measured) at higher concentrations than the partially exposed frogs in comparison (as no fully aquatic frogs were collected at KNP).

An additional factor in comparisons between these different taxa is the fact that for both Tigerfish and crocodiles dietary uptake is the main exposure route to these pollutants based on their status in the food web and behavioural patterns. While exposure pathways in amphibians include both dermal and dietary routes, dermal uptake has been identified as the most important pesticide exposure route for amphibians (Smith et al. 2007; Brühl et al. 2011). This is due to the high permeability of their skin. Terrestrial exposure pathways are likely to include dietary exposure and dermal contact with contaminated soil (or other surfaces such

as house walls). Aquatic exposure includes dietary pathways and dermal contact with contaminated water, but in terms of more hydrophobic pollutants such as *p,p*-DDT dermal contact with contaminated sediment is very likely the main exposure pathway.

3.4.1 Spatial differences

The unique profile of *C. xerampelina* samples from KNP was partly due to being the only dataset from both sites with *α*-HCH/*γ*-HCH ratio > 1. Technical grade HCH contains majority *α*-HCH (60-70 %) and *α*-HCH/*γ*-HCH ratio somewhere between 4 and 7 (Itawa et al. 1993). Under anaerobic conditions HCHs in sediments were shown to follow degradation order of *γ*-HCH > *α*-HCH > *δ*-HCH > *β*-HCH (Buser and Müller 1995). Long range air transport studies of HCHs in oceanic air suggest that the *α*-HCH/*γ*-HCH ratio increases with distance from exposure source with mid oceanic air ratios usually > 7. This increase is attributed to photodegradation of *γ*-HCH to *α*-HCH (Itawa et al. 1993). Contrary to this, a low ratio (close to zero) can be indicative of "pure" lindane as the source (Itawa et al. 1993). This would suggest that uptake of legacy technical grade HCH from sediments would still contain a majority of *α*-HCH and thus *α*-HCH/*γ*-HCH ratio > 1. Diffuse exposure entailing long range aerial transport would further increase the ratio. The *C. xerampelina* from KNP HCH profile shows a slightly lower ratio than expected from technical grade HCH, which could indicate the presence of both technical grade HCH and lindane as combined sources. This is distinct from The NGR samples where all species had ratios < 0.5 indicative of legacy lindane exposure (Itawa et al. 1993). Large scale lindane use historically included cattle dip for tick control, being sprayed on trees in forestry for wood borer control (Koerber 1976; Hauzenberger 2004), and large area spraying for tstetse fly control (Grant 2001). If lindane was in frequent use for tick or tsetse fly control in the NGR region, and remained in use almost a decade after most other OCPs, this could explain the relatively high (compared to DDT that is still in use) levels of *γ*-HCH measured in this region. The upper catchment of the Phongolo River is also highly utilised for subsistence agriculture and forestry (De Necker et al. 2020), which could also be a source of legacy lindane to the region. The dominant presence of *trans*-chlordane in KNP

frogs compared with trace presence of only *trans*-heptachlor epoxide in frogs from NGR is a further indication of different exposure profiles between the regions. While heptachlor itself was also used as insecticide, heptachlor epoxides can be formed through the breakdown of both heptachlor and chlordane parent compounds (Buser and Müller 1993). This suggests that NGR either has a much older chlordane use legacy, or that mainly heptachlor was used in this region. Chlordane use on cotton fields in the Phongolo River region could be a possible historical source. Studies on different fish species have indicated differences in isomeric preferential accumulation of chlordane. Channel catfish (*Ictalurus punctatus*) were shown to accumulate *cis*-chlordane more than *trans*- chlordane and to not accumulate oxychlordane, which usually accumulates as the ultimate metabolite of chlordane in mammals (Murphy and Gooch 1995). On the other hand, Carp (*Cyprinus carpio*) was shown to preferentially accumulate the *trans*- isomer over *cis*-chlordane (Seemamahannop et al. 2005). A study on the degradation of heptachlor through both photo-degradation and mixed function oxidase (MFO) system reactions yielded *cis*-heptachlor epoxide to a larger extent than *trans*-heptachlor epoxide from both degradation methods and the photo-degradation of *trans*-chlordane yielded very low degradation products in comparison to *cis*-chlordane (Buser and Müller 1993). The isomeric ratios of chlordane accumulation in frogs have not yet been assessed in controlled exposure experiments to the best of our knowledge. Data from the current study seem to indicate a non-species specific preference for the accumulation of *trans*-isomers, while also not showing accumulation of oxychlordane as the ultimate chlordane metabolite. However, evidence of oxychlordane accumulation as the major chlordane metabolite has been shown in field samples of two Japanese frog species, *Rana ornativentris* and *Rana japonica* (Kadokami et al. 2004), indicating that species specific differences may exist in this regard.

Both chlordane, lindane, and DDT were produced in the Gauteng Highveld area, approximately 400 km west of KNP with DDT production ending in the 1980's (formulation continued until 2010), and chlordane and lindane production ending around 2001 (DEA 2011).

Industrial air pollution from the Highveld region of South Africa (including Gauteng) has been shown to cause acid rain in KNP (Mphepya et al. 2006), and prevailing winds in this region of South Africa enter the KNP region from Mozambique, and circulate over industrial and agricultural regions of the Highveld back toward KNP (Kruger et al. 2010). Furthermore the rivers flowing through KNP all have catchments closer to Gauteng surrounded by agricultural land where legacy OCP use and production could have attributed to aquatic transport into KNP. The accumulation profiles along with wind patterns and river catchment layout suggest that the chemical profiles of frogs from KNP could largely be due to long range transport (both aerial and aquatic) of legacy OCPs stemming from historical production and use in both the Highveld region of South Africa and southern Mozambique. This hypothesis is supported by the lack of evidence for direct exposure from current IRS around KNP. Only DDE (mostly *p,p*-DDE) was detected in samples from KNP whereas the majority of NGR samples contained both parent *p,p*- and *o,p*-DDT. Results from Bouwman et al. (2019) indicate that parent DDT is unlikely to end up in aquatic systems due to IRS. If IRS is in fact an unlikely source, illegal continued agricultural use of DDT seems a viable scenario that would result in the presence of parent *p,p*-and *o,p*-DDT in aquatic organisms. This is especially likely in cases where *p,p*-DDT concentrations exceed that of *p,p*-DDE, which is considered the more persistent ultimate metabolite. In the current study *p,p*-DDT concentrations only exceeded the sum of *p,p*-metabolite (DDD and DDE) concentrations for *H. tuberlinguis* from NGR during survey 2. As this is not an across-the-board phenomenon for NGR samples the results in no way conclusively indicate illegal continued DDT use in the region. These isomeric ratios do however place DDT input at NGR on a more recent timeline than that of KNP as the presence of only *p,p*-DDE suggests much older input (Ruggirello et al. 2010). Interestingly *o,p*-DDE was only detected in *C. xerampelina* from KNP where it had the largest relative contribution to total OCP concentration (42.5%) in that species. Majority presence of *o,p*- isomers (i.e. higher concentrations than *p,p*- isomers) can in some cases be an indicator of dicofol exposure, which often contains *o,p*-DDT impurities in its commercial formulations, rather than technical grade DDT which contains majority *p,p*-DDT and only around 25% *o,p*-DDT (Qiu et al. 2005;

Quinn et al. 2011). Atmospheric long range transport of technical grade DDT would however be expected to lower the *p,p-/o,p-* ratio as o,p-DDT is more mobile in air (Van Dyk et al. 2010). Quinn et al. (2011) confirmed that dicofol is a registered insecticide in South Africa mainly for fruit cultivation and garden use, and measured the trace presence (≤ 0.06 ng/g) of dicofol in both soil and sediment from the Vaal-Orange River system in South Africa. The region surrounding KNP to the south is used for citrus farming (Gerber et al. 2021) and the tree dwelling *C. xerampelina* may possibly have come in contact with dicofol from these farms, but the extent of migration for this species has not yet been documented and dicofol itself was not analysed in this study, thus this remains conjecture and cannot be confirmed. Long range atmospheric transport of technical grade DDT is the more likely exposure scenario for KNP in this regard.

3.4.2 Species differences

Species categories chosen related in large part to their association with water. Differentiation in OCP concentration at category level that is not seen at species level would therefore substantiate whether OCP exposure routes can be attributed to being mainly through the terrestrial ecosystem or mainly through the aquatic ecosystem.

The unique OCP signature of *C. xerampelina* from KNP along with the significantly higher total OCPs in *C. xerampelina* from the third survey (compared to other species in that survey) indicate that *C. xerampelina* have some tendency to differ in exposure from other frog species. However, these differences are inconsistent between locations. It is possible that the soaking behaviour of *C. xerampelina* females during the mating season (Minter et al. 2004) can explain higher contact with sediment and water and higher exposure in this regard as seen in NGR, but sex data was not recorded and thus this cannot be confirmed. This species also moves toward and away from water sources between summer and winter months, spending winter months hidden beneath bark, in evergreen trees, rock cracks, as well as the rafters of buildings (Minter et al. 2004). This behaviour could bring individuals into closer contact with exposure

sources, but does not necessarily explain the vastly different accumulation profile observed in KNP, as closer contact with current use DDT sources would have resulted in parent *p,p*-DDT accumulation. Aquatic systems (water and sediment) tend to contain higher *p,p*-/*o,p* isomer ratios than soil and air (Ricking and Schwarzbauer 2012). If the tree dwelling habits of *C. xerampelina* makes exposure through air contact more likely than in ground dwelling or aquatic species, it could also contribute to the unique *p,p*-/*o,p*- signature observed in that regard. Another species specific observation is that the concentration of DDx in *P. anchietae* was detected at lower rates than other species from NGR. The low detection rate is somewhat contradicted by the fact that *P. anchietae* individuals that did contain DDx also had high parent/daughter ratios indicating recent exposure to DDT (Rugerillo et al. 2010). The natural behaviour and habitat of this species (Minter et al. 2004) does not provide any sufficient explanation to such high variation in exposure between adult individuals. *Xenopus muelleri*, as aquatic species, had the lowest *p,p*-DDT parent/daughter ratio of all NGR species. Binding to aquatic sediments can lengthen the environmental lifetime of DDT (Chattopadhyay and Chattopadhyay 2015). It is possible that this could result in aquatic (sediment dwelling) species being exposed to lower parent/daughter ratios for *p,p*-DDT (than expected from current DDT use) in areas where historic DDT use has occurred. This is mainly because *p,p*-DDE is resistant to biotransformation leading to build-up of DDE in aquatic systems (USEPA 1979, Chattopadhyay and Chattopadhyay 2015). The parent daughter ratio of *X. muelleri* from NGR are similar to that of *Synodontis zambezensis* (≈ 0.42) from NGR analysed by Volschenk et al. (2019). This fish species has similar sediment dwelling and feeding habits to that of *X. muelleri*. The DDT parent/daughter ratios in the aquatic *X. muelleri* and unique aspects to the OCP profile of the tree dwelling *C. xerampelina* were the only species differences strongly related to the habitat and habit based categories of frogs, although the major presence of chlordane in KNP semi-terrestrial frogs could also be attributed to the habitat category, albeit a location specific occurrence, based on the terrestrial exposure theory presented for this region and contrasting accumulation profile in the tree dwelling *C. xerampelina*. The unique separation of *H. tuberlinguis* from other species based on OCP composition in the DFA

indicates this species also has unique exposure aspects not encompassed by the current habit and habitat based categories. If extensive species specific behavioural observations are made in future studies a more refined classification system may be obtained, which should have better predictive potential for OCP exposure in amphibians.

3.4.3 Amphibian conservation implications

As large scale removal of legacy OCPs from the environment is not viable, there is no immediate action that can be taken to reduce exposure to these pollutants. However, it is important that these levels be monitored continuously. The results from this study indicate that amphibians are susceptible to OCP exposure even inside conservation regions. The presence of parent *p,p*-and *o,p*-DDT in frogs from NGR indicates that proximity to input sources could be an important factor in the accumulation of DDT in amphibians as NGR applies IRS inside the reserve, however lack of data on possible illegal use (or other inputs) prevents attributing the results to IRS directly. The results from Viljoen et al. (2016) lacking *p,p*-DDT in spraying areas also support that the *p,p*- and *o,p*-DDT in frogs from NGR may be from sources other than IRS. Amphibian health was not visibly affected in either of the study regions suggesting that the accumulation concentrations do not pose a serious threat to amphibian populations, but sub-lethal effects of these pesticides in amphibians have not been investigated to the extent where field measured residue levels can be related to specific effects or lack thereof (*see* Wolmarans et al. 2020). The screening data from survey 4 indicated amphibians in the NGR region are not under threat from current use pesticides to the extent that detectable tissue accumulation occurs. The presence of parent DDT does however place amphibians from NGR at a higher inherent risk than those from KNP with regard to legacy pesticides. Conservation managers should take into account the trans-boundary contamination of pesticides and especially persistent organic pollutants and their potential effects on wildlife in conservation areas. Monitoring programs for these pollutants in and around conservation regions should ideally be implemented to track seasonal fluctuations and long term patterns in both abiotic and biotic concentrations. Only with sufficient monitoring data will the extent of

the hazard held by trans-boundary pollutants in conservation regions be known. There is also an important need for investigation into the sub-lethal effects of these pesticides on amphibians. The unique positioning of amphibians in the food web creates the opportunity to use them as indicators for chemical contamination in the wider ecosystem, but the necessary toxicological data is not yet available.

3.4.4 Study limitations

In an effort to convey accurate results within the correct context there are certain limitations to this study that need to be considered when interpreting the data. The first is that only a single survey was conducted in KNP and thus no inferences can be made regarding temporal changes in this region, whereas the multiple surveys from NGR provide a more complete picture of the variation in environmental exposure from that region. The second limitation is that frogs were not sexed during this study. Sexual differences in organohalogen accumulation can occur in amphibians (Kadokami et al. 2002; Viljoen et al. 2016). This is partly due to maternal transfer of lipophilic compounds altering the bioaccumulation in females (Kadokami et al. 2004). In terms of analytical limitations the screening methods used in survey four had relatively high quantitation limits (due to the nature of screening analyses) that could have masked the presence of other pesticides, and thus this does not conclusively eliminate the possibility of those pesticides being present in the amphibian tissue at trace level.

3.5 Conclusion

The data presented in this study indicate that frogs in conservation regions of South Africa accumulate detectable levels of OCPs. Spatial differences were marked by higher detection of OCPs in NGR than KNP. Compositional differences were also significant between regions with OCP accumulation in anurans from KNP representing that of legacy DDT and chlordane exposure, while OCPs in anurans from NGR indicated recent exposure to DDT and legacy lindane exposure. This study indicates that the proximity to exposure sources could have an

impact on the accumulation of current use DDT in frogs from inside conservation regions, but IRS could not be identified as a definitive source for this accumulation. Other, possibly illegal or accidental, sources need to be thoroughly investigated in the NGR region. In terms of species differences it seems likely that the OCP exposure in different frog species from conservation areas in South Africa is driven by both broader spatial exposure factors as well as species specific differences. These differences are not predictable between species, even with the inclusion of habitat and habit based groupings. Such predictions would require further experimental study and long term exposure monitoring data.

CHAPTER 4 SUB-LETHAL EXPOSURE TO MALARIA VECTOR CONTROL PESTICIDES CAUSES ALTERATIONS IN LIVER METABOLOMICS AND BEHAVIOUR OF THE AFRICAN CLAWED TOAD (*XENOPUS LAEVIS*)

This chapter is under review in Comp Biochem Physiol C Toxicol

4.1 Introduction

There has been a recent resurgence of research into levels of persistent organic pollutants in tropical aquatic ecosystems where malaria vector control is actively carried out using pesticides such as DDT. The presence of DDT and its metabolites has been confirmed in fish (Gerber et al. 2016; Volschenk et al. 2019), aquatic invertebrates (Mbongwe et al. 2003; Govaerts et al. 2018), and frogs (Viljoen et al. 2016; Wolmarans et al. 2018; Wolmarans et al. 2021) from areas where indoor residual spraying (IRS) is applied for malaria vector control. The presence and environmental impacts of other World Health Organisation (WHO) recommended pesticides for malaria vector control (16 pesticides from organochlorine, organophosphate, carbamate and pyrethroid pesticide groups; *see* WHO 2006) have not yet been examined in depth in this region (Quinn et al. 2011), as most previous studies have mainly focused on organochlorine pesticides as the major pollutants (Buah-Kwofie and Humphries 2017; Volschenk et al. 2019). Simultaneous DDT and pyrethroid presence has, however, been previously measured in human breastmilk from malaria vector control (MVC) regions in South Africa, with aquatic ecosystems (water and fish) being identified as a possible vector for these pesticides into humans (Bouwman et al. 2006).

Two pesticides were selected as representatives for malaria vector control pesticides. Dichlorodiphenyltrichloroethane (DDT) as representative of the organochlorine pesticide group, and deltamethrin (DTM) as representative of the pyrethroid pesticide group. While DDT is currently only allowed for use in malaria vector control through IRS in some countries (WHO 2006), Deltamethrin has multiple agricultural uses (Mestres and Mestres 1992; ATSDR 2003).

These two pesticides are often used in conjunction with one another for IRS in South Africa (Brooke et al. 2013).

Both DDT and DTM have broadly similar primary modes of action where binding affects cation channels at neural synapses (Davies et al. 2007; Haschek et al. 2013). Secondary binding activity plays a large role in sub-lethal effects, such as the endocrine disruption activity of DDT (Hascheck et al. 2013). Both primary and secondary binding activity for these pesticides are not extensively studied in non-target organisms such as amphibians.

Dichlorodiphenyltrichloroethane is highly persistent and bioaccumulates through the food web due to its lipophilic nature (Bouwman et al. 2011). Deltamethrin is not considered to be persistent in the environment (ATSDR 2003). The wider agricultural use and consequent multiple sources of DTM to the natural environment could, however, theoretically lead to higher exposure peaks to non-target organisms in the wild. Given the potential hazard presented by these pesticides in the environment and uncertainty as to sub-lethal effects in non-target organisms, the need was identified to assess the sub-lethal effects of these pesticides on non-target anurans. In pursuit of this aim, metabolomics and behavioural analysis were incorporated as possible effect indicators.

Metabolomics is the study of metabolic responses in different parts of the body, and use of these metabolic responses as markers for change in homeostasis caused by events such as disease, dietary changes, or intoxication (Nicholson et al. 1999; Bouhfid et al. 2013). The use of metabolomics in an ecotoxicology context has proven useful in determining the biochemical pathways upon which environmental contaminants act (Labine and Simpson 2020). Metabolomics has previously been used in anuran ecotoxicology to assess the changes in leopard frog liver metabolome towards herbicides, fungicides and insecticides (Van Meter et al. 2018). By assessing metabolomics in conjunction with pollutant exposure the secondary effects on biochemical and physiological pathways can be observed. Through analysis of affected pathways inferences can be made to potential system level (organ functioning), or

endocrine and neurological effects. All these changes can in turn result in behavioural change in the affected individual. An example of this metabolomic link to behaviour can be found in the Alaskan Wood Frog (*Rana sylvatica*). Changes in their hepatic glucose and glycogen levels (stemming from adaptations in gene expression) have been linked to their ability to survive temperatures below 0°C (Do Amaral et al. 2013). In the Wood Frog example the metabolomic adaptation has physiological benefit that allows for extreme behaviour to take place. While expected to be less extreme in outcome, the use of metabolomics in contaminant exposure studies can provide a link between the environmental effects on the individual at a biochemical level as a pre-cursor to physiological change that can subsequently lead to behavioural changes.

Behavioural analysis can also be used as a link between effects on the individual and cascading effects on the population, community, or ecosystem through how the behavioural changes affect the animal's ability to forage, escape predation, reproduce, or survive extreme weather events (Peterson et al. 2017). In many cases behaviour has proven to be a more sensitive "early warning" of toxicity than conventional mortality (LC50) testing based predictions (Hellou 2011). Hellou (2011) also stipulated the importance of fate of chemicals and metabolic abilities of the subject organism in understanding behavioural ecotoxicology in broader terms. Peterson et al. (2017) suggested that the use of behaviour in ecotoxicology should focus on a framework that incorporates causation, ontogeny, adaptation, and phylogeny (Tinbergen's four postulates). According to Peterson et al. (2017), it is only through an integration of our understanding of the mechanisms and causation by which contaminants affect behaviours, along with the effects of these behaviours on fitness, that population level effects of contaminants can be fully understood.

There is a distinct lack of sub-lethal toxicity literature related to anurans and malaria vector control pesticides, particularly in African species (Wolmarans et al. 2020). In this study, with the above mentioned integration framework in mind, we combined chemical accumulation, metabolomics, and behaviour as ecotoxicological endpoints to explore the integrated effects

of these pesticides on the fully aquatic anuran *Xenopus laevis* in a laboratory setting. The African clawed frog (*X. laevis*) was selected as test species for being an established model aquatic anuran as well as having a natural distribution that coincides with areas where IRS is applied in Southern Africa (*for distribution range see* De Villiers and Measy 2017; Du Preez and Carruthers 2017). Based on the biological organisation hierarchy the aims were to link exposure, as measured through pesticide bioaccumulation, to changes in the metabolome that in turn could result in behavioural changes. We further determined whether mixtures of the pesticides would result in additive effects.

4.2 Materials and Methods

4.2.1 Animal husbandry and housing conditions prior to exposure

Xenopus laevis used in this study were bred and housed in a XenoPlus Amphibia housing system (Techniplast) at a density of one adult frog per 2.7 L. The housing system was held within a HEPA filtered recirculating central air conditioned laboratory inside the National Aquatic Bioassay Facility (NABF) of the North-West University, South Africa. An automated system continuously monitored water quality variables and maintained reconstituted water according to set parameters using reverse osmosis filtered water, marine salt (Seachem) and sodium bicarbonate (Merck). Housing conditions were set as: temperature = 23°C (housing room temperature also kept at 23°C); pH = 7.4; and electrical conductivity = 1000 µS/cm. The housing conditions and feeding regime used were in line with international guidelines on optimal housing conditions for *X. laevis* as stipulated by Reed (2005). All frogs were 11 months old at the time that experiments were carried out. Frogs were classified as sub-adult for the purpose of this study as sexual maturity had been reached in some, but external sexual morphology was not present in most animals yet.

4.2.2 Experimental setup and design

4.2.2.1 Pesticide treatments

The two pesticides tested, DDT and DTM, were selected in this study as representatives of IRS pesticides. The scope of this study and ethical limitations on the use of vertebrate animals did not allow for extensive LC50 testing on adult frogs, and no literature containing LC50 data on adult *X. laevis* was found in a recent review (*see* Wolmarans et al. 2020). Therefore, the $LC50_{FETAX}$ values (DDT= 35.7 mg/L and DTM = 0.19 mg/L, Channing 1998; Saka 2004) based on toxicity to larval *X. laevis* were used as a starting point for lethality, even though sensitivity between adult and developmental stages of animals can differ. After initial testing showed mortality at higher concentrations, the exposure concentrations for this study were set at to 1% and 0.1% of the respective $LC50\text{'s}_{FETAX}$ for DDT and DTM where no mortality was observed after 96 h. Fractions of the lethal concentrations were used in order to assess mixtures in terms of equal toxic units. The different exposure treatments used in this study are outlined in Table 4.1. For environmental relevance commercially available formulations of these pesticides were used for exposure treatments.

4.2.2.1.1 Exposure chemicals

The technical grade DDT (AVIDDT 750, Avima) consisted of 71.8% ∑DDT (of which 80.5% = *p,p*-DDT and 19.5% = *o,p*-DDT) and concentrations were prepared in terms of active ingredient (i.e. ∑DDT). For DTM a commercial formulation (Decatix 3, Coopers) was also used consisting of 2.5% m/v deltamethrin and exposure concentrations were also prepared in terms of active ingredient. Stock solutions and dilutions were prepared in fresh housing media.

4.2.2.1.2 Exposure setup

The frogs were allowed to acclimate in the experimental tanks for 2 h prior to exposure (3 L clear polycarbonate tanks) with the temperature gradually being lowered from the housing temperature (23°C) to experimental temperature (20°C) during this period. Static exposures

were performed and animals were not fed during the exposure period. Eight replicates of sub-adult *X. laevis* were individually exposed per treatment. Four of these individuals per treatment were selected prior to exposure and were used for behaviour recordings throughout the exposure.

Control animals underwent the exact same procedure and handling as exposed animals. Behaviour was recorded for 2 h immediately at the onset of the exposure period followed by another 2 h recording initiated at 94 h after exposure. At 96 h the frogs were euthanized through means of mechanical stunning followed by double pithing. Chemical euthanasia methods were not used to reduce the possible effect of euthanasia on the liver metabolome. Liver samples were collected weighed and flash frozen at -80°C for metabolome analysis. The remainder of each carcass was frozen at -20°C for bioaccumulation analysis.

4.2.3 Chemical analysis

Quantification of pesticide bioaccumulation and confirmation of nominal dosing concentrations was performed on a HP 6890 Gas Chromatograph coupled with a micro-Electron Capture Detector (GC-µECD). For dosing concentration analysis, 100 ml water samples (n=4 per treatment) were spiked with 100 µL of 100 µg/L chlorinated biphenyl (CB #143) as internal standard. Liquid-liquid extraction was performed with hexane on two 50 mL sample aliquots, each extracted twice with 50 mL of hexane (Honeywell) in a separation flask (i.e. 100 mL of sample extracted in total at a sample to hexane ratio of 1:2 v/v). The filtered (through sodium sulphate) extract was evaporated down to near-dryness under a gentle nitrogen stream at 34°C in a TurboVap® (Biotage) evaporator. The extract was reconstituted to 100 µL *n*-Decane (Sigma-Aldrich) containing 100 µg/L Dr Erhenstorfer (GmbH) Tetrachloro-m-Xylene (LGC Standards) for final volume calculation before analysis.

Tissue sample extraction method was adapted from Wolmarans et al. (2018). Whole frog carcasses (minus the liver; n = 8 per treatment) were cut into ≈2 x 2 mm pieces, weighed (5-10 g wet weight), and mixed with excess amount of diatomaceous earth for desiccation. Each

sample was spiked with 100 µg/L CB #143 as internal standard. Extraction was done using pressurized liquid extraction using an Accelerated Solvent Extractor (ASE 150; Dionex) at 100°C for three static cycles with acetone:hexane (1:3 v/v) as extraction solvent. Extracted sample volume was reduced in a water bath under gentle nitrogen stream at 34°C. A 10% portion of the sample was removed and air dried in a pre-weighed container for gravimetric lipid content determination. Final clean-up on the remaining 90% fraction was done through solid phase extraction using 6 g of 5% deactivated (m/m; ddH_2O) Florisil® (Sigma-Aldrich) tightly packed in a 40 cm column, prepared with hexane. The sample was eluted with 100 mL hexane:dichloromethane (7:3 v/v), evaporated to near-dryness, and reconstituted in 100 µL n-decane containing 100 µg/L tetrachloro-*m*- xylene for final volume calculation, followed by analysis.

Sample analysis was performed on an HP 6890 GC-µECD. Compound separation was achieved using a 250 µm internal diameter, 0.25 µm film thickness, and 30 m length HT8-MS (SGE) column with H_2 as carrier gas. Splitless injection (1 µL) was used with the inlet temperature set at 225°C. The oven program followed initiation at 100°C which was held for 1 min and ramped up at 20°C/min to 200°C where the ramp was changed to 6°C/min until at 260°C and held for 20 min. Target compounds were identified based on retention time (RT in min: 11.862 = *o,p*-DDE; 12.752 = *p,p*-DDE; 13.131 = *o,p*-DDD; 13.788 = CB#143(IS); 13.908 = *o,p*-DDT; 14.321 = *p,p*-DDD; 15.163 = *p,p*-DDT; 32.225 = DTM). Method validation for both extraction and analysis was performed using Dr Erhenstorfer (GmbH) pesticide mix 1037 (LGC Standards) containing 22 organochlorine pesticide isomers including all six major DDT isomers and metabolites as well as Dr Erhenstorfer (GmbH) Deltamethrin standard (LGC Standards). Instrumental calibration for all DDT isomers and deltamethrin were performed on 7-point calibration curves (averaged over 5 runs) ranging between 5 and 1000 µg/L with $R^2 > 0.999$ for all compounds except Deltamethrin that had an $R^2 = 0.998$. Samples were analysed in batches of seven with quality control standards run between batches. Concentrations reported were adjusted according to recovery of the internal standard added to each sample,

which was 68 ± 11% (mean ± standard deviation) for the analysed water samples and 80 ± 14% for the analysed tissue samples. Instrumental limit of detection (LOD) and limit of quantitation (LOQ) was calculated individually for each compound as 3 times and 10 times (respectively) the standard error of the gradient of the calibration curve. All instrumental LODs were below 50 µg/L.

4.2.4 Metabolome analysis

Frog liver metabolome analyses were performed at the North-West University National Metabolomics Platform (NMP). Metabolite extraction from liver samples was done using a Bligh-Dyer method (Bligh and Dyer 1959) as adapted for wide coverage untargeted metabolomics analysis (Blackwell et al. 2013). Approximately 50 mg of liver (n=8 per treatment) was weighed into the 1.5 mL lock microcentrifuge tube (Eppendorf) and homogenized with methanol (Honeywell), ultrapure water and 50 µg internal standard (3-Phenylbutyric acid with the concentration of 50 µg/mL; Sigma Aldrich) solution by shaking the sample mixture in a mixer mill (Retch) at a frequency of 30 Hz for 2 min, after addition of a 3 mm steel bead. After homogenisation, chloroform (Honeywell) was added, the sample vortexed for 30 s, and phase separation was induced by keeping the sample on ice for 10 min. The ratio of methanol, water (ultrapure; Honeywell) and chloroform was 1:0.8:1. Samples were centrifuged at a speed of 5000 rpm for 5 min at 4°C. Both the top and bottom phase were collected into a glass GC vials and were dried under a light stream of nitrogen. The dried materials were methylated and derivatized using 50 µL methoxyamine hydrochloric acid (Sigma Aldrich) in pyridine (200 mg/mL; Merck) at 60°C for 60 min, followed by 50 µL BSTFA (Sigma Aldrich) with 1% TMCS (Sigma Aldrich) at 40°C for 60 min. The extracts were then transferred into a glass vial inserts and capped for analysis.

Whole metabolome untargeted analysis was performed using Gas Chromatograph coupled with Time-Of-Flight low resolution Mass Spectrometry (GC/TOF-MS). Helium was used as carrier gas at constant flow of 1.5 ml/min. Splitless injection (1 µL) was used with the inlet

temperature at 250°C. Randomized injection order was for samples in batches of nine with quality control samples run after five samples and quality assurance and quality control standards analysed in triplicate between each batch. A Rxi-5Sil-MS (RESTEK) column of 30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness was used for separation. The primary oven temperature program was initiated at 70°C and held for 1 min followed by an initial ramp of 7°C/min to a final temperature of 300°C, which was held for 1 min. Ionization was achieved through electron ionization (EI) at 70 eV with the source temperature at 200°C, and transfer line temperature at 225°C. The selective mass range was between 50 m/z and 800 m/z with an acquisition rate of 20 spectra/s, the detector voltage at 50V with a solvent delay of 260 seconds.

Peak finding and mass spectral deconvolution was performed using Leco Corporation ChromaTOF software (version 4.50) at an S/N ratio of 100, with a minimum of three apexing peaks. Using the mass fragmentation patterns generated by the MS, together with their respective GC retention times, the identities of these peaks were determined by comparing it to commercially available NIST spectral libraries (mainlib, replib). Level three compound identification as per Schymanski et al. (2014) was performed.

4.2.5 Behavioural analysis

For the first 2 h post-exposure, experimental treatments (n=4 per treatment) were recorded with a digital camera (Basler monochrome GigE, 1/1.8" CMOS sensor) at 1280 x 720 pixel resolution at a frame rate of 25 fps. During recordings the exposure room was locked, temperature controlled, and sound insulated to minimize external stimuli. Video recordings were analysed using EthoVision XT 13 (Noldus) behavioural tracking software. Upon analysis tanks were split into two zones (top and bottom) for analysis of zone movement metrics. Mobility state and other movement metrics were also analysed (detailed metrics are given in the Statistical analysis – Behavioural analyses section; Table 4.5).

4.2.6 Statistical analysis

4.2.6.1 Chemical accumulation analyses

Initially zero-exclusion was performed on the data disregarding compounds (per treatment group) with detection rates (values above LOQ) less than 50% for each group. For all further statistical analyses remaining values below LOD were replaced with 0.5 LOD values. Comparison groups were set to compare each exposure treatment to the control treatment. Multiple t-tests were performed on each comparison group coupled with Dunn's post hoc test with p-value adjustment for multiple comparison using the Holm-Sidak method with significance set at $\alpha \leq 0.05$.

4.2.6.2 Metabolomics analyses

Non-parametric analyses for metabolome data consisted of a fold change (FC) analysis performed on untransformed MS peak intensity data. Fold change was determined as follows:

$$sign(\overline{X}_1 - \overline{X}_2).2^{|log_2(\overline{X}_1) - log_2(\overline{X}_2)|};$$

Where $\overline{X}_1$ and $\overline{X}_2$ represent the means, and the sign of the difference between the means are added after calculation indicating the direction of the FC (+ up- and – down-regulation). This analysis was performed for each exposure treatment compared to the control treatment for each detected spectral peak. This analysis provides a sense of practical significance to changes in the screening data with significance set at $|FC| \geq 2$.

Prior to parametric analyses a zero-filter was used excluding all compounds with detection rates below 50% in samples from both groups within a comparison. Log transformed ($Log_{10}X+1$) metabolome data were used to achieve normalisation for parametric testing. The parametric analysis consisted of independent samples multiple t-tests with adjustment for multiple testing (Benjamini Hochberg adjustment for the control of false discovery rates; FDR). Significance was set at adjusted $p < 0.1$ because of the number of variables measured. As a

parametric measure of practical significance in the changes observed, effect sizes (Cohen's –d value) were calculated as follows:

$$|\bar{X}_1 - \bar{X}_2| / \max(S_1; S_2);$$

Where $\bar{X}_1$ and $\bar{X}_2$ represent the means of the groups with S_1 and S_2 representing the standard deviations of the respective groups. Significance was set at at $|d| \geq 0.8$ (Lakens 2013).

Both effect size and FC were used as effect size takes into account the standard deviation between samples whereas the FC is based only on the mean per treatment, but more sensitive to changes in this mean.

A partial least squares-discriminant analysis (PLS-DA) was performed on the metabolome data for each of three pesticide groupings (DDT, DTM, and Mix) in order to assess separation variables between treatments in terms of the severity of exposure. Data included in this analysis were filtered based on effect sizes and FC as previously defined. The PLS-DA models were used to identify predictor variables based on their variable importance in projection (VIP) scores for separation between the control, 0.1%, and 1% treatments for each analysis (and overall separation in final PLS-DA). For predictive ability only VIP scores >1 were considered viable. All metabolomics statistical analyses were performed using Metaboanalyst 4.0 (Chong et al. 2019).

Pathways analysis was performed using the Metaboanalyst pathway analysis package (Xia and Wishart 2010). As *X. laevis* is not a species option within this package the zebrafish, *Danio rerio*, was used as the analysis organism in the model. Onjiko et al. (2016), and Portero and Nemes (2019) provide precedent for the use of *D. rerio* as substitute for assessing *X. laevis* metabolic pathways, but the limitations should be recognised. Only general pathways shared between *D. rerio* and *X. laevis* would be detectable in this manner limiting the power of this analysis in assessing any pathways unique to anurans. Pathway analysis algorithms consisted of pathway enrichment analysis by global test and pathway topology analysis by relative-

between-ness centrality. All pathways referred to in this study were confirmed through the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database (KEGG 2020; *also see* Kanehisa et al. 2010; Tanabe and Kanehisa 2012).

4.2.6.3 Behavioural analyses

Various metrics were calculated from the two hour recordings of the frogs based on centre point tracking data (Table 4.5). The total distance moved and the mean velocity was measured as well as the cumulative duration spent moving, the mean acceleration, and meander. Meander is a factor of turn angle and total distance moved and is thus dependent on the direction of movement in the tank with right handed turns resulting in a positive turn angle and left handed turns resulting in a negative turn angle. The frequency of visits to the top zone as well as the cumulative duration spent in the top zone were also measured. Lastly the frequency of being in and cumulative duration spent in each of three mobility states were measured. An immobile state was set a 0-20% of the maximum velocity. A mobile state was set as 20-60% of the maximum velocity, and a highly mobile state being above 60% of the maximum velocity.

The behavioural metrics in this study were analysed individually using Kruskal-Wallis analyses for comparison between groups coupled with Dunn's post-hoc test for mean rank comparison Significance was set at $p \leq 0.05$. Three sets of comparison groups were analysed for the behaviour data. A time factor was assessed consisting of each treatment at 0 h vs that same treatment at 96 h. Two treatment factors were assessed consisting of the control at 0 h vs every exposure treatment at 0 h, as well as control at 96 h vs every exposure treatment at 96 h. For a measure of practical significance effect sizes (Cohen's –d value) were also calculated in the same manner as defined for metabolomics data. Significance was set at $|d| \geq 0.8$.

A principal component analysis was performed on log transformed ($Log_{10}X+1$) behavioural data to determine group separation based on behavioural metrics and identify possible driving metrics behind group separations.

4.2.6.4 Combined effects analyses

To assess whether links of association could be established between the datasets measured at different levels of biological organisation, redundancy analyses (RDAs) were performed for the various combinations of variable sets (exposure treatment, chemical accumulation, 96h behaviour, and metabolomics) using CANOCO v. 5 software. In dealing with the large number of variables within these datasets interactive forward selection of variables was applied to each RDA in order to identify the most important variables for explaining variation in the data in each analysis. P-value cut-off was set at 0.1.

This analysis was first used to identify the most important behavioural metrics in explaining the variation in pesticide accumulation. This was followed by assessing the most important metabolites in explaining the variation in chemical accumulation. Finally, the metabolite data were selected for explaining the variation in behaviour data. Significant variables selected from these RDAs were then re-analysed and priority variables were identified based on their influence in explaining the variation in the data of each group with a p-value cut-off at 0.1.

4.3 Results

4.3.1 Pesticide concentrations in the exposure medium

Analysis of sample water from the exposure tanks taken directly after exposure (from those not used for behaviour analysis) indicate that both DDT and DTM were present in the water column at detectable levels (Table 4.1).

Table 4.1 Experimental treatments with corresponding nominal concentrations of exposures and measured water concentrations taken from the four tanks, where behaviour was not measured, for each treatment. The percentage of the nominal exposure measured is also included

Treatment	Nominal exposure concentration	Measured concentration (% of nominal)
Control	—	<LOD
DDT 0.1%	*DDT 20 µg/L ≈ 0.1% of $LC50_{FETAX}$	19.3 ± 2.7 µg/L (97%)
DDT 1%	DDT 357 µg/L = 1% of $LC50_{FETAX}$	301.6 ± 41.6 µg/L (84%)
DTM 0.1%	DTM 0.19 µg/L = 0.1% of $LC50_{FETAX}$	0.16 ± 0.07 µg/L (87%)
DTM 1%	DTM 1.9 µg/L = 1% of $LC50_{FETAX}$	1.66 ± 0.37 µg/L (87%)
Mix 0.1%	DDT 35.7 µg/L + DTM 0.19 µg/L	DDT 25 ± 2.8 µg/L (70%) + DTM 0.18 ± 0.4 µg/L (95%)
Mix 1%	DDT 357 µg/L + DTM 1.9 µg/L	DDT 265 ± 9.7 µg/L (74%) + DTM 1.69 ± 0.2 µg/L (89%)

Table 4.2 Concentrations of DDT measured in frog tissue (mean ± standard deviation; ng/g wet weight) with calculated metrics. Statistical significant differences ($p \leq 0.05$) from the control are indicated with * and in bold. Isomeric ratio significance is in comparison to the stock DDT used. Superscript lettering ([a] and [b]) indicate significant differences between exposure treatments for parent/daughter (P/D) ratios

Treatment	BCF	∑DDx	P/D ratio (DDT/DDD+ DDE)	Isomeric ratio (*p,p*-/*o,p*-) stock = 4.13 ± 0.1	***o,p*-DDE**	***p,p*-DDE**	***o,p*-DDD**	***p,p*-DDD**	***o,p*-DDT**	***p,p*-DDT**
Control	-	<LOD	-	-	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
DDT 0.1%	1.09 ± 0.25	21.7 ± 5.0	[a]**0.69 ± 0.83**	4.77 ± 2.07	<LOD	0.35 ± 0.42	***2.1 ± 1.89**	***11.62 ± 2.86**	***1.91 ± 1.68**	5.54 ± 6.44
DDT 1%	0.68 ± 0.34	***244 ± 121**	2.40 ± 1.13	***12.8 ± 6.40**	<LOD	***9.29 ± 2.69**	***18.17 ± 4.74**	***39.5 ± 19.5**	<LOD	***176 ± 102**
Mix 0.1%	0.65 ± 0.3	23.13 ± 10.6	[ab]**17.15 ± 16.8**	3.08 ± 2.26	<LOD	0.34 ± 0.46	0.24 ± 0.27	***1.06 ± 0.92**	*5.77 ± 3.44	***15.55 ± 8.34**
Mix 1%	0.36 ± 0.14	***127.16 ± 49.06**	[b]**1.7 ± 0.69**	***1.28 ± 0.20**	<LOD	***3.43 ± 1.57**	***14.45 ± 8.61**	***30.90 ± 13.24**	***41.82 ± 17.65**	***36.39 ± 18.35**

4.3.2 Bioaccumulation

Deltamethrin was not detected in any of the analysed tissue samples. In some of the DTM 1% and Mix 1% tissue samples small GC-µECD peaks were observed on the chromatograms at the appropriate retention time (RT = 32.225), but these were all well below the instrumental LOD and thus excluded from analysis.

All control, DTM 0.1% and DTM 1% samples did not have any DDT isomers present. For this reason, DTM treatments are absent from accumulation analyses. For statistical analyses purposes control sample values and values below LOD within the DDT and Mix treatments were substituted with 0.5 of the LOD values for each respective compound in all analyses.

The DDT isomers were successfully detected at measurable concentrations in samples from the DDT 0.1%, DDT 1%, Mix 0.1% and Mix 1% exposure groups (supplementary Table B1). Multiple t-tests showed significant differences between all DDT exposed treatments (DDT and Mix groups) compared to control (Table 4.2). The *o,p*-DDE isomer was not detected in any of the samples. The *p,p*-DDE isomer was detected in 100% of the DDT 1% and Mix 1% treatment samples. In the 0.1% treatments for DDT and Mix, *p,p*-DDE was also only detected in one sample, respectively. The *o,p*-DDD detection rate was above 87.5% for all treatments that received DDT except the Mix 0.1% treatment where it was only detected in one sample. For *o,p*-DDT the detection rate was above 50% in the DDT 0.1%, Mix 0.1%, and Mix 1% treatments, but was not detected in the DDT 1% treatment. The detection rates of both *p,p*-DDD and *p,p*-DDT were above 50% for all treatments.

The composition of DDT isomers (Table 4.3) measured in each sample was inconsistent between treatments with large variation within the treatments. The prominent compound in the DDT 1% and Mix 0.1% treatments was *p,p*-DDT making up more than 63% of the ∑DDT. The *p,p*-DDD was the major contributor in the DDT 0.1% treatment (58.8%) while *o,p*-DDT made up only 32.8% of the ∑DDT in the Mix 1% treatment.

Table 4.3 Composition of DDT isomers as percentage of total DDT with the top contributing mean per treatment indicated in {bold}

Treatment	*o,p*-DDE	*p,p*-DDE	*o,p*-DDD	*p,p*-DDD	*o,p*-DDT	*p,p*-DDT
DDT 0.1%	1.64 ± 3.26	1.4 ± 1.43	10.8 ± 8.3	**58.8 ± 22.6**	7.5 ± 6.28	19.9 ± 20.6
DDT 1%	0.13 ± 0.15	6.07 ± 7.37	15.1 ± 23.4	15.1 ± 6.67	0.29 ± 0.33	**63.28 ± 25.75**
Mix 0.1%	0.99 ± 0.73	1.5 ± 1.23	1.27 ± 1.28	5.08 ± 4.52	23.88 ± 10.82	**67.29 ± 11.12**
Mix 1%	0.15 ± 0.06	2.63 ± 0.25	11.07 ± 3.11	25.28 ± 8.06	**32.86 ± 5.1**	28.02 ± 5.36

4.3.3 Metabolomics

The whole metabolome screening analysis detected 562 spectral compound peaks in the frog livers. Based on FC and effects sizes a total of 259 spectral peaks with notable up- or down regulation (i.e. practical significance as defined under statistical analysis) between groups were identified. Of these spectral peaks, 41 showed statistically significant differences to control based on multiple t-tests (supplementary Table B2). The DDT 0.1% and DTM 0.1% treatments had the most spectral peaks (18 and 17 in total, respectively) with statistical significance. The least significant changes, only three peaks, were found in the Mix 0.1% treatment. The DDT 1% and DTM 1% treatments showed significant up- or down-regulation in 13 and 10 metabolites respectively. The Mix 1% treatment also had 10 statistically significant changes, but had only two spectral peaks in common with the individual 1% treatments in terms of statistical significance. In addition to the 42 peaks with statistical significance, 13 peaks were also considered important for being either completely absent or only present in the analysed treatments compared to control (supplementary Table B2). Difficulty in compound identification through the screening databases used, resulted in a large number of unidentified analytes among those that showed significant differences.

The PLS-DA analysis across five components for all DDT treatments showed separation between the control and DDT 1% treatment along the first two components (Figure 4.1A) accounting for 27.7% (20.2% and 7.5% for component one and two respectively) of the variation. The DDT 0.1% treatment did not separate from either the control or DDT 1% treatment, but its distribution was

rather uniformly split between the two other treatments resulting in a clear concentration based shift from the control in the bottom left to the DDT 1% in the top right. Cross validation of the model showed the highest Q_2 value (predictive capacity measure) at only one component (0.60) with the highest accuracy (0.83) at three components. The VIP scores were used to identify the top metabolic variables in predicting concentration groups of DDT exposure across the first two components of which 60 had scores >1. The variable with the highest VIP scores was phosphoric acid, with scores >1.76 for both components. The PLS-DA analysis performed for all DTM treatments showed clear separation between all three treatments (Figure 4.1B) across the first two components accounting for 24.8% (15.3% and 9.5% for component one and two respectively) of the variation. The first and third components (not shown) accounted for more variation (component three explained 13% of the variation), but in this combination the DTM 0.1% treatment showed overlap with the control. Cross validation of the model also showed the highest Q_2 value at five components (0.72) with the highest accuracy (0.91) at two components. The VIP scores were again used to identify the top variables in predicting groups across the first two components, of which 77 had scores >1. L-Sorbitol had the highest VIP scores (>1.9 across the first two components). For analysis of both Mix groups the PLS-DA analysis showed less separation between control and mixture exposure treatments than the individual pesticide treatments. There was slight overlap between the Control and the Mix 1% with the Mix 0.1% once again falling in between the two along the first two components (Figure 4.1C). The group separation was skewed resulting in greater separation between groups toward the top left and convergence toward the bottom right. This suggests the two components having an inverse relationship toward each other. Component one accounted for 22.4% of the total variation and component two for 10.4% (32.8% in total). The variable with the highest VIP score (>1.77 on the first two components) was an unknown peak (Analyte 458 UM: 159, RT: 1376.42). The top 62 variables showed VIP scores >1. Cross validation of this model indicated two components as the optimal in terms of predictive value (Q_2 = 0.48) and accuracy (0.74).

When all treatments were analysed together (Figure 4.1D) clear separation from the control could be established for DTM 0.1%, DDT 1%, and DTM 1%. The Mix 0.1% and Mix 1% treatments only showed minor overlap with the control whilst the DDT 0.1% grouped closer to control than the other exposure treatments. All exposure treatments overlapped with each other across the first two components. Only 18.3% of the variation was accounted for. This model however showed poor predictive capacity (Q_2 = 0. 24) and accuracy (0.38). In total 81 variables showed VIP scores > 1 with L-sorbitol showing the greatest predictive capacity (VIP > 2.5 across the first two components).

Table 4.4 Significant results of metabolic pathway analysis for the different exposure treatments. Only Holm-corrected p-values <0.1 were included. Unknown analytes were excluded from analysis. PI = pathway impact

Treatment	Pathway	Hits	p-value	PI	Compound hits
DDT 0.1%	Biosynthesis of unsaturated fatty acids	4	0.012	0	Stearic acid; Oleic acid; Linoleic acid; Eicosapentaenoic acid
	Inositol phosphate metabolism	1	0.016	0.09	Myoinositol
	Ascorbate and aldarate metabolism	1	0.016	0	Myoinositol
	Galactose metabolism	2	0.063	0.002	Alpha-D-Glucose; Myoinositol
DDT 1%	Fructose and mannose metabolism	3	0.017	0.02	Sorbitol; Mannose 6-phosphate; alpha-D-Glucose
	Galactose metabolism	4	0.035	0.04	alpha-D-Glucose; Glycerol; Sorbitol; Myoinositol
DTM 0.1%	Propanoate metabolism	1	0.026	0	2-Hydroxybutyric acid
	Biosynthesis of unsaturated fatty acids	2	0.032	0	Stearic acid; Eicosapentaenoic acid
DTM 1%	Pentose phosphate pathway	4	0.014	0.08	6-Phosphogluconic acid; Beta-D-Glucose-6-phosphate; Beta-D-Glucose; Gluconolactone
	Pyruvate metabolism	2	0.046	0.17	Pyruvic acid; L-Malic acid
	Propanoate metabolism	4	0.047	0	Succinic acid; Hydroxypropionic acid; Beta-Alanine; 2-Hydroxybutyric acid
	Biosynthesis of unsaturated fatty acids	4	0.072	0	Stearic acid; Oleic acid; Eicosapentaenoic acid; Alpha-Linolenic acid
	Citrate cycle (TCA cycle)	4	0.088	0.17	Succinic acid; L-Malic acid; Pyruvic acid; Fumaric acid
Mix 0.1%	Biosynthesis of unsaturated fatty acids	3	0.023	0	Stearic acid; Oleic acid; Eicosapentaenoic acid
	Valine, leucine and isoleucine degradation	3	0.087	0	L-Valine; L-Isoleucine; L-Leucine
	Glycine, serine and threonine metabolism	3	0.093	0.26	Glyceric acid; L-Serine; L-Threonine
	Valine, leucine and isoleucine biosynthesis	4	0.093	0.99	L-Threonine; L-Leucine; L-Valine; L-Isoleucine
Mix 1%	Biosynthesis of unsaturated fatty acids	4	0.058	0	Stearic acid; Oleic acid; Eicosapentaenoic acid; Alpha-Linolenic acid

The metabolic pathway analysis results (Table 4.4) indicated four pathways were significantly influenced in the DDT 0.1% treatment frogs. The inositol phosphate metabolism and galactose metabolism pathways showed pathway impact (PI) > 0 (*see* Xia and Wishart 2010 for description of pathway impact). In the DDT 1% treatment the fructose mannose metabolism pathway and galactose metabolism pathways showed PI > 0. The DTM 0.1% influenced two pathways significantly, but the net impact was zero for both. Five different pathways were significantly affected in the DTM 1% treatment. Three of which (pentose phosphate pathway, pyruvate metabolism and the citrate cycle) showed PI > 0. Four pathways were significantly affected in the Mix 0.1% treatment. The glycine, serine and threonine metabolism pathway as well as the valine, leucine and isoleucine biosynthesis pathway both had PI > 0. The Mix 1% treatments showed only one pathway affected, but with zero PI. Using a less strict cut-off (false discovery rate adjusted $p < 0.1$) affected pathways were placed into their respective categories. All exposure treatments resulted in carbohydrate-, lipid- and amino acid metabolism changes along several pathways. The aminoacyl-tRNA biosynthesis pathway (under translation pathways) was uniquely affected by the DDT 1% treatment and both Mix treatments with 11 amino acids affected in each treatment and a 12th, L-proline, also affected in Mix treatments.

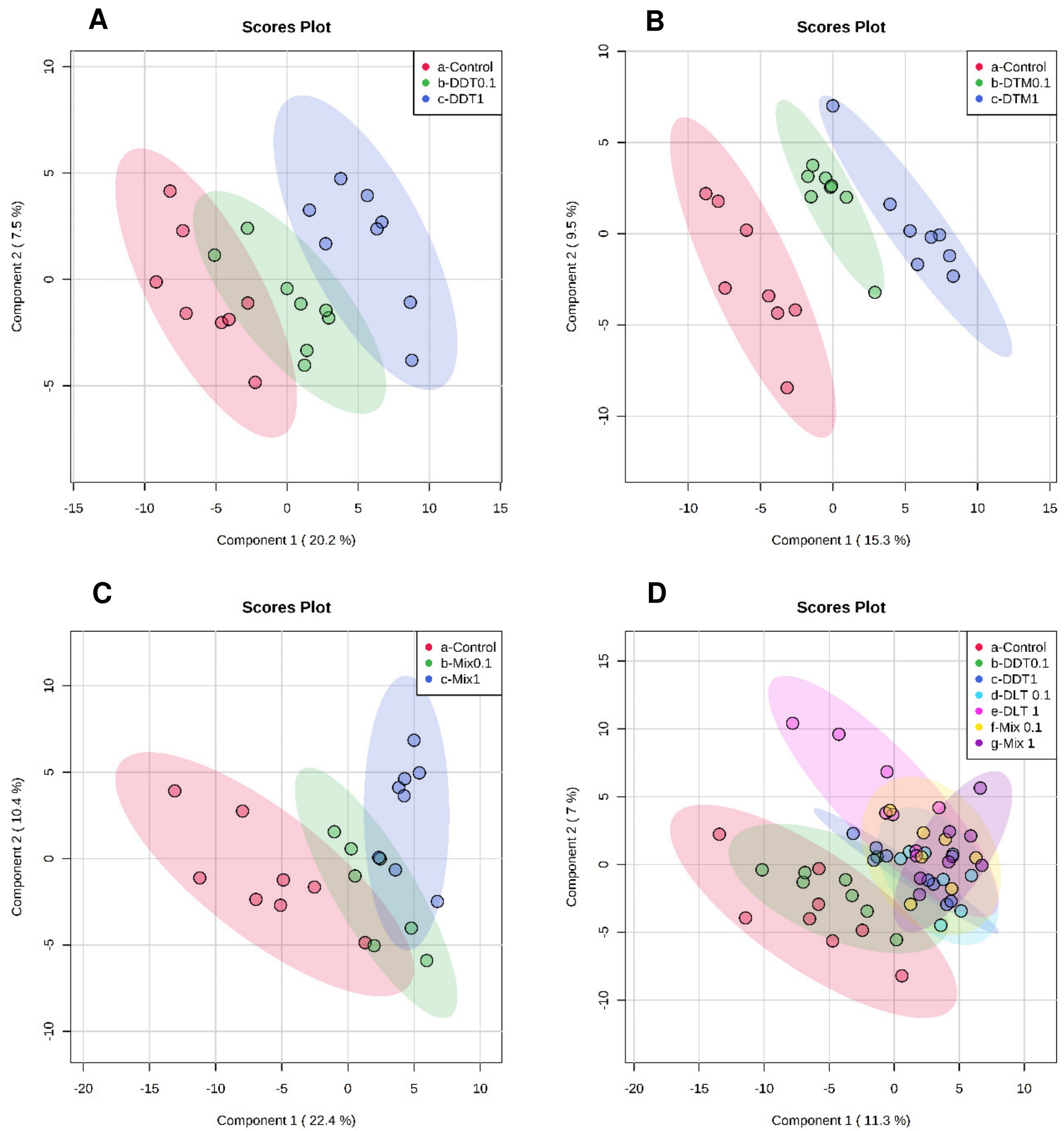

Figure 4.1 **PLSDA scores plots for each exposure group (DDT (A), DTM (B), Mix (C)) individually and all groups assessed together (D). Colours are indicated in each legend**

4.3.4 Behaviour

Analysis of behavioural data showed no statistical differences between immediate (0 h) and 96 h post-exposure recordings per treatment groups. Effect sizes (Cohen's-d value) did, however, show large changes for most treatments (Table 4.5; Visual representations of all behavioural data are provided in supplementary Figures B1 and B2). Control frogs showed a temporal decrease in the frequency of being in either a highly mobile, mobile or immobile state, with the cumulative duration spent in a highly mobile and mobile state, and the cumulative duration in the top zone also decreased at 96 h compared to 0h. For the DDT 0.1% treatment no changes were observed. For the DDT 1% only a decrease in the frequency of being in an immobile state along with an increase in the cumulative duration spent in an immobile state was observed. The DTM 0.1% treatment showed a decrease in the distance moved, mean velocity, and frequency of visits to the top zone. It also showed an increase in the frequency of being in a highly mobile state. The DTM 1% treatment showed a decrease in the total distance moved, mean velocity, and cumulative duration spent moving, with an increase in the cumulative duration in an immobile state. The Mix 0.1% treatment showed a decrease in the frequency of being in a mobile state, and a decrease in the cumulative duration of being both in a mobile state and in an immobile state. The Mix 1% treatment showed a decrease in the frequency of being in both a highly mobile, mobile, and immobile state as well as the cumulative duration of being in highly mobile and mobile states.

For treatment factor comparisons (Table 4.6) statistical significance was shown in some group comparisons at 0 h. The DDT 0.1% treatment had significant decreases from the control for the frequency of visits to- and the cumulative duration spent in the top zone. There was also a significant decrease ($p<0.05$) in the frequency of being in a mobile state and immobile state as well as in the percentage of the total duration spent in a mobile state. The Mix 1% treatment showed significant decrease from the control in the cumulative duration spent in the top zone. There were multiple changes based on large effect sizes (Table 4.6). Markedly the cumulative duration spent at the top, the frequency in a highly mobile state, and the cumulative duration in a

highly mobile state were lower for all exposure treatments at 0 h compared to the control treatment. The frequency and duration in a mobile state were also lower for all exposure treatments except the Mix 1% treatment. The frequency of visits to the top zone was lower for the DTM 1% and both of the Mix treatments. The frequency of being in an immobile state was lower for the DTM 1% and Mix 0.1% treatments while the cumulative duration in an immobile state was lower for the DDT 1%, DTM 0.1% and DTM 1% treatments. The only metrics that increased at 0 h were the cumulative duration in an immobile state for the Mix 1% and the mean meander for the Mix 0.1% treatment.

Table 4.5 Summary of time factor (differences between 0 h and 96 h) results for the analysed behavioural metrics. Increase (↑), and decrease (↓) from 0h to 96h indicated for each metric measured through an effect size (Cohen's-d value) > 0.8. None of the data showed statistical significance only practical changes are included

Treatment	Distance moved	Velocity	Cumulative duration moving	Frequency of visits to top zone	Cumulative duration in top zone	Frequency in highly mobile state	Cumulative duration in highly mobile state	Frequency in mobile state	Cumulative duration in mobile state	Frequency in immobile state	Cumulative duration in immobile state	Mean Acceleration	Mean Meander
Control					↓	↓	↓	↓	↓	↓			
DDT 0.1%													
DDT1%										↓	↑		
DTM 0.1%	↓	↓		↓		↑							
DTM 1%	↓	↓	↓								↑		
Mix 0.1%								↓	↓		↓		
Mix 1%						↓	↓	↓	↓	↓			

After 96 h no exposure treatment groups showed significant differences to the control group. Effect sizes did however once again show multiple notable changes, but in less treatments than at those measured at 0 h. The mean meander showed a notable increase for the DDT 0.1%, DDT 1%, Mix 0.1% and Mix1% treatment groups. The cumulative duration in an immobile state and the mean acceleration both showed a decrease for the Mix 0.1% treatment, but an increase for the Mix 1% treatment. Along with the prior changes mentioned, the Mix 1% treatment also showed a decrease in the frequency of visits to the top zone, cumulative duration spent in the top zone, frequency of being in a mobile state, frequency of being in an immobile state and cumulative duration spent in a mobile state. Furthermore, the DTM 1% treatment showed a decrease in total distance moved, mean velocity, cumulative duration spent moving, frequency of visits to the top zone, frequency of being in a mobile state, frequency of being in an immobile state and the cumulative duration spent in a mobile state.

Table 4.6 **Summary of results of treatment factor (differences between exposure and control treatments) for the analysed behavioural metrics. Increase (↑), and decrease (↓) from control for each treatment is indicated for each metric measured through an effect size (Cohen's-d value) > 0.8. Statistical significance from the Kruskal-Wallis analyses are indicated with an asterisk (*)**

Exposure treatment		Distance moved	Velocity	Cumulative duration spent moving	Frequency of visits to top zone	Cumulative duration in top zone	Frequency in highly mobile state	Frequency in mobile state	Frequency in immobile state	Cumulative duration in highly mobile state	Cumulative duration in mobile state	Cumulative duration in immobile state	Mean Acceleration	Mean Meander
0h; Control vs:	DDT 0.1%	↓	↓	↓	↓*	↓*	↓	↓*	↓*	↓	↓*			
	DDT1%					↓	↓	↓		↓	↓	↓		
	DTM 0.1%					↓	↓	↓		↓	↓	↓		
	DTM 1%				↓	↓	↓	↓	↓	↓	↓	↓		
	Mix 0.1%				↓	↓	↓	↓	↓	↓	↓			↑
	Mix 1%				↓	↓*	↓			↓		↑		
96h; Control vs:	DDT 0.1%													↑
	DDT1%													↑
	DTM 0.1%													
	DTM 1%	↓	↓	↓	↓			↓	↓		↓			
	Mix 0.1%											↓	↓	↑
	Mix 1%				↓	↓		↓	↓		↓	↑	↑	↑

A principle component analysis (PCA) of the behavioural metrics (not included) did not show any clear group separation along the first two axes (Eigen values 0.50 and 0.17 respectively) of the total variation in the data. Meander was shown to have a negative correlation to all other metrics along the first axis.

4.3.5 Association between MVCP accumulation and metabolome

An RDA was performed on the bioaccumulation data with the metabolome data as selection variables. Forward selection of variables (based on raw p-values) indicated 20 variables of importance in explaining the variation in accumulation data. Forward selection with FDR adjusted p-values only showed conditional significance (adjusted $p < 0.1$) of 16 variables under the inclusion of all 20 (Figure 4.2). In total 99% of the variation in chemical accumulation was explained by the selected variables (56.3% was explained by significant variables) with 81.3% explained across the first two axes (eigenvalues of 0.63 and 0.18 for axis one and two respectively). The DDT 0.1% and 1% treatments as well as the DTM 1% treatment separated from the control based on 95% confidence intervals.

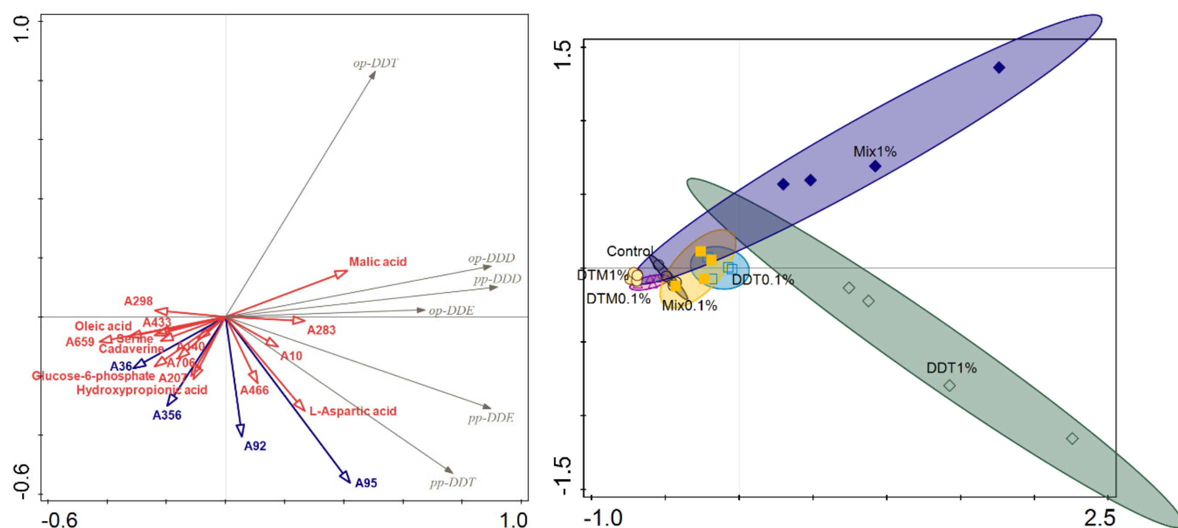

Figure 4.2 **Redundancy analysis of tissue accumulation data with metabolome data as selection variables (left). Red arrows indicate significant explanatory variables ($p < 0.1$), with blue arrows indicating non-significant explanatory variables ($p > 0.1$) Sample distribution with 95% confidence interval ellipses displayed separately (right)**

4.3.6 Association between MVCP accumulation and behaviour

A second RDA performed on the 96 h behaviour metrics with the individual compound bioaccumulation data as selection variables (Figure 4.3) did not indicate pesticide bioaccumulation as having a significant influence on the separation of behaviour data. There was a quadrant based separation of the Mix 1% treatment samples from all the other treatment samples on both the first and second axis. The Mix 1% treatment showed strong association with increased *o,p*-DDT, *o,p*-DDD, and *p,p*-DDD which correlated positively with mean acceleration and the cumulative duration spent immobile. This RDA however only explained 10.5% of the total variation in the data cumulatively between the first two axes.

4.3.7 Association between metabolome and behaviour

A third RDA was performed on the behaviour data using the metabolome data as selection variables. In order to reduce the number of variables an initial analysis was performed where after metabolome data were filtered based on raw p-values < 0.1 from that analysis. This resulted in 23 remaining variables. From a second round analysis using only these variables and applying forward selection, nine spectral peaks were identified as significant based on FDR p-values < 0.1 (Figure 4.4). The nine selected variables accounted for 75.5% of the total variation in behaviour data with 58.5% explained across the first two axes (eigenvalues of 0.44 and 0.14 for axis one and two respectively). No group separation was visible due to very large spatial variation between individuals within each treatment (Figure 4.4). Of the nine variables selected six were unknown analytes. Alpha-D-glucopyranose, mannobiose, and L-arabiniose were the only known metabolites of significance.

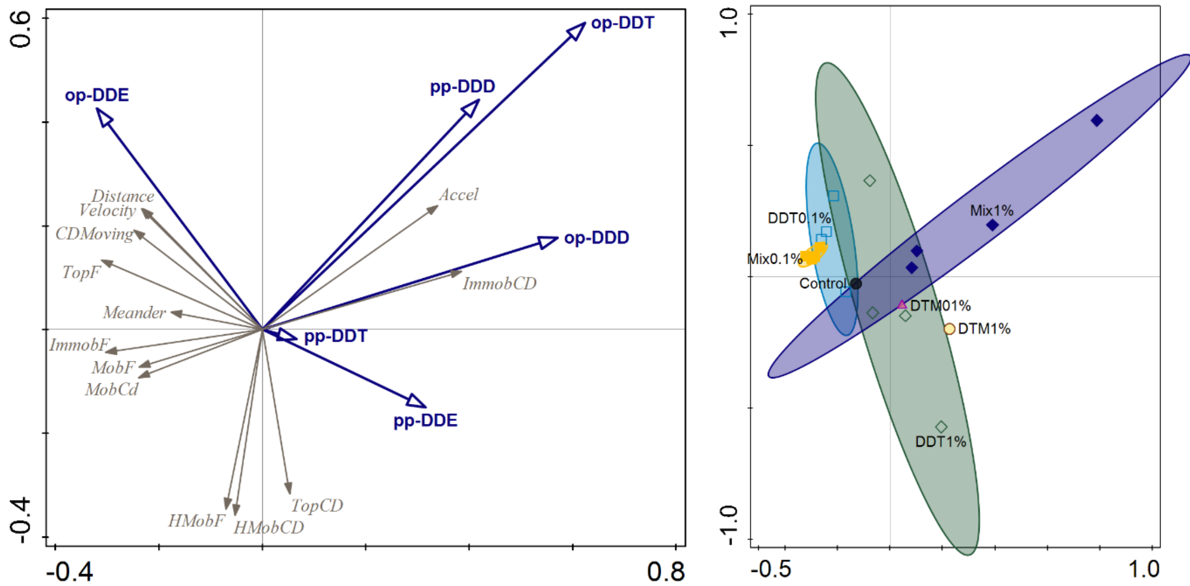

Figure 4.3 Redundancy analysis of behavioural data with tissue accumulation data as explanatory variables (left). Eigen values across first and second axis are 0.06 and 0.05 respectively. Blue arrows indicate non-significant explanatory variables ($p > 0.1$). Behavioural metrics short-labels are as follows: Distance = Total distance moved; Velocity = Velocity; Accel = Mean acceleration; CDMoving = Cumulative duration spent moving; TopF = Frequency of visits to top zone; TopCD = Cumulative duration in top zone; Meander = Mean meander; ImmobF = Frequency in Immobile state; ImmobCD = Cumulative duration in immobile state; MobF = Frequency in mobile state; MobCD = Cumulative duration in mobile state; HMobF = Frequency in highly mobile state; HMobCD = Cumulative duration in highly mobile state. Sample distribution with 95% confidence interval ellipses are displayed separately (right)

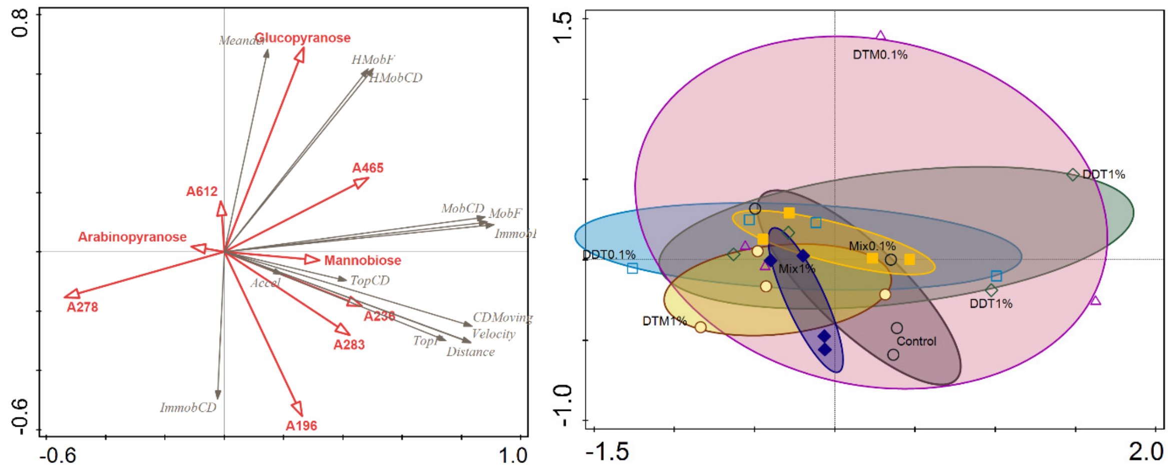

Figure 4.4 Redundancy analysis of behavioural data with metabolome data as explanatory variables (left). Eigen values across first and second axis are 0.44 and 0.14 respectively. Red arrows indicate significant explanatory variables ($p < 0.1$). Behavioural metrics short-labels are as follows: Distance = Total distance moved; Velocity = Velocity; Accel = Mean acceleration; CDMoving = Cumulative duration spent moving; TopF = Frequency of visits to top zone; TopCD = Cumulative duration in top zone; Meander = Mean meander; ImmobF = Frequency in Immobile state; ImmobCD = Cumulative duration in immobile state; MobF = Frequency in mobile state; MobCD = Cumulative duration in mobile state; HMobF = Frequency in highly mobile state; HMobCD = Cumulative duration in highly mobile state. Sample distribution with 66% (due to high variation) confidence interval ellipses are displayed separately (right)

4.4 Discussion

In attempting to discern the sub-lethal effects of DTM and DDT on *X. laevis*, this study used pesticide accumulation, liver metabolomics and behaviour as assessment metrics at biochemical and organism level (i.e. levels of biological organisation. These components were assessed individually and in conjunction with one another to gain an understanding of the effect mechanisms at play. Metabolomic and behavioural shifts were observed under exposure, combination

4.4.1 Bioaccumulation

The bioaccumulation of DTM in frog tissue could not be confirmed through the methods used in this study. The validation results of the extraction and analysis methods for DTM used in this study indicated that the method was effective for the extraction and analysis of DTM. No published literature is available on the accumulation of DTM in *Xenopus* tissue (*see* Wolmarans et al. 2020). Studies on fish such as Szegletes et al. (1995) also failed to measure DTM residues in tissue samples. Kim et al. (2008) did successfully measure DTM residues in various tissues of rats when orally exposed to 2 and 10 mg/Kg DTM. The authors calculated the maximum accumulation time to be ≤ 6 h for all tissue types except skin (12 h) and the terminal elimination half-life was only > 96 h for fat, muscle and skin. These values are not comparable to a constant immersion exposure scenario with aquatic organisms where skin permeability plays a major role in uptake, but do speak to the tendency of DTM to eliminate from tissue rapidly.

Accumulation of DDT was successfully measured in all individuals exposed to DDT, both individually and in a mixture. The accumulation data showed large variation in terms of total accumulated concentration, as well as isomeric and breakdown product contribution. Large variation in DDT accumulation data is consistent with field results in *Xenopus sp.* from areas of South Africa where DDT is applied for MVC (Viljoen et al. 2016; Wolmarans et al. 2018). The mean ∑DDT tissue concentration in the DDT 0.1% (21.7 ng/g ww) and Mix 0.1% (23.13 ng/g ww) correspond to the highest survey mean for *X. muelleri* from Wolmarans et al. (2018) of 17.79 ng/g ww. Frogs from Viljoen et al. (2016) had higher levels with male *Xenopus* sp. having a mean

∑DDT concentration of 206 ng/g ww making it comparable to the DDT 1% treatment from this study (244 ng/g ww), but these concentrations from Viljoen et al. (2016) were measured in lipid bodies only and not whole organism tissues. It should also be noted that there is limited value in a direct comparison of accumulation between laboratory exposure data and long term field exposure data where the latter is subjected to long-term diffuse exposure. Interestingly the fraction of the total accumulated DDT consisting of *p,p*-DDT itself was lower than expected for such a short exposure period relative to the general half-life of *p,p*-DDT, which is 28 days in river water and 56 days in lake water (USEPA 1989). The accumulation of ∑DDT was very similar between the DDT 0.1% treatment and Mix 0.1% indicating that the addition of DTM did not affect the accumulation of DDT at the lower concentration. At higher concentration though the Mix 1% treatment only accumulated approximately half of the ∑DDT accumulated in the DDT 1% treatment. This suggests some interaction in either the uptake or storage of these two chemicals at the higher exposure concentrations tested in this study. Although literature in this regard is scarce, interactions between chemicals in a mixture have been shown to affect its biological uptake in plants in some cases. Measured in aquatic macrophytes (*Juncus effuses L.*), the variability in chlorpyrifos uptake was shown to be affected in mixtures whereas for atrazine it was not (Lytle and Lytle 2002). In the current study, uptake could only have occurred through dermal absorption. While *X. leavis* skin is known to be highly permeable, there is very little published literature on how chemical interactions might affect the selectivity in permeability. The skin of *X. laevis* contains peptides, such as magainin, that can form ioniphores in bilipid layers as part of the neurosecretory system (Duclohier et al. 1989). Atrazine has been shown to reduce the protein content of *X. laevis* skin peptides, but with no effect on the antimicrobial properties these peptides have (Gibble and Baer 2011). Both permethrin and DTM have been shown to interact with ion transport pathways in *Rana esculenta* skin causing absorption of Na^+ and excretion of Cl^- ions, but the mechanism behind this activation could not be confirmed (Cassano et al. 2009). The

possibility that DTM acts on frog skin and subsequently alters dermal DDT uptake in *X. laevis*, therefore cannot be excluded at this time.

4.4.2 Metabolomics

In the current study, the PLS-DA results showed that the metabolic profiles of *X. laevis* liver can be used to identify exposure to pesticides under laboratory conditions, however, only at the higher concentration range assessed in this study due to separation from the control observed for both the DDT-, DTM-, and Mix 1% treatments, but not at their respective 0.1% treatments. Distinguishing between frogs exposed to different pesticides based on their metabolic profiles was not possible with the metabolome screening methods used due to large overlap in metabolite changes observed.

While some unique responses could be identified through univariate comparisons, a lot of overlap in metabolic profiles emerge during multivariate analysis indicating some general response to exposure independent of the pesticide. This becomes clear from the pathway analyses where carbohydrate, amino acid, and lipid metabolism are all affected across the board. This is similar to the results of Van Meter et al. (2018) that found carbohydrate and amino acid metabolism affected in leopard toads exposed to individual and mixtures of herbicides, fungicides, and insecticides. Van Meter et al. (2018) also attributed this to a general exposure response, which is supported by the current study results, even though different pesticide classes were used on a different species. Alanine appears to be a suitable biomarker for insecticide exposure. Increased alanine was found in frogs exposed to malathion and malathion containing mixtures in the study by Van Meter et al. (2018), but not in exposures only containing herbicides and fungicides. The increased alanine to glycine ratio measured by McKelvie et al. (2009) in earthworms for both DDT and endosulfan, and the alanine increase observed in mussels exposed to lindane (Tuffnail et al. 2009), indicate that this increase upon exposure occurs across different classes of organisms. Li et al. (2014) found an increase in alanine in brain tissue of goldfish exposed to λ-cyhalothrin, but

a decrease in kidney tissue. Tissue specific differences in metabolomic response or variability is common (Simmons et al. 2015). In the current study, several derivatives of alanine were spectrally detected with the major peak (RT: 310.19) indicating an increase (based on effect size) in the DTM 0.1% treatment and both mixture treatments. The DTM 1% treatment, however, showed a decrease. The ratio between alanine and glycine could not be calculated from the untargeted method used, but based on the results from McKelvie et al. (2009) we suspect this ratio may hold greater promise as a biomarker of insecticide exposure across different species than alanine changes alone.

The alanine changes discussed, along with similarities in sugar and amino acid metabolism, between the current study and Van Meter et al. (2018) suggest a secondary effect of exposure stress regardless of the pesticide, which could affect a wide range of organisms. A possible explanation for changes observed in carbohydrate and lipid metabolism could be a part of a stress response not unlike general adaptation syndrome (GAS), initiated through activation of the hypothalamus-pituitary-interrenal (HPI) axis, increasing cortisterone levels (Selye 1950*).* Direct evidence of cortisol or corticosterone changes in the liver itself could not be confirmed in the current study, but the down-regulation of cholesterol observed in DTM and Mix treatments could be the result of liver response toward HPI axis activation, as it serves a precursor for cortisone and corticosteroid synthesis through the steroid hormone biosynthesis pathway (KEGG 2020). DDT exposure did not cause cholesterol changes, but squalene was down-regulated in all exposures except the Mix 0.1% treatment. Squalene serves as a precursor to cholesterol formation through the steroid biosynthesis pathway (KEGG 2020). It should be noted that cholesterol hydroxylase, involved in the breakdown of cholesterol into primary bile acids (KEGG 2020), can be induced by pregnane X receptor (PXR) activity, which is known to be activated by DDE (You 2004).

Based on the current evidence the aminoacyl-t-RNA biosynthesis pathway is uniquely affected by DDT at the higher exposure concentration, with the responses exacerbated in mixture exposure. The majority of the amino acids involved in the aminoacyl-t-RNA biosynthesis pathway were down-regulated. The decrease in amino acids could indicate increased protein synthesis depleting amino acid resources, but aminoacyl-t-RNA has also been linked to other biochemical processes including aminoacylation of phospholipids in the cell membrane, and antibiotic biosynthesis (Raina and Ibba 2014). Evidence of nucleotide metabolism changes was seen in all individual exposures and the higher concentration mixture exposure through changes in the purine metabolism and pyrimidine metabolism (only in DTM 1%) pathways. This suggests that both pesticides have the potential to cause some form of DNA or RNA alteration, and that this potential persists in mixture exposures. DNA damage correlating to DDT exposure in humans is well documented (ATSDR 2002; Yáñez et al. 2004), and Petrovici et al. (2020) indicated the DNA damaging potential of DTM on Zebrafish gonads. Damage to DNA (both genome and epigenome) links back to oxidative stress and oxidative damage (Sies et al. 2017), which increases due to increased mitochondrial activity (Barzilai and Yamamoto 2004). The redox system of the body can also be triggered by any disruption to the metabolic state of equilibruim, as redox signalling occurs through changes in a multitude of metabolic pathways interconnected (Sies et al. 2017). Based on the evidence we hypothesise that, similar to GAS, a general stress response reaction occurs upon exposure to low concentrations of pesticides leading to exhaustion of key components (e.g. L-Sorbitol was down-regulated to the point of depletion in all but one exposure treatment). General stress can also lead to redox signalling changes causing oxidative damage and subsequent DNA damage (Sies et al. 2017).

A limitation in assessing the metabolomics data from this study was the large number of unidentified analytes, which form the majority of important variables identified. This is a common issue with untargeted metabolomics methods, but is a compromise against the cost of chemical

standards and specificity (requires marker metabolites) in using targeted methods (Cajka and Fiehn 2016).

4.4.3 Behaviour

When considering the behavioural responses, the changes over time have to be considered in order to distinguish persistence of effects. The differences to control observed at 0 h are drastically reduced at 96 h. For the mobility state metrics, the reduction over time seen in the control could be a possible reason for this shift as the Mix 1% treatment also shows some time differences for mobility state metrics and thus also shows differences to control at 96 h. The only persistent differences were in the distance moved, velocity, cumulative duration spent moving and frequency of visits to the top from the Mix 1% treatment, and the increase in meander seen in DDT exposures at both concentrations. There thus seems to be an initial response at the onset of all the exposure treatments. A general decrease in activity is observed. Organophosphate pesticides are known to elicit a concentration dependent decrease in swimming behaviour and heart rate on *X. laevis* and *D. rerio* larvae (Watson et al. 2014). The effects of chronic DTM exposure between 0.25 and 2 µg/L on *D. rerio* behaviour showed increase in aggression after 6 days at all concentrations indicating that time dependent responses can occur and that longer term exposure may be required for conclusive analysis of behavioural effects of pesticides (Strungaru et al. 2019). If the reduction in activity observed in the current study was an anti-threat response based on human activity when initiating the exposures, then this should be reflected in the control organisms as well, but it is not. It thus seems logical that the initial behavioural responses are due to the chemical change in the environment and may be a direct or indirect result of toxicological interaction of the pesticides. From an ecological perspective this behaviour is unlikely to lead to increased predation. Because the acceleration, velocity and distance moved are unaffected (except in DDT 0.1% for the latter two factors) the ability to escape predation would likely remain unaffected. While the ability to recognise and detect predators may be impaired under DDT exposure, the reduction in mobility and duration spent at the top zone would likely

increase camouflage and reduce the chance of being spotted by a predator (South et al. 2019). *Xenopus laevis*' dorsal eye position means threats are mainly perceived from above (Reed 2005), making the bottom zone the safest position for the frog. *X. laevis* tadpoles also show reduced activity in the presence of predators (Kruger et al. 2019). That being said, literature indicates that pesticides generally reduce escape responses of frogs (Sievers et al. 2019). The reduction in activity seen in the control after 96 h is possibly due to acclimatisation toward the exposure conditions over time (exposure tanks were clear, not smoke coloured like housing tanks, to facilitate behaviour recording), or due to stress from interruption of the feeding routine and acute starvation. In marine fish starvation can encourage movement into colder temperatures in search of food (Sogard and Olla 1996) and increase foraging activity (Miyazaki et al. 2000). Hunger stress also increases the aggression of *D. rerio* females (Ariyomo and Watt 2015). This suggests that hunger stress would be more likely to increase than decrease animal activity, and that the reduction in activity over time observed in control frogs could be due to complete acclimatisation to exposure conditions. The fact that the exposed animal activity is more similar to the control after 96 h suggests that while there was an initial behavioural reaction, some adaptation to the initial chemical stressor occurred over the 96 h period. The time dependent variability in behaviour of DTM exposed *D. rerio* observed by Strungaru et al. (2019) could also explain the temporal variation observed in the present study. The persistence of these effects at the Mix 1% treatment indicates some exposure specific reaction in this specific case. The mixture at the higher concentration possibly enhances individual toxicological effects to the degree that persistent behavioural change is observed, but as previously noted chronic behavioural analysis may reveal different results. The increase in meander with no change in velocity, seen in the DDT exposures, indicates the changes are due to turn angle changes (meander is calculated using turn angle and velocity). An increase in right angled turns (positive meander) could be an indication of disorientation effects by the DDT exposure. Pesticide induced disorientation may originate from neurotoxic effects. In a study on the swimming pattern differences between aquatic and terrestrial

frogs Jizhuang et al. (2017) noted that not only foot shape, but also movement patterns such as ankle rotation and lateral leg rotation play a role in the efficiency of swimming behaviour. Changes to nerve firing due to pesticide activity has the potential to disrupt these movement patterns. From a meta-analysis by Sievers et al. (2019) insecticides predominantly increase abnormal swimming behaviour and decrease general activity in frogs. Granted that the majority of studies analysed used tadpoles, these data correspond to what was observed in the current study. Data on surface activity caused by insecticides was widely varied (Sievers et al. 2019). South et al. (2019) showed that the immediate behavioural changes seen at the DDT 0.1% treatment of the current study can alter predator-prey interactions between *X. laevis* and *Culex* sp. larvae. These interaction effects described by South et al. (2019) may be expressed similarly at the higher DDT exposure concentrations and could probably also be evident in both DTM exposures based on the similarity in behavioural changes (from control) observed between these treatments. More importantly we predict that, as the mixture exposure treatments showed persistent changes in behaviour after 96 h, ecological interaction effects as those shown by South et al. (2019) may also be persistent after 96 h in mixture exposure scenarios.

4.4.4 Association between datasets

When integrating the data from all analyses, the metabolomics data were separated into responses unique to a pesticide (i.e. occurring in either the DDT or DTM treatments, but not both) and shared responses. This distinction identified adenine as metabolite of interest in DTM exposure both in individual and mixture exposure. Up-regulation of adenine played an important role in exposure identification (treatment group separation) and pathway analysis (purine metabolism), and may prove a useful biomarker in identifying DTM (or similar pyrethroid) exposure in frogs. In the DDT exposures l-arabinopyranose proved useful in identifying DDT exposure (separation from control) in *X. laevis*. However, the effects seem concentration dependent and are not markedly visible in mixture exposures. L-Arabinopyranose also showed a notable statistical relationship to the variation in behavioural data. L-Arabinopyranose plays an

important role in the interconversion of sugars and is linked to the ascorbate and aldarate metabolism, pentose and glucoronate interconversions, and amino sugar and nucleotide sugar metabolism pathways (KEGG 2020). DDT is known to affect carbohydrate metabolism, such as the inhibition of glycolytic pyruvate production in insects (Agosin et al. 1961).

Shared responses between pesticides indicate metabolites that may be used as general stress response markers, whereas unique metabolites may indicate exposure to a specific pesticide. In this regard further identification of unknown metabolites will also illuminate the possible mechanisms behind the general response observed. L-sorbitol and myo-inositol was shown to be important in both pathway analysis and general exposure identification (i.e. separation of exposed from control regardless of pesticide or concentration). Myo-inositol plays a role in mitigating the oxidative damage in plants where it inhibits lesion formation, and cell death due to increases in H_20_2 and salicylic acid (Chaouch and Noctor 2010). When the fish cells were treated with myo-inositol after copper exposure it even reversed the oxidative damage in the form of cell injury, lipid peroxidation, and protein oxidation (Jiang et al. 2013). We suggest that myo-inositol production may be activated in *X. laevis* as a means to combat oxidative damage due to pesticide exposure. Sorbitol degradation has been shown to be down-regulated in (*Carassuis auratus*) by exposure to a mixture of fungicides and herbicides (Gandar et al. 2017). Such impairment of the degradation pathway could result in elevated sorbitol levels, as observed in this study, and subsequently force interconversion into fructose, glucose or glycogen (Gandar et al. 2017). Oleic acid, cadaverine and two unknown compounds (analyte 433 and analyte 706) were shared responses that showed importance in explaining variation in accumulation. The increase in fatty acids due to exposure can lead to disruptions in redox signalling and increase oxidative stress (Mels et al. 2011) and may stem from endocrine disruption (Strong et al. 2015). Cadaverine levels were down-regulated. Theoretically this could be the result of increased GSH metabolism into glutathionylaminopropylcadaverine (Wagner et al. 2012), but GSH oxygenation through GST

seems more likely under increased oxidative stress. Cadaverine is produced though lysine decarboxylation (KEGG 2020), but no lysine changes were observed.

The carbohydrate metabolism pathways affected in this study are likely to alter the cellular energy production and storage. Changes in energy sources available to the organsism at cellular level are likely to form the connection between metabolomic and behavioural responses this case. Such alterations have been shown in mammals, where high calorie, lipid based, diets can alter mouse circadian rythms (Kohsaka et al. 2007). Identifying direct causal pathways between the unique metabolome response for each exposure treatment in this study and subsequent behaviour change would first require further investigation into the valibity of possible biomarkers observed.

While the evidence presented in the present study cannot outright prove causality for any of these responses, the controlled conditions under which the study was performed increases the probability of a causal relationship between exposure and measured effect (Adams 2003). In-depth targeted analysis of the metabolites of interest identified in this study, as well as further correlation analyses between metabolites and exposure in real-world or simulated real-world settings, may be necessary to provide robust biomarkers of exposure for these pesticides in frogs. The variation explained by the selected variables in the RDA analyses was higher (< 50%) for the two consecutive levels of organisation (i.e. accumulation vs. metabolome, and metabolome vs. behaviour) compared to the accumulation vs behaviour analysis where only 10.5% of the variation in data was explained by the selected variables. This indicates the importance of multi-level approaches in assessing the effects of pesticides as it shows the variation increase when levels of organisation are skipped, reducing the efficacy of such analysis. It is for this reason that many unknowns still exist regarding the mechanisms involved in pesticide toxicology, and that adverse outcome pathways (AOPs) and computational modelling are increasing in popularity in ecotoxicological studies (Wu et al. 2020).

4.5 Conclusion

This study successfully showed that acute exposure to DDT and DTM can result in sub-lethal effects on *X. laevis* in terms of changes to the liver metabolome, with some of the effects enhanced in exposure to a mixture of these pesticides. The behaviour of *X. laevis* exposed to DDT and DTM at sub-lethal concentrations showed initial responses in the form of decreased activity, less alterations between mobility states, and less time spent at the top with persistence of this response in mixture exposure after 96 h. This shows that sub-lethal exposure to pesticides can alter the biochemical homeostasis of frogs with the potential to cascade onto behavioural changes in mixture exposure scenarios. Further targeted analysis, and perhaps a multi-analysis approach to enhance identification of metabolites, is required in order to fully link the metabolomic effects to accumulation and uptake. The potential shown for behavioural changes with links to known ecological interactions to persist, validates further study into pesticide mixtures and ecological interactions in both laboratory and field settings. Potential unique marker metabolites including adenine (DTM), L-arabinopyranose (DDT), and glycerol-3-phosphate (DDT) were identified. General pesticide exposure markers possibly linked to a general stress response were also identified. These included L-sorbitol, myo-inositol, oleic acid, and cadaverine. These metabolites of interest may serve as a useful starting point for future targeted metabolomics research. This study provides necessary toxicological data on frogs, as non-target victims of malaria vector control pesticide exposure, which can be used improve *in silico* models for future research into AOPs associated with these pesticides.

CHAPTER 5 ACCUMULATION AND METABOLOMIC EFFECTS OF EXPOSURE TO SUB-LETHAL LEVELS OF DDT AND DELTAMETHRIN ON *XENOPUS LAEVIS* ADULTS UNDER SIMULATED FIELD CONDITIONS

5.1 Introduction

Liver metabolome changes in *Xenopus laevis* exposed to malaria vector control pesticides were previously assessed in a laboratory environment (chapter 4). Results showed changes in metabolomics of the frogs that were associated with exposure to malaria vector control (MVC) pesticides. In addition, a mixture of dichlorodiphenyltrichloroethane (DDT) and deltamethrin (DTM) also showed sustained behavioural changes in terms of frog activity after 96 h of exposure. Another study by South et al. (2019) indicated that behavioural changes in adult *X. laevis* caused by exposure to DDT could possibly have ecological implications in terms of changes in their feeding behaviour. South et al. (2019) observed lower mobility in adult *X. laevis* exposed to 20 µg/L DDT. This resulted in less frequent, but longer lasting, top water column visits by the frogs where they would breathe and actively forage for *Culex* sp. larvae. Results from these studies prompted inquiry into the environmental relevance of the observed sub-lethal effects of MVC pesticides on amphibians.

Outdoor microcosms and mesocosms are often used to simulate field environmental conditions for experimental study and provide a bridge between the controlled conditions of a laboratory and real-world scenarios. Additionally it offers the opportunity to study biotic environmental changes at population and ecosystem level (Schäfer et al. 2011). The use of simulated field experiments include toxicity testing for environmental pollutants. Bioaccumulation and environmental fate of pollutants can also be effectively assessed in these systems. Ecosystem functioning can be altered at multiple trophic levels and through multiple direct and indirect pathways (Rumschlag et al. 2020). Organisms are usually more sensitive to pesticide effects at a physiological and

individual level with complex ecosystems being less sensitive (*see* Schäfer et al. 2011) due to high plasiticy at ecosystem level to adapt to change.

While many outdoor simulated field studies focus on the macro-invertebrate communities, published guidelines such as OECD (2006) also allow for studies focussing on individual taxa and the addition of vertebrates (fish and amphibians in particular) in this regard. Adult amphibians are not often used as subjects for simulated field studies. From a practical standpoint this could partly be due to the complexity required in a system where animals need to be able to enter and leave the water at free will without compromising exposure efficacy and system security (preventing escape). Amphibians have, however, formed part of mesocosm and microcosm based toxicological studies since the late 90's (Boone and James 2005). Various metals, nitrate fertilisers, herbicides (atrazine, chlorpyriphos and glyphosate) and insecticides (carbaryl, malathion, and recently several neonicotinoids) have been studied in this manner (Boone and James 2005; Relyea et al. 2005; Cothran et al. 2011; Robinson et al. 2019). None of these studies have included DDT or DTM. The majority of these studies have also focused on tadpole life stages while adult life stage data seem to be lacking in literature.

We used adult *Xenopus laevis*, a fully aquatic species, as model in this study to assess the effects of pesticides used in MVC. The use of fully aquatic species simplifies the experimental design, but it should be noted that results will not be directly applicable to other amphibian species due to differences in habitats and habits. Some of these differences were outlined in Chapter 3 (Wolmarans et al. 2021). As in the laboratory exposures of chapter 4, DDT and DTM were selected as representative MVC pesticides from the organochlorine and pyrethroid pesticide groups, respectively. The MVC pesticides were used in this study to assess their accumulation in, and effects on, *X. laevis* adults when exposed individually and in mixture. The liver metabolomic profiles were assessed as a measure of sub-lethal effects and for comparison to laboratory based results. The impact (both direct and indirect) of DDT and DTM exposure on the

aquatic macro-invertebrate community was also assessed through family level community structure analysis. To the best of our knowledge, this study is the first assessment of frog liver metabolome changes associated with pesticides executed in a simulated field environment.

5.1.1 Aims

This study aims to assess 1.) the sub-lethal effects of MVC pesticides on *X. laevis* in terms of accumulation and liver metabolomics when exposed in a simulated field setting, 2.) how the accumulation and metabolomic effects relate to that of a more controlled (laboratory based) exposure and 3.) what factors may be driving any changes observed. 4.) The potential for ecological impact will also assessed through macro-invertebrate community structure effects. The study further aims to 5.) relate accumulation levels measured in this study to that measured in frogs from a natural environment (chapter 3), and a laboratory exposure setting (chapter 4) in order to link observed effects with potential real-world exposure and accumulation scenarios.

5.1.2 Hypotheses tested

It is expected that DDT will accumulate in frogs exposed in a simulated field setting and that this accumulation will be lower than observed in a laboratory setting (chapter 4) due to added environmental matrices for sequestration of the pesticide.

It is expected that DTM will not accumulate in frogs exposed in a simulated field setting based on the lack of DTM observed in field samples (chapter 3).

Based on laboratory exposure results (chapter 4), both DDT and DTM are expected to affect changes in the liver metabolome of the frogs in a field exposure setting. The variation in observed effects is expected to be higher in a simulated field exposure with higher environmental complexity than in a laboratory based exposure.

5.2 Materials and Methods

5.2.1 Animal husbandry and housing conditions prior to exposure

The standard test species *Xenopus laevis* is indigenous to South Africa, and is commonly found in the North-West province of South Africa where the study was performed, as well as in MVC regions in Southern Africa making the species a perfect candidate test subject for this study. The adult animals used in this study were laboratory bred (F2 generation) through induced ovulation (using human chorionic gonadotropin) followed by natural mating (amplexus) between male and female individuals. Egg clutches from three separate breeding pairs were combined to ensure genetic variation in the test population. Separate feeding regimes were used for different developmental stages, with tadpoles fed a fine ground mixture of spirulina and Tetra Prima twice a day. Between developmental stage 61 and 66 (Nieuwkoop and Faber 1994) froglets were fed Tetra Prima pellets twice a day and adults were fed Tetra Wafer mix once a day. Dietary nutitrion was in line with guidance on *X. laevis* housing standards as given by Reed et al. (2005). During development, frogs were separated regularly based on individual growth rate to prevent cannibalism. All Frogs used in the study were 12 months old at the time of the study and considered full adults. Breeding and development was done at the National Aquatic Bioassay Facility (NABF) at the North-West University, South Africa. Animal housing consisted of a XenoPlus Amphibia housing system (Techniplast), with 27 L smoke coloured polycarbonate tanks, within a HEPA filtered recirculating central air conditioned laboratory. Animals were held at a density of one frog per 2.7 L. The Laboratory housing system used reverse osmosis filtered water which was reconstituted automatically with marine salt (Seachem) and sodium bicarbonate (Sigma Aldrich) to maintain set water quality parameters. These parameters included water temperature at 23°C, pH at 7.4, electrical conductivity at 1000 µS/cm, and a light/darkness regime of 12h each, which all fall within acceptable parameters for housing *X. laevis* (Reed 2005). Water filtration consisted of mechanical filtration (50 micron pressurized filter), activated carbon and UV with continuous water changes replacing 20% of the total system water every 24h hours. The

frogs were removed from the housing system 2 days prior to initiation of the experiment in order to acclimate to natural temperature cycles and undergo a gradual change to exposure medium through a series of 5 x 20% water replacements.

5.2.2 Experimental setup and design

5.2.2.1 Pesticide treatments

The pesticide treatments were chosen on a similar basis as previous laboratory trials (*see* chapter 4 for details). Two pesticides, DDT and Deltamethrin, were selected in this study for being insecticides associated with malaria vector control. Concentrations were chosen as 0.1% and 1% of the $LC50_{FETAX}$ values (embryo toxicity derived through the Frog Embryo Teratogenesis Assay – *Xenopus*) for both DDT (35.7 mg/L; Channing 1998) and DTM (0.19 mg/L; Saka 2004) as adult LC50 values were not available. Experimental treatment nominal concentrations consisted of Control = no added chemicals; DDT 0.1% = 35.7 µg/L; DDT 1% = 357 µg/L; DTM 0.1% = 0.19 µg/L; DTM 1% = 1.9 µg/L; Mix 1% = DDT 1% + DTM 1%. All treatments were performed in triplicate microcosms.

5.2.2.2 Exposure chemicals

The DDT and DTM used were the same as in previous laboratory trials (*see* Chapter 4). Technical grade DDT (AVIDDT 750, Avima) and a commercial DTM formulation (Decatix 3, Coopers) were used with exposure concentrations prepared in terms of active ingredient. Stock solutions and dilutions were prepared with the same borehole water used to maintain microcosms (*see* next section).

5.2.2.3 Exposure trials

This microcosm study was designed to primarily obtain an environmentally realistic partitioning of the MVC pesticides between the different compartments and subsequent exposure of *X. laevis*. The inclusion of invertebrates in the microcosm study was to ensure that there was sufficient food

sources for *X. laevis* during the assay. This did however provide an opportunity to undertake a preliminary study into the role that MCV exposure may have on the invertebrate food sources and act as a potential co-stressor with the direct pesticide exposure.

Outdoor lentic microcosms were constructed in accordance with OECD guidelines for field exposure trials (OECD 2006) at the North-West University Unit for Environmental Sciences and Management METSI field research facility. The 18 microcosms used (dimensions: 1.5 m L, 40 cm W, and 30 cm H) were lined with clean polyethylene sheeting to ensure future reuse without contamination. Sediment was collected from a nearby, slow flowing stream (Gerrit Minnebron River, North-West Province, South Africa). Sediment was collected near the river source in an effort to limit prior contamination with pollutants. Each microcosm was filled with 5–7 cm of sediment, six stones (10–20 cm diameter) collected from the river, and filled with 200 L borehole water. Local sedge (*Schoenoplectus* sp.) were added to each microcosm for macrophyte coverage over ±20% of the surface area of each microcosm. Water levels were sustained within 20% of the original level throughout the experiment as per OECD (2006) guidelines. Borehole water was used to maintain water levels and was added via a gentle spray simulating light precipitation. After setup the microcosms were kept for eight weeks to allow invertebrate and diatom colonization prior to initiation of the experiment. Exposures were conducted at the start of the summer season for increased invertebrate activity.

After the colonization period, pre-exposure sediment, water, and invertebrate samples were collected (for invertebrate sampling method *see* section 5.2.6). After initial invertebrate sampling a 24 h recovery period was given to further reduce the impact of disturbance on the systems. Four adult *X. laevis* were then added to each microcosm with another 24 h acclimatization period before initiation of exposure. With addition of the frogs, microcosms were covered in bird netting to prevent predation on frogs. Upon initiation of exposure, microcosms were dosed with treatments as described under the pesticide treatments section. Dosing concentrations were

confirmed by chemical analysis of stock solutions. After 96 h two frogs were sampled from each microcosm (six frogs per treatment) and samples for chemical analysis and metabolomics analysis collected. Sediment (500 g) and water samples (2 x 500 mL) were also collected for analysis. A second sampling occurred 28 d after initialization where the remaining two frogs, sediment, water, and invertebrates from each microcosm were collected.

Upon collection water and sediment samples were frozen at -20°C until analysis. Frogs were euthanized by mechanical stunning and double pithing to exclude chemical euthanasia interference on metabolome. Once euthanized the body mass was weighed and the liver removed, weighed, and flash frozen in liquid N_2. The carcass was wrapped in aluminium foil and frozen at -20°C until analysis. Flash frozen Liver samples were transferred from the liquid N_2 to a -80°C freezer until analysis.

The Hepatosomatic index (HSI) was calculated as the ratio LM/BM between the liver mass (LM) and the body mass (BM) of each frog (Leão et al. 2020).

5.2.3 Physico-chemical water parameters

For each outdoor microcosm *in situ* water quality measurements were recorded prior to each sampling session. Measurement were taken at 9:00 h for each sampling session. Nutrient analysis was performed on one of the two water samples collected at each sampling session. *In situ* measurements for temperature and dissolved oxygen were measured using an Extech DO610 meter (Extech instruments) and electrical conductivity, pH, salinity, and total dissolved solids (TDS) were recorded using and Extech EC610 meter (Extech instruments). Water nutrient analysis was performed using a Spectroquant® Pharo 300UV-VIS (Merck) and associated test kits (with kit numbers) for ammonium (#114752), nitrate (#109713), nitrite (#114776), total phosphate (#114848), and sulphate (#114791).

5.2.4 Chemical analysis

Pesticide residues were measured in collected water, sediment, and frog carcass samples excluding the liver (as these were used for metabolomic analysis as in Chapter 4).

The extraction procedure for water samples consisted of 100 mL water samples extracted through liquid-liquid extraction with 200 mL hexane. Extracts were evaporated to near-dryness under gentle N_2 at 34°C and reconstituted in 100 µL of *n*-decane (Sigma Aldrich).

For sediment analysis, the extraction procedure was adapted from the method of Yohannes et al. (2013). Freeze-dried sediment (5 g) was mixed with 10 g anhydrous sodium sulphate (Na_2SO_4) along with 1g of activated copper for desulphurization. This sample mixture was sonicated three times with 30 mL of hexane/acetone mixture (1:1 v/v). Clean-up of extract was performed using 6 g Florisil. Eluent was 200 mL hexane/dichloromethane mixture (7:3 v/v). The elution was concentrated to near-dryness under a gentle stream of N_2 at 34°C in a TurboVap® (Biotage) evaporator and reconstituted in 100 µL *n*-decane.

For tissue analysis a modified version of the tissue extraction method from Wolmarans et al. (2018) was used. Whole frog carcasses were cut into small (≈2 x 2 mm) pieces and mixed with diatomaceous earth (2:1 Diatomaceous earth:sample) for desiccation. Pressurized liquid extraction was used (three static cycles at 100°C) with acetone:hexane (1:3 v/v). A 10% aliquot of extract was set aside air dried and weighed for gravimetric lipid content determination. Clean-up of the sample extract (remaining 90% consisted of solid phase extraction using 5% deactivated Florisil® (hexane prepared) in a 40 cm column eluted with 100 mL hexane:dichloromethane (7:3 v/v). The sample was then evaporated to near-dryness under gentle N_2 at 34°C and reconstituted in 100 µL n-decane.

In all samples 100 µg/L chlorinated biphenyl (CB #143) was added as internal standard at the start of extraction, with 100 µg/L tetrachloro-m-xylene (T*m*X) added to the *n*-decane for final reconstitution used as final volume confirmation

Quantification of the pesticides was done using a Gas Chromatograph coupled with a micro-Electron Capture Detector (GC-µECD). The oven program was initiated at 100°C held for 1 min followed by a 20 C/min ramp to 200°C directly followed by a 6°C/min ramp to 260°C which was held for 20 min. Splitless injection of 1 µL was used with an inlet temperature of 225°C. Detector temperature was set at 300°C with N_2 as make-up gas. Separation was achieved on a 30m HT8-MS (SGE) column with a 250 µm internal diameter and 0.25 µm film thickness with H_2 as carrier gas 9constant flow 1.5 mL/min. Using Dr Erhenstorfer (GmbH) pesticide mix 1037 (LGC Standards) containing 22 organochlorine pesticide isomers and Dr Erhenstorfer (GmbH) Deltamethrin standard (LGC Standards) compounds were identified based on retention time. Calibration was performed using a series of seven concentrations (5 and 1000 µg/L) of these standards containing the internal standard CB#143 at a constant concentration of 100 ug/L. All calibrated compounds had $R^2 > 0.999$ except Deltamethrin ($R^2 = 0.998$). Internal standard recovery was 61 ± 17% (mean ± standard deviation) for all analysed water samples, 73 ± 20% for the analysed sediment samples, and 79 ± 10% for the analysed tissue samples. Instrumental limit of detection (LOD) and limit of quantitation (LOQ) was calculated as 3 times and 10 times (respectively) the standard error of the gradient of the calibration curve. The LODs were below 50 µg/L for all compounds. Nominal dosing concentrations were confirmed through analysis of water samples taken immediately post initialisation of exposure and were (mean ± SD in µg/L) DDT 0.1% = 33.8 ± 2.2; DDT 1% = 296.3 ± 13.4; DTM 0.1% = 0.16 ± 0.005; DTM 1% = 1.86 ± 0.2; Mix 1%: DDT = 289.2 ± 10.5 and DTM = 1.7 ± 0.1

5.2.5 Metabolomic analysis

Metabolome analysis on the frog liver samples were performed at the North-West University National Metabolomics Platform (NMP) in the same manner as in Chapter 4. The Bligh-Dyer method (Bligh and Dyer 1959) as applied for whole metabolome screening untargeted metabolomics (Blackwell et al. 2013) was used for metabolite extraction. Approximately 50 mg of liver was homogenized in a mixer mill (Retch) at 30 Hz for 2 min with methanol, ultrapure water and 50 µg internal standard (50 µg/mL 3-Phenylbutyric acid; Sigma Aldrich). Chloroform was added after homogenisation (total extraction solvent ratio of methanol:water:chloroform was 1:0.8:1 v/v) and the sample vortexed for 30 s after which and phase separation was induced by keeping the sample on ice for 10 min. Samples were then centrifuged (5000 rpm for 5 min at 4°C), the top and bottom phases collected separately, and dried under gentle N_2. Methylation and derivatization was done using 50 µL methoxyamine hydrochloric acid in pyridine (200 mg/mL) at 60°C for 60 min, followed by 50 µL BSTFA with 1% TMCS at 40°C for 60 min.

Untargeted whole metabolome analysis was performed using a GC coupled with Time-Of-Flight Mass Spectrometry (GC/TOF-MS) using electron ionization (EI) at 70 eV (source temperature of 200°C and transfer line temperature of 225°C). Separation was achieved using an Rxi-5Sil-MS (RESTEK) column of 30 m length, 0.25 mm internal diameter, and 0.25 µm film thickness with helium as carrier gas (constant flow of 1.5 mL/min). The oven program was initiated at 70°C held for 1 min, followed by a 7°C/min ramp to 300°C held for 1 min. Splitless injection of 1 µL was used with an inlet temperature of 250°C. Mass selection was between 50 m/z and 800 m/z at an acquisition rate of 20 spectra/s with the detector at 50V and a solvent delay of 260 seconds.

Peak finding and mass spectral deconvolution was performed using Leco Corporation ChromaTOF software (version 4.50) at an S/N ratio of 100, with a minimum of three apexing peaks. Using the mass fragmentation patterns generated by the MS, together with their respective GC retention times, the identities of these peaks were determined through commercially available

NIST spectral libraries (mainlib, replib). Level three compound identification was performed as per Schymanski et al. (2014).

5.2.6 Macroinvertebrate community assessment

For invertebrate collection, sweep sampling of sediment, water and macropyte regions (10 minutes in each biotope) of the microcosm were performed using 1 mm mesh invertebrate nets. Live invertebrate samples were collected into a white tray, identified to family level, counted, and returned to their respective microcosms.

5.2.7 Statistical analysis

Data were tested for normality using the Shapiro-Wilk test prior to analysis and the appropriate analyses were assigned accordingly as descried below. All chemical analysis data did not show normal distribution.

5.2.7.1 Water quality data

A Kruskal Wallis analysis coupled with Dunn's adjustment for multiple comparisons was performed on water quality variables for comparison between treatments and control within each time group (96 h and 28 d) with significance set at $\alpha \leq 0.05$. Wilcoxon rank tests (for repeated measures) were performed separately per compound for each treatment to assess differences between the two exposure times.

5.2.7.2 Accumulation data

Prior to statistical testing values below LOD were replaced with 0.5 x LOD. For each chemical compound a Kruskal Wallis analysis coupled with Dunn's adjustment for multiple comparisons was performed for comparison between treatments and control within each time group (96 h and 28 d) with significance set at $\alpha \leq 0.05$. Mann-Witney tests were performed per compound for each treatment to assess differences between the two exposure times. As different individual frogs were analysed in different time points these were not treated as repeated measures. Differences

in HSI were assessed in the same manner as chemical accumulation. Comparisons made to laboratory based accumulation results from Chapter 4 were assessed using a two way ANOVA coupled with Sidak's multiple comparison test. Significance was set at adjusted $p < 0.05$.

5.2.7.3 Metabolome data

Fold Change (FC) analysis was performed on untransformed MS peak intensity data as non-parametric analysis using the equation: $sign(\overline{X}_1 - \overline{X}_2).2^{|log_2(\overline{X}_1) - log_2(\overline{X}_2)|}$;

Where $\overline{X}_1$ and $\overline{X}_2$ represent the means, and the sign of the difference between the means are added after calculation indicating the direction of the FC (+ up- and – down-regulation). The cut-off was set at $|FC| \geq 2$.

Parametric analysis performed on Log transformed ($Log_{10}X+1$) metabolome data consisted of independent samples multiple t-tests with adjustment for multiple testing (Benjamini Hochberg adjustment for false discovery rates; FDR). As a part of data filtering, compounds with detection rates below 50% in samples from both groups within a comparison were excluded. Significance was set at adjusted $p < 0.1$ because of the number of variables measured and the relatively small sample size. This practise is considered within reason based on the original work of Fisher (1995), but this produces a false positive rate of 10% that should be kept in mind upon data interpretation. In order to take into account variation within the data relative to the change in means, effect sizes (Cohen's –d value) were also calculated using the equation: $|\overline{X}_1 - \overline{X}_2| / \max(S_1; S_2)$;

Where $\overline{X}_1$ and $\overline{X}_2$ represent the group means, and with S_1 and S_2 representing the standard deviations of the respective groups. Cut-off was set at $|d| \geq 0.8$ (Lakens 2013).

A partial least squares-discriminant analysis (PLS-DA) was performed on the metabolome data (within the defined FC and Cohen'-d cut-off ranges) for each of pesticide groupings (DDT, DTM, and Mix) at each respective sampling time (96 h and 28 d) in order to assess separation variables between treatments in terms of the severity of exposure. From the PLS-DA models, variable

importance in projection (VIP) scores for separation between the control, 0.1%, and 1% treatments for each exposure group were used to identify important variables for identifying exposure. Only VIP scores >1 were considered viable. All metabolomics statistical analyses were performed using Metaboanalyst 4.0 (Chong et al. 2019).

Pathway analysis was performed using the Metaboanalyst 4.0 pathway analysis package (Xia and Wishart 2010). *Danio rerio*, was used as substitute for *X. laevis* in the model due to the absence of an amphibian representative (for precedent in using this method *see* Onjiko et al. 2016; Portero and Nemes 2019). Pathway enrichment analysis by global test and pathway topology analysis by relative-between-ness centrality was used. All pathways referred to in this study were confirmed through the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database (KEGG 2020; *also see* Kanehisa et al. 2010; Tanabe and Kanehisa 2012).

A secondary data filtering was performed for the 96 h dataset based on the similarity in univariate response (treatment vs control) to laboratory based results from Chapter 4. The spectral hits with up- or down-regulation corresponding to up- or down-regulation observed in the laboratory based exposures were selected and this secondary refined dataset was used for univariate and pathway analysis once more in the same manner as described for the full dataset.

A redundancy analysis (RDA) was performed on this secondary refined dataset to assess association with accumulation of pesticides in the frog tissue. Cut-off for significance based on explanation of variance in accumulation was set at FDR adjusted $p < 0.05$.

5.2.7.4 Invertebrate community structure

Non-metric multi-dimensional scaling (NMDS) plots were produced using Bray-Curtis similarity coefficients as distance measure to assess shifts in the invertebrate community structure due to exposure treatment and time. Rank abundance graphs were plotted based on relative abundance as $\log_{10}(X)$ with X being the abundance count of a taxa. Shannon-Wiener diversity index was

determined as H = $-\Sigma p^{s}_{i} \ln(p_i)$ where s is the total number of taxa in the sample, i is the total number of individuals in one species and p_i is the number of individuals of one species in relation to the number of individuals in the population. Taxa evenness scores were calculated as E = H/ln(s) where H is the calculated diversity index score and s is the total number or taxa. Taxa used for these calculations are at family level.

A redundancy analysis was performed on $Log_{10}(x+1)$ transformed invertebrate abundance data using physicochemical water quality data as explanatory variables to assess associations between the datasets.

5.3 Results

5.3.1 Water quality parameters

Physico-chemical water quality parameters and water nutrient levels remained consistent between treatments throughout the duration of the study with no statistical differences between treatment groups and control for either 96 h or 28 d sampling (supplementary Table C1). In terms of temporal changes, nitrites showed statistically significant increase over time for all treatments including control (28 d vs. 96 h $p < 0.05$), while total phosphate showed a significant decrease over time for all treatments including control.

5.3.2 Pesticide residue analysis

5.3.2.1 Water

Analysis of pesticide concentrations in microcosm water sampled at both 96 h and 28 d post-exposure indicated depletion of both pesticides in the water column of all treatments as no quantifiable concentrations were measured for DDT or DTM. The presence of DTM was confirmed in two DTM 1% treatment replicates and one DTM 0.1% treatment at 96 h, but concentrations were below instrumental LOQ.

5.3.2.2 Sediment

No DTM was measured in any of the sediment samples at 96h or 28d post-exposure. Dichlorodiphenyltrichloroethane metabolites were detected in all treatments that received DDT (i.e. DDT 0.1%, DDT 1%, and Mix 1%) after 96 h and in both DDT 1% and Mix 1% after 28 d. The DDT metabolites detected consisted predominantly of *p,p*-DDD with no parent *p,p*- or *o,p*-DDT itself detected in any of the sediment samples. The ∑DDT concentrations are given in Table 5.1.

Table 5.1 The mean (± standard deviation) of pesticide concentrations (µg/kg) measured in sediment samples from different treatments at 96h and 28d post-exposure

Treatment	96h		28d	
	∑DDx	DTM	∑DDx	DTM
Ctrl	< LOD	< LOD	< LOD	< LOD
DTM 0.1%	< LOD	< LOD	< LOD	< LOD
DTM 1%	< LOD	< LOD	< LOD	< LOD
DDT 0.1%	3.1 ± 1.38	< LOD	< LOD	< LOD
DDT 1%	49.3 ± 40.2	< LOD	5.6 ± 4.4	< LOD
Mix 1%	9.8 ± 2.2	< LOD	11.9 ± 6.3	< LOD

5.3.2.3 Tissue accumulation

Accumulation results are provided in Table 5.2. Both the DTM 0.1% and DTM 1% treatment samples had no detectable levels of any of the analysed pesticides present. The 96 h Control treatment detected trace levels (>LOD) of *p,p*-DDD at a detection frequency of 66%, but these were still below the LOQ and thus not quantified. The 28 d Control samples had no detectable levels of any of the analysed pesticides. The DDT 0.1 % treatment samples contained no detectable parent DDT isomers, but *p,p*-DDD, *p,p*-DDE and *o,p*-DDE were detected after 96 h. Levels for *p,p*-DDD and *p,p*-DDE increased after 28 d, while the *o,p*-DDD levels decreased to below LOD. The DDT 1%, and Mix 1% treatment samples contained all analysed DDT isomers and breakdown products, except for *o,p*-DDE which was not detected in any samples. For both the 96 h and 28 d datasets, statistical differences (compared to control) were only found for the

DDT 1% and Mix 1% treatments (Table 5.2). At 96h the DDT 1% treatment frogs had levels significantly higher than control for *p,p*-DDE, *o,p*-DDD, *p,p*-DDD, *o,p*-DDT, and *p,p*-DDT. The Mix 1% treatment frogs contained residue levels significantly higher than control for *p,p*-DDE, *o,p*-DDD, and *p,p*-DDD. At 28 d the DDT 1% treatment frogs contained levels significantly higher than control for *p,p*-DDE, *o,p*-DDD, and *p,p*-DDD while the Mix 1% treatment only had levels significantly higher than control for *p,p*-DDE and *p,p*-DDD.

In terms of temporal change, ∑DDT accumulation in frogs showed statistically significant increase in the DDT 1% treatment between the 96 h and 28 d samplings. At 28 d all treatments containing DDT had higher variability in ∑DDT accumulation than at 96 h.

A compositional shift was also observed with significant loss of *o,p*-DDT in DDT 1% and Mix 1% treatment samples from 96 h to 28 d. The *o,p*-DDD levels were also significantly reduced in the DDT 0.1% and Mix 1% treatments over time, but not in the DDT 1% treatment. The *p,p*-DDD and *p,p*-DDE levels in both the DDT 1% and Mix 1% treatment frogs showed significant increase over the exposure period.

5.3.2.4 Comparison to laboratory based exposure

The two way ANOVA results for comparison of ∑DDT between simulated field exposures from the current study and laboratory based exposures from Chapter 4 indicated significant differences in terms of both treatment, setting and interaction ($p < 0.001$). Sidak's multiple comparison test for differences between settings (per treatment) indicated significantly lower ∑DDT accumulation in the outdoor microcosm setting for the DDT 1% treatment. The DDT 0.1% and Mix 1% treatments both also had lower means in the outdoor microcosm study (Figure 5.1).

Table 5.2 **The mean (± standard deviation) values for Body mass (BM; g), Hepatosomatic index (HSI), lipid content (as percentage of BM), and concentrations of pesticides for frogs from each exposure treatment sampled at 96h and 28d. * = Significant difference between treatment compared to control per timeframe (i.e. per compound within the 96h exposure set or within the 28 d exposure set); ‡ = Significant difference between timeframes per treatment (i.e 28 d – 96h difference per treatment per compound, only indicated on the 28 d dataset). a = three treatments have n = 5 at 28 d as three escapee frogs were excluded from the study**

t	treatment	*n*	BM	HSI	%Lipids	*o,p*-DDE	*p,p*-DDE	*o,p*-DDD	*p,p*-DDD	*o,p*-DDT	*p,p*-DDT	∑DDx	DTM
96h	**Ctrl**	6	2.35 ± 0.81	0.023 ± 0.004	4.99 ± 2.39	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	–	<LOD
	DTM 0.1%	6	2.92 ± 0.45	0.025 ± 0.004	3 ± 1.39	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	–	<LOD
	DTM 1%	6	6.02 ± 5.17	0.022 ± 0.01	3.45 ± 0.98	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	–	<LOD
	DDT 0.1 %	6	4.89 ± 1.68	0.028 ± 0.005	3.39 ± 1.43	<LOD	1.9 ± 0.62	1.43 ± 0.8	5.38 ± 2.02	<LOQ	<LOD	10.42 ± 3.3	<LOD
	DDT 1%	6	4.71 ± 1.32	0.032 ± 0.007	3.45 ± 1.61	<LOD	*7.28 ± 2.54	*10.41 ± 0.98	*38.93 ± 12.48	*7.93 ± 2.61	*20.04 ± 15.32	84.75 ± 31.38	<LOD
	Mix 1%	6	5.68 ± 2.53	0.031 ± 0.005	4.01 ± 0.77	<LOD	*8.26 ± 4.53	*17.34 ± 6.54	*50.18 ± 19.45	3.36 ± 2.27	3.35 ± 1.63	82.64 ± 30.6	<LOD
28d	**Ctrl**	5	3.68 ± 1.02	0.016 ± 0.002	2.52 ± 0.6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	–	<LOD
	DTM 0.1%	6	6.33 ± 2.19	0.016 ± 0.002	1.92 ± 0.65	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	–	<LOD
	DTM 1%	5	3.58 ± 1.24	0.017 ± 0.003	2.35 ± 0.7	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	–	<LOD
	DDT 0.1 %	6	7.63 ± 3.74	0.018 ± 0.001	2.14 ± 0.68	<LOD	4.5 ± 2.25	‡<LOQ	10.11 ± 11.82	<LOD	<LOD	15.84 ± 14.07	<LOD
	DDT 1%	5	5.46 ± 2.42	0.017 ± 0.004	3.25 ± 1.83	<LOD	*‡54.17 ± 24.99	*12.03 ± 11.5	*‡142.91 ± 72.75	‡<LOQ	5.73 ± 7.14	216.09 ± 110.5	<LOD
	Mix 1%	6	5.86 ± 1.97	0.017 ± 0.003	2.45 ± 0.85	<LOD	*‡37.61 ± 17.99	‡4.12 ± 2.66	*84.04 ± 40.31	‡<LOQ	6.49 ± 7.95	133.15 ± 66.78	<LOD

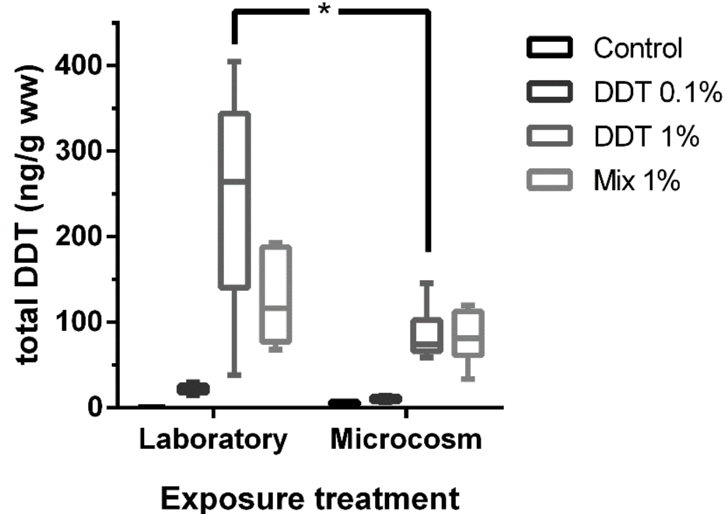

Figure 5.1 Comparative Box plot of total DDT accumulation in DDT containing treatments from a simulated field exposure in the current study vs laboratory based exposure data from Chapter 4. Significant difference between the two groups (per treatment) from a two-way analysis of variance (ANOVA) with Sldak's multiple comparison test ($p < 0.05$) is indicated with *

5.3.3 Metabolomics

A total of 562 spectral peaks were detected in the *X. laevis* liver samples. The group differences (based on FC and effects sizes) between exposed and control treatments filtered a total of 337 spectral peaks with measureable up- or down regulation. Based on multiple t-tests 13 different spectral hits showed significant differences from control (FDR $p < 0.1$; Table 5.3). At 96 h the DDT 0.1% treatment showed significant down-regulation of two spectral hits (unknown analyte 356 and glycine derivative). At 28 d unknown analyte 500 and cholesterol were significantly down-regulated while unknown analyte 5 was significantly up-regulated. The only other treatment that showed statistically significant change was the DTM 0.1 % treatment at 96h with three up-regulated spectral hits (unknown analytes 197, 576, 92), and five down-regulated spectral hits (unknown analytes 209, 458, 58, and Guanosine and L-Alanine derivatives).

Table 5.3 ***Metabolome data filtered to only show spectral hits with statistical significance (indicated with an asterisk*) in comparison to control for each exposure group with up- (↑) and down-regulation (↓) indicated based on fold change > 2, and effect size (Cohen's-d value) > 0.8. All hits only present (p) or absent (a) in the exposure treatment compared to control are also indicated. RT = retention time***

		DDT 0.1%		DDT 1%		DTM 0.1%		DTM 1%		Mix 1%	
		96 h	28 d	96 h	28 d	96 h	28 d	96 h	28 d	96 h	28 d
Statistically significant changes ($p < 0.1$)	Up-regulated	0	1	0	0	3	0	0	0	0	0
	Down-regulated	2	2	0	0	5	0	0	0	0	0
	Total	2	3	0	0	8	0	0	0	0	0
3 alpha Mannobiose isomer 1 (RT:1809.63)		↑p	↑p		↑p					↑p	
9-Octadecenoic-Acid (RT:1538.36)										↑p	
Analyte 140(RT:986.21)			↑	↑p							↑
Analyte 144(RT:1088.09)										↑p	
Analyte 163(RT:683.94)							↓a		↓a		
Analyte 167(RT:1234.74)		↑							↑p		
Analyte 19(RT:601.39)					↑p				↑p		
Analyte 191(RT:722.72)		↓		↓						↓a	
Analyte 196(RT:1167.4)		↑	↑p			↓a			↑	↑	
Analyte 197(RT:1036.64)		↑		↑	↑	↑**	↑		↑		
Analyte 209(RT:966.33)		↑		↓	↑	↓**					
Analyte 228(RT:773.65)		↑p								↑p	
Analyte 262(RT:992.07)		↑p	↑p							↑p	
Analyte 264(RT:916.56)					↓	↓a	↓				↓
Analyte 295(RT:1417.22)			↓a		↓				↓a		↓a
Analyte 303(RT:1175.28)		↑p	↓								
Analyte 327(RT:1203.13)									↓a		
Analyte 353(RT:1712.99)					↓		↓		↓a		
Analyte 356(RT:1165.45)		↓**a****	↑	↓				↓			
Analyte 361(RT:1080.38)		↑				↓a	↑				
Analyte 387(RT:2051.93)						↓a					
Analyte 398(RT:1002.91)			↑p								
Analyte 4(RT:261.56)			↑p								
Analyte 426(RT:1338.3)										↑p	
Analyte 458(RT:1376.42)						↓**					↓
Analyte 46(RT:969.27)		↓a				↓a		↓a		↓a	
Analyte 465(RT:1414.48)			↑p								
Analyte 483(RT:4539.56)			↓a		↓a		↓a		↑		↓a
Analyte 498(RT:1509.06)		↑p	↑p							↑p	
Analyte 499(RT:2196.48)			↓a			↑			↓a		
Analyte 5(RT:482.66)		↑	↑*				↑			↑	
Analyte 500(RT:1326.6)				↑						↓a	
Analyte 507(RT:1848.86)							↑p				
Analyte 509(RT:1672.95)					↓a		↓a		↓a		
Analyte 545(RT:1498.39)		↑		↓a		↓			↑		
Analyte 55(RT:591.61)				↓a		↓				↓a	

Table 5.3 (continued)

	DDT 0.1%		DDT 1%		DTM 0.1%		DTM 1%		Mix 1%	
	96 h	28 d	96 h	28 d	96 h	28 d	96 h	28 d	96 h	28 d
Analyte 552(RT:1502.57)			↓		↓				↑	↓
Analyte 566(RT:1248.89)					↓a				↓a	
Analyte 569(RT:1546.09)	↓a				↓		↓			
Analyte 576(RT:1493.67)	↑	↑	↑		↑*	↑	↑			↑
Analyte 577(RT:2804.2)		↑p								
Analyte 58(RT:711.96)			↓		↓**					
Analyte 605(RT:1712.71)		↓						↓a		↓
Analyte 628(RT:1837.65)	↓		↓	↓				↓a		
Analyte 641(RT:1348.79)	↑p									
Analyte 65(RT:698.58)		↓a		↓		↓		↓a		
Analyte 657(RT:1371.37)	↑			↓a			↑			
Analyte 674(RT:2634.78)	↑	↑p	↑			↑p				
Analyte 678(RT:2288.63)	↓	↓*		↓	↑			↓	↓a	↓
Analyte 681(RT:2068.24)	↓a	↓a						↓		
Analyte 72(RT:705.63)					↓a					
Analyte 729(RT:2197.61)		↓a								
Analyte 91(RT:742.94)					↑	↑p			↑	
Analyte 92(RT:648.11)	↑				↑**					
Analyte 95(RT:544.82)			↓a						↓	
Cadaverine (RT:1925.51)	↑				↓a					
Cholesterol (RT:1763.86)		↓*		↓		↓		↓		↓
D-Tagatose (RT:1739.39)		↑p							↑p	
Galactose (RT:1794.54)		↓a		↓		↓		↓		↓
D-Galactose (RT:1256.08)	↓						↓		↓a	↓
Gluconolactone (RT:1806.47)				↓a					↑	
Gluconolactone (RT:1712.37)	↓a	↓	↓a	↓a						↓
glucose (RT:1783.15)	↑p								↑p	
Glucose-6-phosphate (RT:1283.1)		↑p								
Glycine (RT:776.28)	↓*	↓	↓	↓	↓	↓			↓	↓
Guanosine (RT:1884.01)	↑				↓**				↓	
L-Alanine (RT:310.19)	↑p								↑p	
L-Alanine (RT:1072.22)	↓	↓	↓		↓**	↓	↓	↓		↓
D-Maltose (RT:2120.56)	↑p	↑p							↑p	
Maltose (RT:1818.21)	↑p									
N-Acetylglucosamine (RT:2564.38)	↑p		↑p						↑p	
Oleic acid (RT:1609.05)	↑p									
Piperidine (RT:262.72)	↑p	↑					↓		↑p	
Ribitol (RT:1227.07)									↑p	
Campesterol (RT:1420.13)	↑p	↑p								
Phosphoric acid (RT:1221.32)		↓a	↓	↓	↑	↓	↑			↓

The PLS-DA analyses (not included) showed effective separation from control (based on 95% confidence interval) for both concentrations in the DDT 96 h and DDT 28 d exposure groups. The Mix 1% treatment also separated effectively from the control at both 96 h and 28 d. The DTM 1%

treatment at 28 d was the only DTM treatment to effectively separate from the control. Combined PLS-DA analysis of all treatments did not show effective group separation at either 96 h or 28 d. The accuracy and predictive capacity of the PLS-DA models were very low overall with only the DDT 96 h (Accuracy 0.7 and Q_2 0.2 at 4 components) and Mix 28 d (Accuracy 0.63 and Q_2 0.22 at 3 components) being the only models showing positive predictive values.

5.3.3.1 Pathway analysis

Pathway analysis results (Table 5.4) yielded statistically significant results in the DDT 0.1% treatment at both 96 h and 28d, the DDT 1% treatment at 96h, the DTM 0.1% treatment at 96h and the Mix 1% treatment at both 96 h and 28 d.

Both the DDT 0.1% and Mix 1% treatments at 28 d showed impact on the steroid biosynthesis, steroid hormone biosynthesis, and primary bile acid biosynthesis pathways. These pathways were affected mainly through the down-regulation of cholesterol. At 96 h the DDT 0.1% and DDT 1% had effects on the galactose metabolism and pyruvate metabolism pathways respectively, both forming part of carbohydrate metabolism. Although the Selenocompound metabolism pathway was affected in the DTM 1% treatment at 96h, the pathway impact (PI) was zero indicating low chance of cascading effects within that pathway resulting from the changes in L-alanine observed. This was also the case for the Porphyrin and chlorophyll metabolism pathway effects due to glycine changes observed in the Mix 1% treatments at 96h. This glycine change did however indicate an effect on the Glutathione metabolism.

Table 5.4 Significant results of metabolic pathway analysis for the different exposure treatments. Only Holm-corrected p-values <0.1 were included. Unknown analytes were excluded from analysis. PI = pathway impact. N/A = Not Applicable (no affected pathways within the cut-off criteria)

Treatment	Pathway	Hits	p-value	PI	Compound hits
DDT 0.1% 96 h	Galactose metabolism	2	0.085	0.08787	alpha-D-Galactose; D-Glucose
DDT 0.1% 28 d	Steroid biosynthesis	2	0.016	0.028	Cholesterol; Campesterol
	Primary bile acid biosynthesis	1	0.018	0.212	Cholesterol
	Steroid hormone biosynthesis	1	0.018	0.008	Cholesterol
DDT 1% 96 h	Pyruvate metabolism	2	0.077	0.115	(R)-Lactate; (S)-Malate
DDT 1% 28 d	N/A				
DTM 0.1% 96 h	Selenocompound metabolism	1	0.095	0	L-Alanine
DTM 0.1% 28 d	N/A				
DTM 1% 96 h	N/A				
DTM 1% 28 d	N/A				
Mix 1% 96 h	Glutathione metabolism	1	0.054	0.089	Glycine
	Porphyrin and chlorophyll metabolism	1	0.054	0	Glycine
Mix 1% 28 d	Primary bile acid biosynthesis	1	0.058	0.212	Cholesterol
	Steroid biosynthesis	1	0.058	0.028	Cholesterol
	Steroid hormone biosynthesis	1	0.058	0.008	Cholesterol

5.3.3.2 Comparison to laboratory based exposure

A secondary data filter was applied to include only the spectral hits with similar responses compared to control as seen in the laboratory based study of Chapter 4. This resulted in a total of 49 hits over the five treatments (Table 5.5). DDT 1% had the most metabolomic changes in common with the laboratory exposure with a total of 22 compounds affected in both studies. The Mix 1% was the second most affected with 18 compounds similarly affected in both studies. The DTM 0.1% treatment followed with 13 spectral hits similarly affected in both studies. DTM 1% and DDT 0.1% were the least affected with six and two shared responses for both studies respectively.

In terms of pathway analysis the galactose metabolism pathway affected by the DDT 0.1% treatment was the only affected pathway in common with the laboratory based exposures from chapter 4.

Table 5.5 Metabolome data filtered to only show spectral hits with up- (↑) and down-regulation (↓) corresponding to laboratory based results of Chapter 4. Statistical significance (compared to control) is indicated with * for false discovery rate adjusted p < 0.05. All hits only present (p) or absent (a) in the exposure treatment compared to control are also indicated. RT = retention time

	DDT 0.1%	DDT 1%	DTM 0.1%	DTM 1%	Mix 1%
up-regulated	2	8	1	4	8
down-regulated	0	14	12	2	10
total	2	22	13	6	18
1-Aminocyclopropane-1-carboxylic acid (RT:773.9)					↓
Cis-Oleic acid (RT:1762.96)					↓
beta-alanine (RT:869.06)				↑	
Analyte 140(RT:986.21)		p↑			
Analyte 155(RT:1105.59)		↓			
Analyte 197(RT:1036.64)		↑			
Analyte 202(RT:885.83)		↑			
Analyte 209(RT:966.33)		↓	*↓		
Analyte 213(RT:1098.94)			↓		
Analyte 236(RT:1170.8)		↑	↑		
Analyte 361(RT:1080.38)	↑				
Analyte 387(RT:2051.93)			a↓		
Analyte 392(RT:1426.25)			↓		
Analyte 393(RT:1523.4)		↓	↓		
Analyte 446(RT:1540.58)		↑			
Analyte 458(RT:1376.42)			*↓		
Analyte 466(RT:1519.9)					↓
Analyte 5(RT:482.66)					↑
Analyte 541(RT:1828.53)				↓	
Analyte 570(RT:1705.38)		↑			
Analyte 58(RT:711.96)		↓			
Analyte 612(RT:1656.57)	↑				
Analyte 638(RT:1647.02)				↓	↓
Analyte 678(RT:2288.63)					a↓
Analyte 706(RT:1804.92)				↑	

Table 5.5 (continued)

	DDT 0.1%	DDT 1%	DTM 0.1%	DTM 1%	Mix 1%
Analyte 72(RT:705.63)			a↓		
Analyte 728(RT:2108.23)					↓
Analyte 9(RT:382.56)		↓			↓
Analyte 94(RT:696.65)		↓			↓
Analyte 95(RT:544.82)					↓
Cadaverine (RT:1610.51)				↑	
Cadaverine (RT:1925.51)			a↓		
D-Tagatose (RT:1739.39)					p↑
Guanosine (RT:1884.01)					↓
l-Alanine (RT:593.51)		↓			
l-Alanine (RT:310.19)					p↑
L-Asparagine (RT:1248.77)		↓	↓		
L-Aspartic acid (RT:945.94)		↓			↑
L-Isoleucine (RT:741.42)		↓	↓		
L-Methionine (RT:1055.21)		↓	↓		
l-Threonine (RT:674.66)		↑			↑
L-Valine (RT:877.31)		↓			
L-Malic Acid (RT:1275.31)					↑
D-Maltose (RT:2120.56)					p↑
Phenylalanine (RT:1106.65)		↓	↓		
Piperidine (RT:262.72)					p↑
Serine (RT:850.53)		↓			
Phosphoric acid (RT:674.35)		↑			
Boric acid (RT:447.26)				↑	

5.3.3.3 Supplementary analysis

A redundancy analysis (RDA) of 96 h chemical accumulation data and filtered metabolome data, selected as explanatory variables for variation in chemical accumulation (cut-off raw $p < 0.1$) indicated 68% of total variation in chemical accumulation could be explained by nine selected metabolites. The first two axes explained 62% of the total variation (Figure 5.2). Using false discovery rate adjustment only *cis*-Oleic acid showed $p < 0.05$. *Cis*-Oleic acid accounted for 22% of the variation in chemical accumulation in the frog tissue with moderate negative correlation to the DDT isomers detected in the frog tissue.

5.3.3.3.1 Secondary filtered pathway analysis

Based on raw p-values < 0.1 only two pathway results corresponded with significantly affected pathways from laboratory exposure. For the DTM 1% treatment the propanoate metabolism pathway was indicated as possibly affected, which corresponded with laboratory based results. For the Mix 1% treatment the biosynthesis of unsaturated fatty acids pathway was also indicated as possibly affected that corresponded with the laboratory based exposures. Accounting for multiple comparisons, however, no significantly affected pathways (Holm corrected $p < 0.1$) were identified in the analysis of secondary filtered 96h metabolome data from this study.

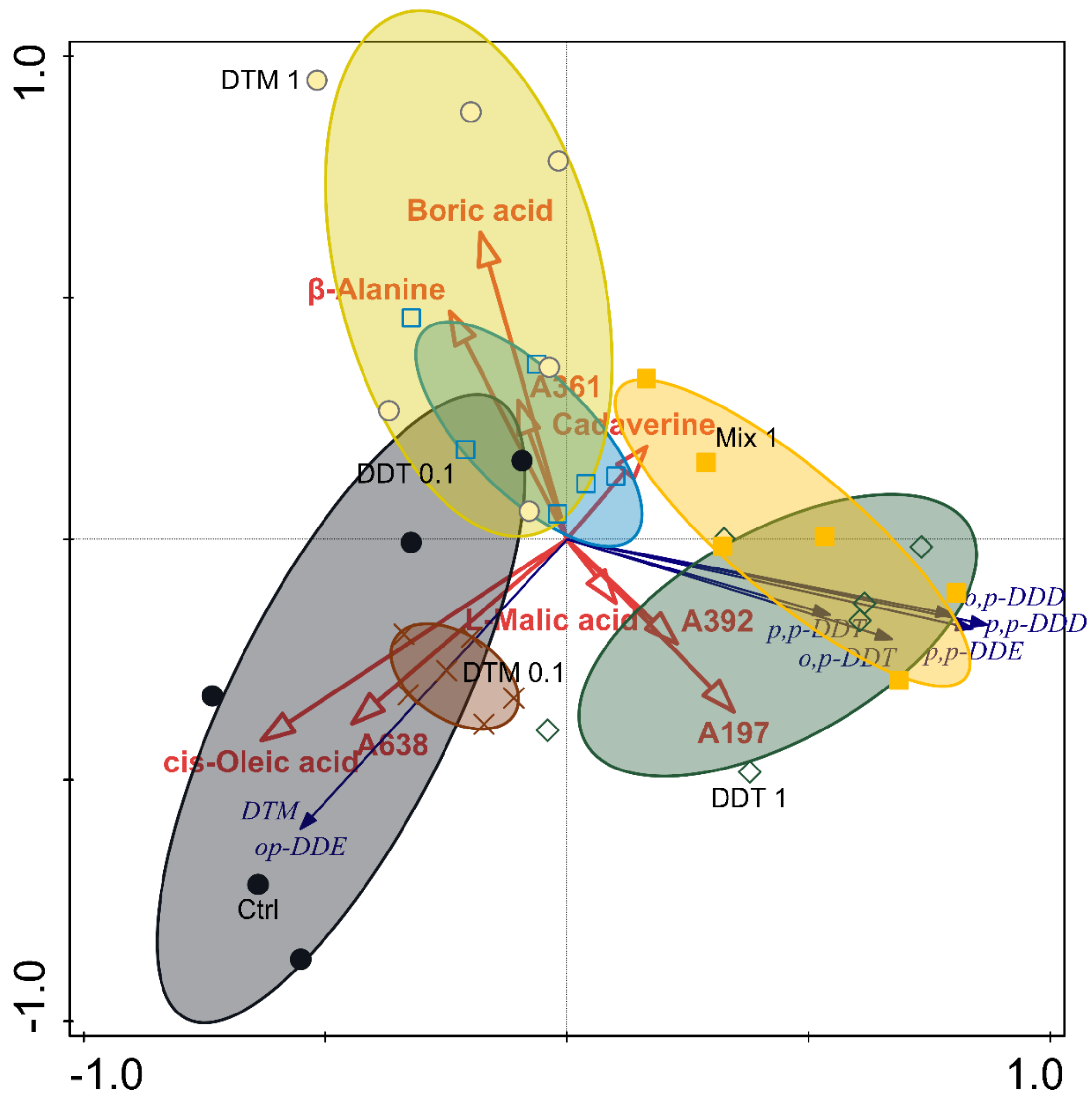

Figure 5.2 **Biplot of axis one and two of the redundancy analysis (RDA) performed on the chemical accumulation data of 96h exposure and corresponding metabolome data filtered by response correlation with laboratory exposure data from Chapter 4 with forward selection of liver metabolites as explanatory variables for variation in chemical accumulation (cut-off raw p < 0.1). Ellipses indicate 66% confidence intervals for each treatment. Red arrows indicate metabolites and blue arrows indicate chemical accumulation. Explanatory variables accounted for 68% of total variation with 62% explained across the first two axes. Only *cis*-Oleic acid had false discovery rate adjusted p < 0.05 and accounted for 22% of the total variation in accumulation data**

5.3.4 Invertebrate effects

Initial invertebrate assessment did not reflect an even distribution of taxa at family level and was highly dominated by *Culicidae* and *Chironomidae* (Tables 5.6 and 5.7). The invertebrate community structure data show increased taxa diversity and evenness in the control over the 28 d exposure period (Table supplementary Figure C1). While not significantly different, a temporal increase in taxa diversity and evenness was also observed in the DDT 0.1% and DTM 0.1% treatments with The DDT 0.1% treatment showing a very similar abundance (based on families) pre-and post-exposure. The DTM 1% treatment showed almost no change in taxa diversity and evenness over the 28 d exposure period. Both the DDT 1% and Mix 1% treatments showed a large increase in variation in diversity and evenness between replicates over the 28 d exposure period. This is attributed to each having one replicate where only a single organism was found during the 28 d sampling. Changes in the control treatment were driven by a sharp decrease in *Culicidae* abundance to the point where the abundance was halved, even with the next four most abundant families showing an increase in abundance over time between 0 d and 28 d (Tables 5.6 and 5.7). The control treatment was subject to predation effects by *X. laevis* alongside the natural temporal change in macroinvertebrate composition over the 28 day period. The significant loss of diversity in the Mix 1% treatment vs Ctrl at 28 d was the most prominent treatment-based effect observed (Table 5.8). The number of families in the Mix 1% treatment reduced by 12 from pre- to post-exposure (Tables 5.6 and 5.7).

The influence of physico-chemical water quality on invertebrate diversity (Figure 5.3) assessed through a RDA with water quality as explanatory variables indicated no treatment based separation of data groups, but control treatment did show greater varation along the first axis whereas all exposed treatments showed greater variation along the second axis. Nitrites (NO_2) had the greatest influence accounting for 11% of the variation in the invertebrate data (41% of the total variation is explained in Figure 5.3), however this influence was not considered statistically significant (Holm correction adjusted $p = 0.53$).

Table 5.6 **Aquatic macroinvertebrate count totals per treatment for pre-exposure survey (0d). Individual counts from all three replicates for each treatment were summed. Identification was performed to family level. Grey fill indicates families present in both pre-and post-exposure surveys**

	Notonectidae	*Chironomidae*	*Gerridae*	*Culicidae*	*Corixidae*	*Atydae*	*Libellulidae*	*Ghomphidae*	*Caenidae*	*Hydrophilidae*	*Dytiscidae*	*Belostomatidae*	*Hydrachnellae*	*Hirudinea*	*Oligochaeta*	*Ceratopogonidae*	*Tabanidae*	*Helodidae*	*Psychodidae*	*Thiaridae*	*Lymnaeidae*	Abundance per family	No. of families	Total abundance
Ctrl	2	86	0	527	1	0	0	0	0	9	16	0	1	2	0	0	0	0	0	0	0	80.5	8	644
DTM 0.1%	1	316	0	356	6	0	0	0	0	13	1	0	0	2	0	0	0	0	0	0	7	87.8	8	702
DTM 1%	0	58	0	238	17	0	0	0	0	33	10	0	0	0	0	2	0	0	0	0	0	59.7	6	358
DDT 0.1%	2	516	4	600	87	1	0	2	4	8	0	0	1	0	0	0	0	0	0	0	0	122.5	10	1225
DDT 1%	0	84	0	339	15	0	0	0	0	8	4	0	2	0	0	0	0	0	0	0	0	75.3	6	452
Mix 1%	12	364	0	687	139	0	2	0	0	17	1	0	2	4	2	2	1	1	1	1	0	82.4	15	1236

Table 5.7 Aquatic macroinvertebrate abundances per treatment for post-exposure survey (28d). Individual counts from all three replicates for each treatment were summed. Identification was performed to family level. Grey fill indicates families present in both pre-and post-exposure surveys

	Notonectidae	*Chironomidae*	*Gerridae*	*Culicidae*	*Corixidae*	*Atydae*	*Ghomphidae*	*Caenidae*	*Hydropsychidae*	*Dytiscidae*	*Belostamatidae*	*Hydrachnellae*	Abundance per family	No. of families	Total abundance
Ctrl	56	120	22	49	38	0	5	0	0	32	6	1	36.6	9	329
DTM 0.1%	179	281	58	8	11	1	0	0	0	0	0	0	89.7	6	538
DTM 1%	28	27	4	0	3	0	23	0	3	9	0	0	13.9	7	97
DDT 0.1%	48	98	5	0	31	0	10	1	0	0	0	0	32.2	6	193
DDT 1%	41	119	0	16	8	0	0	0	0	7	1	0	32	6	192
Mix 1%	11	27	0	0	0	0	14	0	0	0	0	0	17.3	3	52

Table 5.8 Invertebrate community species diversity (Shannon-Wiener) and (Pielou's) evenness scores. a = Significant difference (Sidak's corrected $p < 0.05$) between Mix 1% and Ctrl in diversity at 28 days

	0 days		**28 days**	
	Diversity index (± SD)	**Evenness score (± SD)**	**Diversity index (± SD)**	**Evenness score (± SD)**
Ctrl	0.59 ± 0.15	0.37 ± 0.06	1.56 ± 0.35	0.87 ± 0.06
DTM 0.1%	0.64 ± 0.23	0.39 ± 0.11	0.93 ± 0.1	0.75 ± 0.08
DTM 1%	1.02 ± 0.31	0.68 ± 0.25	1 ± 0.41	0.73 ± 0.22
DDT 0.1%	0.98 ± 0.13	0.55 ± 0.07	1.03 ± 0.24	0.75 ± 0.07
DDT 1%	0.68 ± 0.16	0.46 ± 0.07	0.81 ± 0.77	0.55 ± 0.48
Mix 1%	0.84 ± 0.45	0.41 ± 0.21	[a]0.35 ± 0.32	0.5 ± 0.46

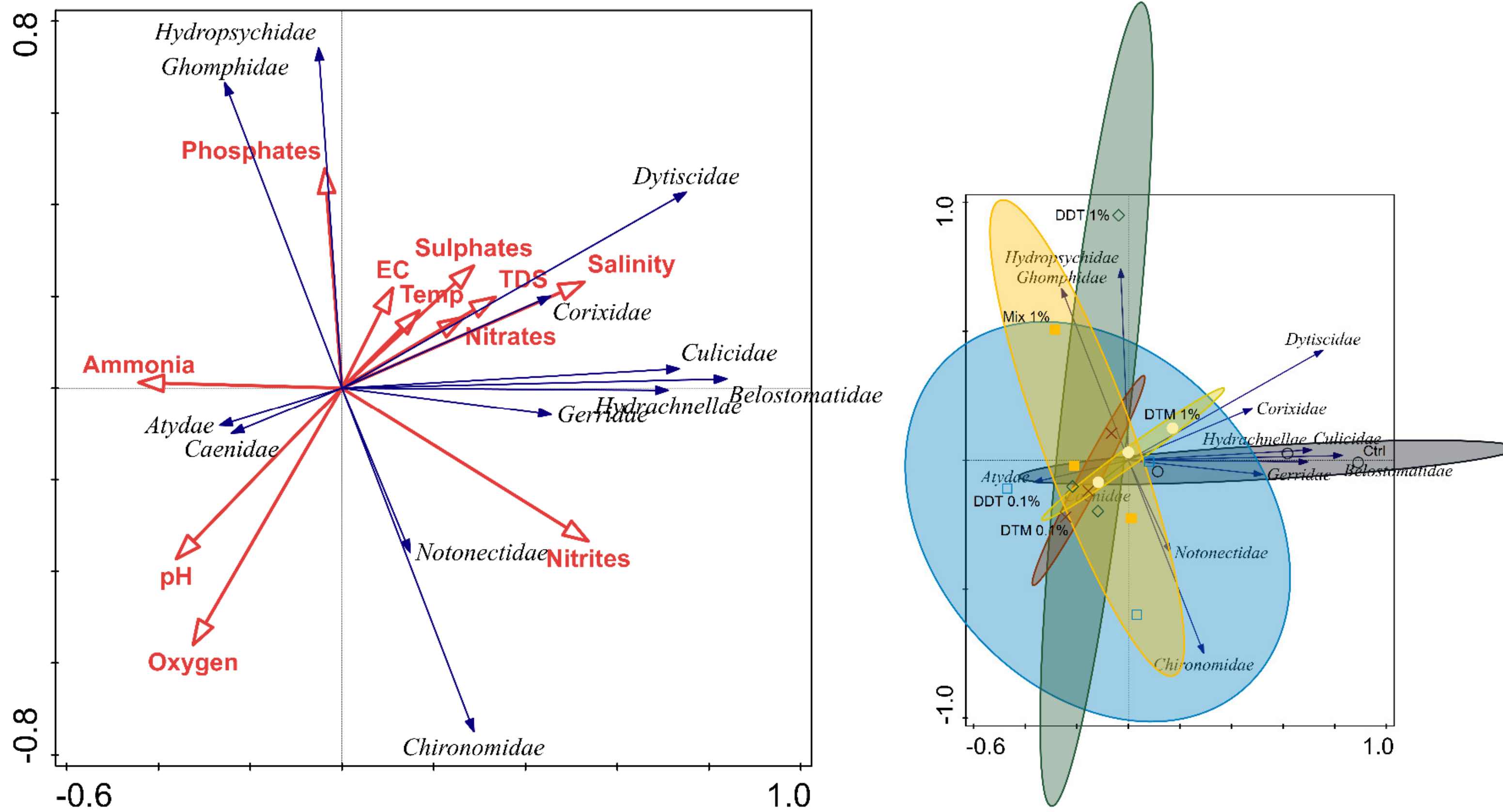

Figure 5.3 **Redundancy analysis of invertebrate community structure data with water quality variables used as explanatory variables. Only 41% of the variation is explained across the first two axes. Right-hand side plot indicates 95% confidence interval ellipses for exposure treatment groupings.**

5.4 Discussion

The study successfully measured the environmental partitioning and accumulation of DDT in amphibians from outdoor microcosm based simulated field exposures. The DDT tissue accumulation was lower in frogs in the simulated field setting compared to laboratory based exposure. Deltamethrin levels were not detectable. The metabolomics assessment indicated acute exposure affecting carbohydrate metabolism, but with low correlation to laboratory based exposures, and 28d exposure to DDT affecting cholesterol changes linking to steroid and steroid hormone biosynthesis pathways. In terms of ecological effects, the mixture exposure showed significant decrease in invertebrate diversity and abundance.

5.4.1 Accumulation

The successful detection of DDT breakdown products in frogs from all DDT containing exposure treatments (DDT 0.1%, DDT 1%, and Mix 1%) indicates the active accumulation of DDT in a simulated field exposure. As the presence of DTM could not be confirmed in any of the frog samples collected at either 96h or 28d post-exposure it seems likely that DTM does not accumulate in the frogs, however the instrumental limitations should be taken into account and it is possible that low level accumulation could occur below the LOD of the analysis procedure used in this study. This cannot be confirmed through the current data and would require higher concentration exposures to confirm, which would run the risk of mortality in the frogs during exposure.

A temporal pattern was seen in the accumulation of DDT where the *o,p-* isomers showed a consistent decrease over time while the *p,p-* isomer metabolites (DDD and DDE) showed increase. The loss of *p,p*-DDT over time was also observed, but to a lesser extent than that of *o,p*-DDT. The *o,p*-DDT/*p,p*-DDT ratio in the aquatic environment can change slightly for various reasons. Firstly *o,p*-DDT is more likely to partition in air than in sediment and water, and the two compounds have different half lives in the environment (Qiu and Zhu 2010). Furthermore the

elimination of *o,p-* isomers from animal tissues are often higher than that of *p,p*-isomers due to differences in lipophilicity (Di et al. 2017). Some microorganisms have also been shown to catalyse isomeric transformation between *o,p*-DDT and *p,p*-DDT, but at negligible rates when taking into account chemical degradation rates according to Qiu and Zhu (2010). The mixture exposure in the current study resulted in slightly lower (but not statistically different) overall DDT accumulation compared to the DDT 1% treatment within the 28 d exposure timeframe. As observed in the laboratory based exposure from Chapter 4, this lower accumulation may be a result of either competitive uptake between the two pesticides, or enhanced elimination in the presence of both pesticides. No significant compositional differences were observed at 96 h between Mix and DDT treatments as was seen in a laboratory exposure setting (chapter 4). It should be noted that in Chapter 4 study frogs were not fed during the 96 h extent of those exposures. The fact that these animals were free to hunt for food can affect direct comparisons of accumulation levels between the two. Dietary uptake is however not considered the main environmental pesticide exposure route for amphibians, but rather dermal uptake (Brühl et al. 2011).

The lower ∑DDT accumulation observed in the current study compared to laboratory based results are justified when taking into account the sediment sequestration of the pesticides in the simulated field setting. This, however, indicates that dermal uptake plays a large role in the acute exposure (96 h) accumulation of DDT in *X. laevis*. Frogs from laboratory based exposures had no food intake whereas frogs from the current study had food available in the form of macro-invertebrates that were actively consumed. The increase in accumulation at 28 d compared to 96 h can be due to both dermal and dietary uptake, but if dietary uptake played a larger role than dermal uptake in the accumulation over the initial 96 h period the expected accumulation would be similar or higher than that seen in the laboratory. Dermal uptake is known as a major exposure route for amphibians for agricultural pesticides, including DDT and pyrethroids (Shah et al. 1983),

and is considered a more effective exposure route in amphibians than it is in invertebrates or other vertebrates (Smith et al. 2007; Brühl et al. 2011).

Accumulation of DDT in *X. laevis* from this study after 28 d exposure to the DDT 0.1% treatment compares to levels of DDT found in *Xenopus muelleri* in May of 2016 (chapter 3) from Ndumo Game Reserve, a conservation area in South Africa where DDT is actively used as MVCP for indoor residual spraying. In the current study ∑DDT accumulation had means of 10.42 and 15.84 ng/g ww for the DDT 0.1% 96 h and 28 d treatments respectively. In the field survey from Chapter 4, *X. muelleri* had a mean accumulation of 27.3 ng/g ww for ∑DDx. Two prior field surveys showed lower DDT accumulation, ranging between 0.03 and 9.7 ng/g ww, but this gives an indication that the lower exposure concentrations used in the current study are within the same range as the environmental exposure for frogs from conservation regions where DDT is currently still in use. With no accumulation of DTM measurable in the frogs there is no measurement of how relevant DTM levels were to current environmental exposure concentrations. Deltamethrin is in use for MVC in the same region where amphibian samples from chapter 3 and Wolmarans et al. (2018) were collected in northern KwaZulu-Natal (Goodman et al. 2001). Only the presence of cypermethrin was previously been confirmed in sediment and water from this region (Sereda and Meinhardt 2005), but DTM was detected in human breastmilk in this same area (Bouwman et al. 2006) confirming the possibility of simultaneous exposure to both DDT and DTM (including other pyrethroids) for frogs from MVC regions.

5.4.2 Metabolomics

Univariate analysis revealed very few statistically significant changes. There is an expected natural increase in data variability from a simulated field exposure as opposed to a laboratory based exposure with no substrate (sediment or vegetation). This is complicated further through dietary changes in a simulated field exposure with the presence of invertebrate prey compared to a non-feeding laboratory based exposure. Dietary change such as fasting has been shown to

directly affect the liver metabolomic profile of *Rana omeimontis* tadpoles (Zhu et al. 2019). However, in tadpoles, lipid storage is dependent on the liver, where adult frogs store lipids in fat bodies (Zhu et al. 2019). Food withdrawal has also been shown to change the serum metabolome of mice (Zheng et al. 2018). In the current study the feeding changes were partially solved for by the secondary refinement of the 96 h metabolome dataset to corresponding results from the laboratory based exposure. The remaining metabolites are indicative of changes most likely to have a causal relationship with each exposure treatment regardless of secondary exposure circumstances. These remaining metabolites however contained only two unknown compounds that showed changes of statistical importance, both in frogs from the DTM 0.1% treatment. While the pathway analysis showed low probability correspondence with laboratory based exposure, the variation in field based exposure data is too high to accurately describe effects on the liver metabolome in this manner.

It is worth noting that the laboratory based exposures in Chapter 4 indicated many shifts in energetics (carbohydrate and lipid metabolism) attributed to a general adaptation syndrome or similar effect to the stress of exposure. This same response is not seen in the current study. The lack of these universal energetics changes to exposure could possibly indicate that the frogs from laboratory based exposure experienced stress, either due to fasting or lack of hiding spots in the experimental tanks. The addition of chemicals then exacerbated this stress response resulting in a notable difference to the controls in this regard. The field exposure setting offered feeding opportunity and camouflage for the frogs, and thus the exposure does not effectively exacerbate any underlying stress and distinguish carbohydrate and lipid metabolism from the controls to the same extent. This argument is in line with hypothalamic-pituitary-adrenal (HPA) axis activation evidence given in Chapter 4 for the laboratory based exposure. Chemical stressors are known to exacerbate underlying physical and psychological stresses (Friedman and Lawrence 2002). Mouse liver metabolome response to polychlorinated biphenyl (PCB) exposure has also been shown to be altered by dietary changes that cause liver injury (Deng et al. 2019). The observation

that this difference exists between laboratory and field based exposure responses is important to take into account for future metabolomics studies when attempting to tie lab based responses to real-world exposure scenarios.

5.4.3 Invertebrate effects

The changes observed in the control in terms of increased taxa diversity and evenness over the 28 d period indicate that the invertebrate community structure stabilised. The total abundance of invertebrates was greatly reduced in the control, mainly through loss of *Culicidae*. This could be due to natural progession as larvae emerge, but with mosquitoes constant repopulationis expected and such a dramatic loss has to include predation by *X. laevis*. This is in line with results by South et al. (2020) indicating that *X. laevis* actively hunt *Culex* sp. larvae from the top water column. With *X. laevis* being visual predators (South et al. 2020) the most abundant food source (Culicidae in this case) would likely be targeted. The loss of Culicidae is seen in all exposures and is considered a consistent aspect in all treatments. There was however an increase in abundance for *Notonectidae*, *Gerridae*, *Chironomidae* and *Corixidae* in both the control and DTM 0.1% treatments, whereas the abundance for these familes were reduced in all other exposure treatments indicating a pesticide effect rather than a predation effect on these taxa. Based on the loss of taxa in the Mix 1% treatment, the direct combined pesticide effects on invertebrate community increased predation pressure to such an extent where abundance and diversity could not be maintained. Rico et al. (2018) assessed the effects of a neonicitinoid pesticide mixture containing imidacloprid, acetamiprid, thiacloprid, thiamethoxam and clothianidin on aquatic invertebrate communities in a mesocosm setting. Their results also indicated the mixture as having a greater effect on invertebrate abundances compared to imidacloprid alone. The study by Rico et al. (2018) showed the greatest decrease in abundance at 28 days. At 56 days abundance recovery was seen in the imidacloprid exposure. In mixture exposure however the concentrations with significant abundance reduction at 28 days did not result in complete recovery in 56 days. Although not assessed in this study, given the abundance and diversity reduction seen

in the DDT and DTM mixture at 28 days, the persistence of DDT could potentially distrupt such recovery even further.

5.4.4 Ecological implications

Based on the effects observed in the aquatic invertebrate community, chronic exposure to environmentally relevant levels of DDT and Deltamethrin can significantly affect the food web structure in the presence of *Xenopus* as predators. This is most prominent in mixture exposure and suggests small ponds and pans may be significantly altered by the introduction of mixture pesticides at currently relevant levels. This is supported by the comparison between experimental and field measured DDT concentrations in section Accumulation5.4.1. Recent studies from MVC regions in South Africa do not mention clear large scale disruptions to invertebrate ecology or particularly low diversity in small ponds and pans (*see* Dube et al. 2017). Dube et al. (2020) has, however, shown that invertebrate diversity in pans inside the Ndumo Game Reserve conservation area are higher than in pans that are outside the reserve and in close proximity to human settlements. Pesticide use is one of the stressors expected to be elevated outside the conservation area, but Ndumo Game Reserve also makes use of vector control inside the reserve and thus this difference in biodiversity cannot be attributed to pesticides alone. The fact that large ecological shifts are not currently witnessed in small pans from these regions could indicate resistance build-up from generational exposure to low levels of these pesticides in both target and non-target organisms. The target organism (mosquito) resistance build-up towards pyrethroids and DDT has been proven (Lumjuan et al. 2011; Ranson et al. 2011), but non-target organisms' resistance build-up has not yet been assessed for these pesticides to the best of our knowledge. Genomic assessments of field sampled invertebrates, amphibians and other non-target organisms could provide insight into the potential for resistance to the effects of these pesticides. Such non-target resistance could manifest similarly to that seen in a study by (Wirgin et al. 2011) where Atlantic tomcod (*Microgadus tomcod*) populations from the Hudson River (notorious for historical PCB pollution) showed visible resistance to PCB toxicity, and contained a different

variant of the aryl hydrocarbon receptor (AHR2) allele responsible for initiating biotransformation responses to these pollutants. This feature naturally occurred in a small percentage of Atlantic tomcod populations from less polluted rivers and it is theorised by the authors that selection pressure from the long term introduction of these pollutants changed the genetic variation of the Hudson River populations resulting in a sustainable CB resistant population. A similar scenario could possibly be at play for one or more non-target organisms from malaria risk regions where decades of chronic pesticide input could have forced genetic evolution in a specific direction via toxicity resistance. Much further study is required in this regard before the true effects of MVC pesticides on non-target organisms can be fully understood.

5.5 Study limitations

Intrumental limitations to this study could have masked some of the pesticide residue and metabolomic results obtained. For instance, improved analytical techniques such as use of high-resolution mass spectrometry may have resulted in better identification of metabolites lowering the number of unknown analytes observed in the study. Lower limits of quantitation for pesticide residue analysis would have offered better opportunity to assess the fate of DTM in the aquatic environment.

The absence of any effect on macroinvertebrate availability as food source is possibly due to the study design that was too robust and that invertebrate colonisation time was not sufficient to ensure that the community structure in the microcosms reflect what would be expected under natural conditions. The colonisation period used in this study was predicted to be effective based on data from a sub-tropical floodplain climate where invertebrate diversity and abundance in mesocosms stabilised in 5 weeks (*see* De Necker et al. 2019). In the current study that timeframe did not allow for a stable invertebrate community structure to develop likely due to climate differences, missing peak summer time invertebrate activity, and low initial abundance seeding during set-up of microcosm ponds.

5.6 Conclusion

This study aimed to assess the accumulation patterns and associated liver metabolome changes in amphibians exposed in a simulated field setting. Accumulation was successfully measured in sediment and frog tissue for DDT, one of the two pesticides tested, at both 0.1% and 1% of the $LC50_{FETAX}$ concentration. This study indicates that simulated field assessments can be used effectively to bridge accumulation trends in laboratory based exposures to field observed accumulation patterns in aquatic frogs. Metabolomic profile of the liver of *X. laevis* showed great variability in response that could be attributed to environmental and dietary changes apart from pesticide exposure itself. The resulting effects could not be used to accurately deduce exposure effects in a simulated field exposure scenario. That being said, there is some evidence in terms of overlap between laboratory and field based exposure results to indicate that a mixture of DDT and DTM at 1 % of the $LC50_{FETAX}$ could affect the liver energetics of *X. laevis* in terms of altering fatty acid biosynthesis. The aquatic macro-invertebrate community structure was also shown to be significantly affected as a secondary effect of the exposure to a mixture of these pesticides and the possibility that current environmental exposure levels in MVC areas could drive wider ecological change in this regard could not be ruled out.

5.7 Recommendations

In terms of improvements to the current study for future analysis, a longer colonization period for invertebrates would be suggested prior to initiation of experiment, with maximal abundance seeding during pond set-up. For maximal invertebrate activity pond set-up should be considered in mid-summer. Assessing the effects of pesticides on invertebrate communities without the predation pressure of amphibians would provide and indication of what extent of the ecological effects are direct on the invertebrate community and what extent are enhanced due to predation. If possible, studies where individual frog behaviour can be assessed in a simulated field

assessment would offer excellent opportunity further link metabolomic and behavrioual endpoints in frogs exposed to MVC pesticides.

CHAPTER 6 INCORPORATING REGIONAL RISK ASSESSMENT AND ECOSYSTEM SERVICES TO INFORM AMPHIBIAN CONSERVATION MANAGEMENT STRATEGIES IN THE PHONGOLO RIVER FLOODPLAIN

6.1 Introduction

In northern Kwa-Zulu Natal, the Pongolapoort Dam creates an artificial barrier separating the upper and lower Phongolo River. The lower Phongolo River flows northeast from the dam to form the largest inland floodplain in South Africa. On a continental scale the Phongolo River floodplain is fairly small, but it is considered one of the most important floodplains in South Africa for hosting the highest biodiversity of any floodplain system in the country. The system is made-up of a combination of riverine and pan (colloquial term for a shallow basin endorheic wetland) habitats covering about 20% of the surface area (Heeg and Breen 1982). The nutrient rich soil with seasonal flooding and warm climate results in a highly productive system. The local human communities utilise the depressions and pans for cultivation of subsistence crops and are highly dependent on the environment for materials, food, and water. After the Pongolapoort dam was built in the early 1970's, the annual floods in the floodplain were simulated though artificial dam releases on a regime designed around social and ecological needs (Brown et al. 2018, De Necker et al. 2020).

Due to the use of artificial flooding to manage such an important ecosystem for biodiversity in South Africa, the Phongolo River floodplain has been the focus of quite a few ecological risk studies. These include the original work by Heeg and Breen (1982; 1994) on the ecological functioning of the flooding regime itself followed by studies by Jaganyi et al. (2008), Lankford et al. (2011) and more recently Smit et al. (2016) and Acosta et al. (2020). Each of these studies have built on previous work and incorporated aspects of prior studies thereby increasing the confidence in data generated. A section of the study by Smit et al. (2016) was developed into a regional risk assessment by O'Brien et al. (2021). These studies formed the basis from which the

current study was conceived. Dube et al. (2015) identified the need for inclusion of environmental flow assessments in the region, after which Brown et al. (2018) developed desk-top based flow requirements for vegetation and fish. However, amphibian responses to alterations in water quality and flow were not considered.

In the study by Smit et al. (2016) certain shortcomings in data were identified at the time. One of the main areas where lacking data was identified was environmentally relevant toxicity data for African amphibians. The use of DDT as malaria vector control (MVC) pesticide was included as a chemical stressor in the conceptual model of O'Brien et al. (2021), but toxicity data used to calculate hazards to amphibians did not include local species. Other currently used pesticide inputs were not assessed alongside DDT, and the environmental relevance of exposure data could not be verified. These factors effectively add uncertainty to the chemical hazard data used in the original assessment. As part of the Relative Risk Model (RRM) methodology used by Smit et al. (2016) one of the assessment steps includes hypothesis generation for future testing (the RRM steps are discussed in more detail in section 6.2.1). This was the motivation for undertaking the current study to investigate certain aspects of amphibian well-being within the floodplain in more detail with regard to chemical stressors.

The RRM is a robust ecological risk assessment tool that fits within the regional risk assessment framework as set out by (Wiegers and Landis 2005). Its use focusses on summarising complex interactions between stressors and ecological endpoints on a spatial scale (O'Brien et al. 2018; O'Brien et al. 2021). Bayesian Networks (BN) are incorporated into modern RRM to provide holistic analysis of stressor effects on ecosystem functioning (O'Brien et al. 2021; Wade et al. 2021). The endpoints assessed with the Bayesian Network Relative Risk Model (BN-RRM) can be adapted to the specific ecological risk assessment needs. For instance, Wade et al. (2021) assessed the Thukela River system in South Africa from the perspective of legally enforceable

Resource Quality Objectives (RQOs), whereas O'Brien et al. (2021) assessed the Phongolo floodplain from a biodiversity and utilisation interaction perspective.

Ecosystem service assessments address socio-ecological interactions from a human perspective focussing on sustainable use of environmental resources. These assessments can also be used to assess the effects of stressors on socio-ecological endpoints as outlined by Maltby et al. (2017) and Faber et al. (2019). Often ecosystem service assessments focus on specific protection goals as outcomes using the framework of Niensted et al. (2012). The specific protection goals set for a region aim to protect ecological drivers responsible for important or impacted ecosystem services in that region. Maltby et al. (2017) provides methodology for the assessment of chemical risks on ecosystem services. This method focusses on stressor impacts on habitats and services provided within a habitat to derive concern rankings for ecosystem services. The inclusion of ecosystem services into ecological risk assessment with a focus on chemical stressors is still in its infancy and an implementation roadmap still in development (Faber et al. 2019). Exploring integration between these assessment types is therefore important to derive functional datasets that can be used for refinement of existing implementation frameworks. To associate chemical risk assessment on amphibians with ecosystem services, understanding links between the two are important. As described in Chapter 1, the most important contribution amphibians make to ecosystem services are in terms of alterations to ecosystem functioning through food web interactions. In Table 6.1 the specific contributions made by amphibians relevant to the Phongolo floodplain are described. Although locally significant in being the only amphibian seen as a food source in South Africa, human consumption of *P. edulis* was not specifically observed during this study in the Phongolo River floodlpain and its contribution as a food source is included as a delicacy rather than a staple for this region. The contribution of adult amphibians to mosquito control is evident from the results of South et al. 2020 and reduction in *Culicidae* abundance seen in the outdoor microcosm experiments of Chapter 5. In the current study these service contributions by amphibians are assessed in terms of habitats within the floodplain and risks toe

habitats are related to services in that manner. The main global cause for loss of amphibian species is loss of habitat (Acosta et al. 2020). This means the risk of ecosystem service loss cannot be separated from the risk of habitat loss when to quantifying a connection between amphibians and ecosystem services (Hocking and Babbit 2014).

Previous investigations into aspects of this study (Chapters 2, 3, 4 and 5) focussed on experimental work and developing baseline data for improving interactions in the existing amphibian well-being model as defined by O'Brien et al. (2021). The ultimate goal of this chapter was to validate and strengthen linkages between risks posed to amphibian well-being by MVC pesticides and other stressors in the Phongolo River floodplain, and compare with risks to human livelihood in terms of ecosystem service delivery in the floodplain. To attain this goal the existing conceptual model for amphibian well-being (as provided in Smit et al. 2016 and O'Brien et al. 2021) was combined with an ecosystem services assessment in this study. Ecosystem services were included to place risk to amphibian well-being in context of the socio-ecological complexity of the floodplain. Comparison between the ecosystem services assessment and amphibian well-being RRM was enabled through habitat as a common link in the stressor – effect conceptual model design for both assessments. In combining the assessments in this manner the specific aim was to identify habitats of concern for both amphibian well-being conservation and ecosystem service provision (based on the associated risks) which may be prioritised for future conservation management efforts.

6.2 Methods

6.2.1 Regional Risk Assessment methodology

The methods set out by O'Brien and Wepener (2012) were followed for this risk assessment. The relative risk model RRM consists of ten steps as originally set out by Landis and Wiegers (1997). O'Brien and Wepener (2012) and Wade et al. (2021) placed the RRA method within the context

of ecological risk assessment in South Africa establishing it as a useful management tool within the Integrated Water Resource Management (IWRM) framework of South Africa.

The ten RRM method steps are: 1 - Listing the important management goals for the region; 2 - Generating a map on which the potential sources and habitats relevant to the established management goals are indicated; 3 - Demarcating the map into regions based on a combination of the management goals, sources and habitats; 4 - Constructing a conceptual model linking sources of stressors to receptors and to assessment endpoints; 5 - Decide on a ranking scheme to calculate the relative risk to the assessment endpoints; 6 - Calculate the relative risks; 7 - Evaluate uncertainty and sensitivity analysis of the relative rankings; 8 - Generate testable hypotheses for future field and laboratory investigations to reduce uncertainties and to confirm the risk rankings; 9 - Test the hypotheses that were generated in Step 8; 10 - Communicate the results in a fashion that effectively portrays the relative risk and uncertainty in response to the management goals.

This chapter addresses steps 8 and 9 of the assessment by Smit et al. (2016) and O'Brien et al. (2021) and as such the base justifications for the amphibian well-being interactions within the conceptual model originated from Smit et al. (2016) and O'Brien et al. (2021) as well.

6.2.2 Study region and risk regions

In keeping with the original assessment of O'Brien et al. (2021) the study region stretched from the Pongolapoort Dam to the Mozambique border and included the river and bordering floodplain covering 130 km^2 (Figure 6.1). The floodplain was divided into three risk regions based on the geographical floodplain boundaries and changes in human population density within the floodplain according to exisitng municipal boundaries. This division allows for comparison to previous work by Lankford et al. (2011), Smit et al. (2016) and O'Brien et al. (2021). The level of human interaction with the floodplain is seen as decreasing from Risk region 1 to Risk region 3 as the third region borders the Ndumo Game Reserve, which has low direct human interaction

(O'Brien et al 2021). Risk regions are indicated in Figure 6.1. Risk region 1 (RR1) is demarcated as stretching from Pongolapoort Dam to the northern edge of the Othobothini ward boundary on the Phongolo River (-27.386289-S, 32.142684-E). Risk Region 2 (RR2) extends from the boundary of RR1 to the southern boundary of the Ndumo Game Reserve (NGR; -26.929993-S; 32.324131-E). This region includes the Nondabuya, Mboza, Bhenindoda, Kwazamazama, Madonela Shemula, Shemula Gata, Lukwane, Kwa-Ndaba, Makhane, Ndumu and Kwamzimna wards. Risk Region 3 (RR3) includes the Phongolo River and associated pans within NGR which extends from RR2 to the Mozambique border.

6.2.3 Stressors

Two factors have been repeatedly shown by previous studies as major stressors influencing this area. Chemical input and water availability. Water quality, in terms of e.g. nutrient input was not deemed to be a driving factor for amphibian well-being based on temporal consistency seen in the floodplain since construction of the Pongolapoort dam (De Necker et al. 2020). Chemical input is largely driven by pesticide input. Being a malaria risk region the historical and current use of DDT plays a major role in this region. Other agricultural pesticides have not been assessed frequently in this region since the study by Sereda and Meinhardt (2003), but given the high productivity and utilisation of the floodplain (Lankford et al. 2011) and the concurrent use of some pesticides for both malaria vector control and crop protection, the input of these pesticides cannot be ignored. Water availability really came to the forefront as a stressor during the recent droughts experienced in the region between 2015 and 2018 (De Necker et al. 2020). With climate change predictions estimating higher variability in weather patterns in South Africa in the future with extreme circumstances accentuated in climate model predictions (Jury 2019), the importance of water availability for ecosystem service delivery and for habitat stabilisation must be included in a risk assessment of the region.

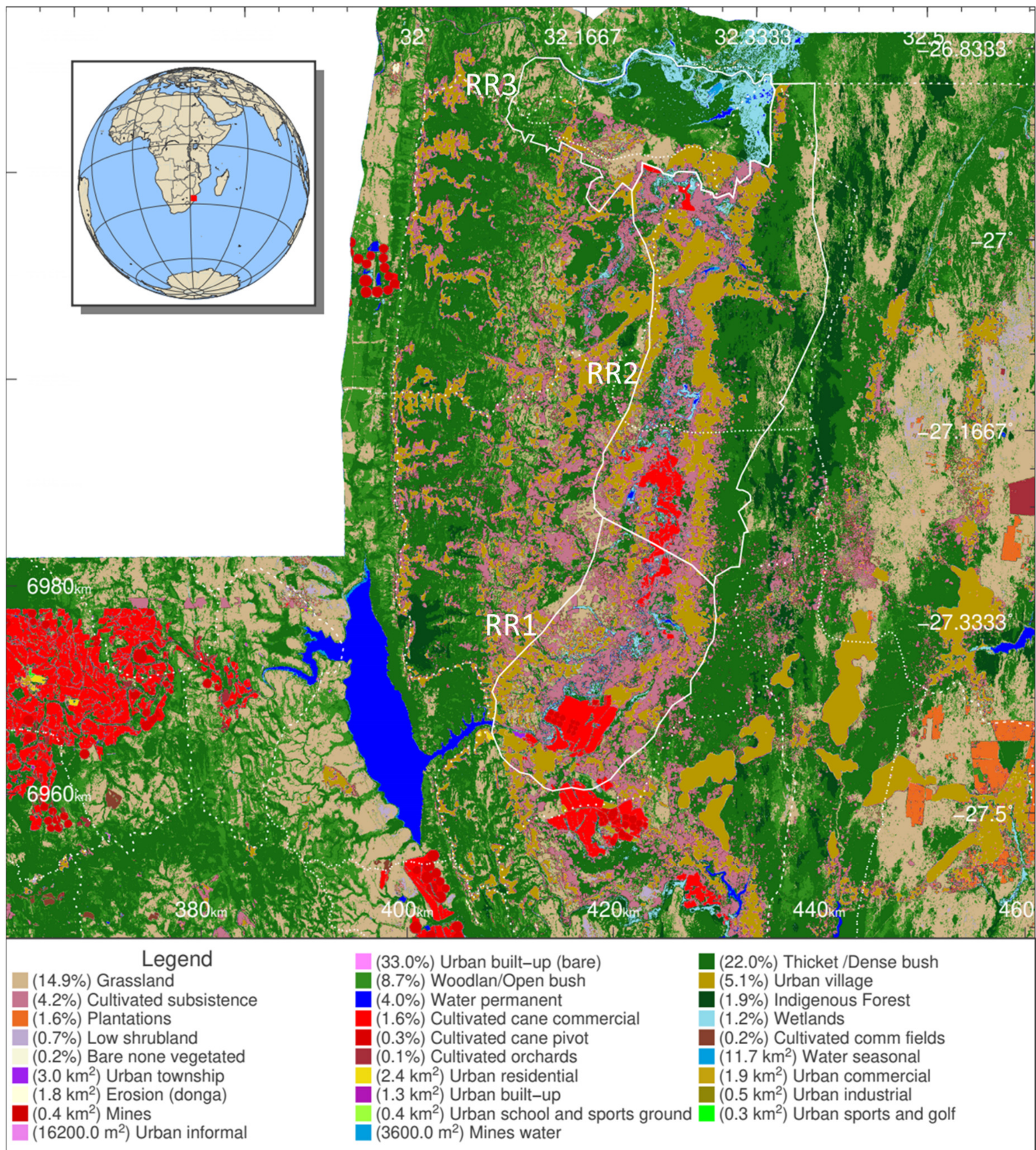

Figure 6.1 **Land cover map of the study region with the demarcated risk regions indicated as RR1 = risk region 1, RR2 = risk region 2 and RR3 = risk region 3. 2014 Land cover data was used in this assessment (DEA 2014).**

6.2.4 Stressor states

The influence of these stressors on amphibians were defined into four states namely zero, low, moderate, and high. Zero was set as having no influence from the stressor, low as having subtle or perhaps sub-lethal chronic influence, moderate was defined as having acute effects, and high as observing morphological effects, mortality or large scale ecological changes. Input levels were derived from a combination of historical input (Sereda and Meinhardt 2003) and the relation between amphibian field accumulation measurements (Wolmarans et al. 2018; Chapter 3, Wolmarans et al. 2021), and exposure concentrations based on data generated in Chapters 4 and 5 of this thesis. The lower concentrations of DDT and DTM assessed in Chapters 4 and 5 were considered to have biochemical effects only whereas the higher concentrations assessed were considered to have behavioural effects with the combined higher concentrations considered as having cascading ecological effects. Where data were available, these concentrations were adjusted based on the sensitivity of *Xenopus laevis* (model amphibian used in these assessments) compared to other amphibian species as assessed in Chapter 2 (Wolmarans et al. 2020). The relationship between accumulation and exposure (Chapter 5) was used to link field based accumulation to input exposure levels. All justifications and concentrations used to define stressor states in this study are given in supplementary Tables D1 and D2.

6.2.5 Habitats

Habitat delineation was derived from a combination of land cover data (DEA 2014), overlapping vegetation habitat data (SANBI 2018), and habitat categories from Lankford et al. (2011). Habitats assessed were categorised as urban, cultivated land, grassland, woodland and forest (including riparian forest), low shrubland, rivers, permanent (flood connected) pans and wetlands, and temporary (ephemeral) pans and wetlands.

6.2.6 Model development

A basic study outline was designed to help set the scope and desired outcome for this assessment (Figure 6.2). Chemical input and water availability were identified as two major stressors to habitats in this assessment based on results from Smit et al. (2016) and O'Brien et al. (2021). The designation of priority habitats to inform future protection goals was set as the desired outcome. The risk to different habitats would be determined through the relative risk to amphibian well-being within specific habitats and risk to ecosystem services in corresponding habitats to compare these model outcomes. Using the basic study outline, conceptual models from O'Brien et al. 2021 were adapted for use in this study. Secondary conceptual models were developed for habitat risk (Model A; supplementary Figures D1 and D2) followed by amphibian well-being (Model B; Supplementary Figure D3) and ecosystem services (Model C). Models B and C were assessed for each habitat identified in Model A to determine risk distributions to each habitat in terms of both amphibian well-being and ecosystems services. The risk scores calculated from the sum of the probability distribution between zero, low, moderate, and high risk catergories were used to prioritise habitats for the protection of amphibian well-being and ecosystem service delivery.

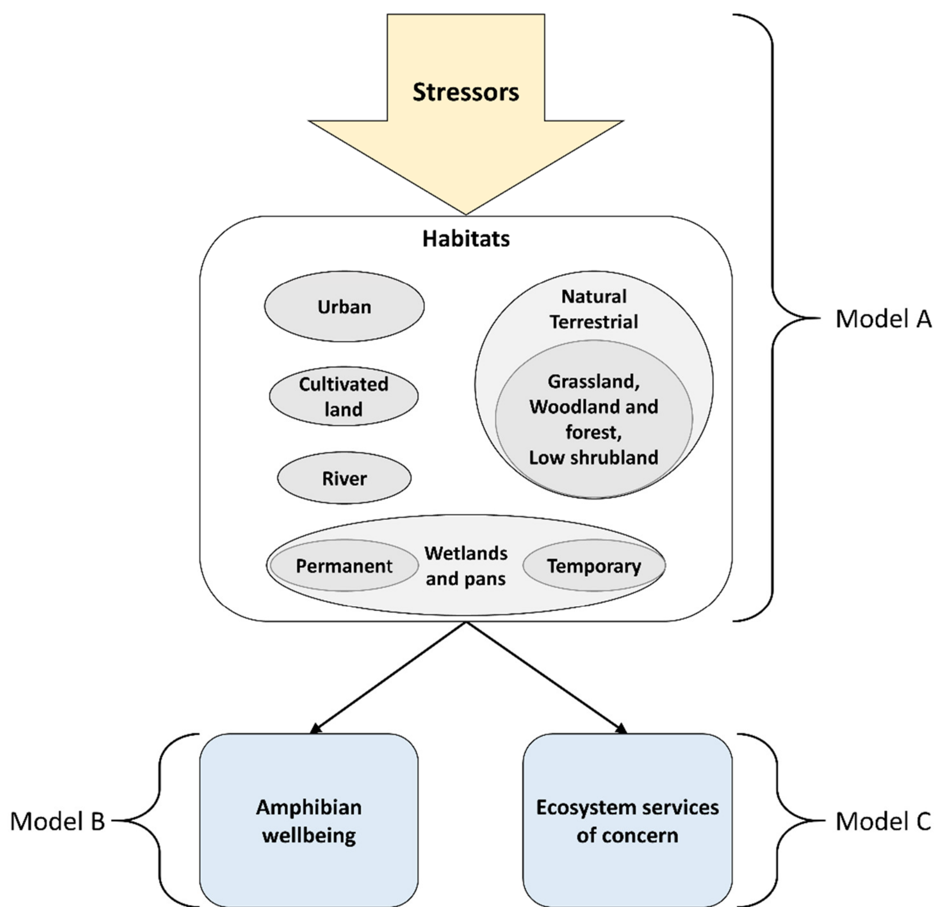

Figure 6.2 **Basic study outline indicating the analytical models used for each step of the assessment. Models A and B used bayesian network models while model C was based on a matrix model**

6.2.6.1 Amphibian well-being

Chemical risks to habitat (Model A) and amphibian well-being (Model B) were determined through BNs constructed using Netica (supplementary Figures D2 and D3). Relationships between input and output variables within the BNs were based on historical data from previous studies in the Phongolo River floodplain region as in O'Brien et al. 2021. The quantification of node interactions was based on mathematical relationships described by Wade et al. (2021) and O'Brien (2021) where stressors and habitats are ranked and weighted through likelihood filters on the interactions

between the two factors. The conceptual model that formed the basis for Model B to assess the influence of stressors on amphibian well-being within the region was adapted and refined from the original study by Smit et al. (2016) with adjustments made to toxicant exposure based on experimental work performed in Chapters 4 and 5 and potential for frog occurrence specified for each habitat assessed in this study.

6.2.6.2 Ecosystem services

The ecosystem services assessment used in Model C and the interactions between ecosystem services, habitats and chemical stressors were determined following the methods of EFSA (2010) and Maltby et al. (2017). These methods were designed around the assessment of ecosystem service impacts with regard to chemical stressors.

The method essentially consists of four steps. The provision of services is first calculated for each available habitat within the study region and a scoring of zero, low, moderate, and high importance is given to each (Table 6.1). Using the methods of Maltby et al. (2017), in the current study these rankings were based on the findings of Lankford et al. (2011) and expanded upon with findings from Smit et al. (2016) and O'Brien et al. (2021). The second step is associating the potential impacts of stressors with habitats and specific services via a conceptual model. Model A quantified this association as a risk distribution. The third assessment step is prioritisation of each service within each habitat. In this step services were ranked based on a matrix of importance (supplementary Table D3) combining the ranking of step 1 and step 2 habitat in providing the service and the potential impact of the stressor on the habitat (Table 6.2). Monte Carlo permutation on the final risk scores provided a risk distribution. The final step in the ecosystem service assessment methodology consists of setting specific protection goals (SPGs) for priority services and habitats. This final step was modified to identify priority habitats for conservation of ecosystem services with specific focus on services where amphibians are considered service providing units.

Within the larger context of the regional risk assessment methodology the models described above were applied to each of the three designated risk regions. The habitat risks determined via Model A, were used as input for Model B and C and were weighted based on relative availability of each habitat within the different risk regions. Land cover data (DEA 2014) were used as measure of habitat availability within each region. Figure 6.1 indicates overall land cover contributions to the floodplain, but this was assessed separately for each risk region and correlated to existing habitat categories as described in section 6.2.5.

6.2.7 Scenario analysis

In addition to prioritising habitats based on current data, future scenarios were also incorporated to determine the extent to which risks to priority habitats would change under different circumstances. The scenarios assessed were derived from Smit et al. (2016) and consisted of three resource management states. Firstly, the current state as determined by current water availability and chemical input data. Secondly a natural state defined as prior to industrial development influence (the assumption here is that indigenous communities prior to the industrial era, lived in harmony with the ecosystem and utilization of resources was based on subsistence living for extended periods). The third scenario assessed was a desired or realistic ideal state. This involves the optimal combination of governance and utilisation of the floodplain to best ensure the sustainability of subsistence and commercial agriculture while also ensuring critical ecological preservation. Monte Carlo permutation tests were incorporated into the RRM as in Landis (2005) to produce probability distribution estimates for output variables from assigned input variable ranks. Designations of zero, low, moderate and high were assigned to endpoints for each scenario. Monte Carlo permutation was then conducted (1000 iterations) and output distributions for each scenario derived.

As the relative risk model makes use of interactions between components in the conceptual model there is an inherent uncertainty to the analysis affected by factors such as quality of the prior data

used. Sensitivity analysis in the form of entropy reduction was used on nodes within the analytical models as measure of uncertainty. Entropy reduction measures the influence of an input variable on a response variable as such that a greater entropy value equals greater influence (Marcot et al. 2006). The information gained from the sensitivity analysis on endpoint variables was used to determine the input parameters that had the greatest influence on risk estimates and the associated uncertainty.

Habitat priority rankings were determined based on the current state risk score plus the difference in risk score between the current state and desired state scenarios, with the highest to lowest resulting scores ranked 1 to 8 respectively.

6.3 Results

6.3.1 Amphibian well-being

The relative risk to amphibian well-being for the whole floodplain was derived from the combined risk distribution for all eight habitats assessed (supplementary Figures D4-D6). Figure 6.2 6.3 indicates the amphibian well-being relative risk distribution for each Risk region from each of the three scenarios assessed as the average risk over all habitats. The current state scenario indicated moderate risk as the most likely outcome ranging between 25% and 52% probability for all three risk regions. Risk region 1 and 2 had a 20% to 30% probability of being at high risk while Risk region 3 had a 10% to 25% probability of being at high risk. The historical state indicated Low risk as the most likely outcome in all three risk regions. The desired state scenario resulted in an even spread between low and moderate risk for all three risk regions.

6.3.2 Ecosystem services

The importance rankings of habitats indicated all habitats as crucial to the provision of ecosystem services (Table 6.2). Concern levels (Table 6.3) indicated river and temporary pan and wetland

habitats as the only habitats of moderate concern. Genetic resources, provision of fresh water, biodiversity, recreation and ecotourism, aesthetic values, and sense of place were identified as service categories of moderate concern within these habitats. No habitat or service of high concern was identified. Contributions by amphibians under the categories identified as moderate concern, include their specific contribution to biodiversity and genetic resources within the floodplain, which affects both terrestrial and aquatic habitats. Alongside this, their potential for contribution to ecotourism, contribution to the aesthetic value and sense of place for the human communities living in the floodplain, as well as to visitors to NGR in Risk region 3 are also regarded to be at moderate risk.

Table 6.1 The specific contributions of amphibians to ecosystem services derived from Hocking and Babbit (2014) and Phaka et al. (2017), with the associated relevance in the Phongolo River floodplain as observed in this study. Only categories with amphibian contribution are included

Ecosystem Service		Amphibian contribution	Relevance in the Phongolo River floodplain
Provisioning services	Food production	Consumption of amphibians as food source. Poisonous amphibian skin secretions are used in some cultures to hunt larger food sources.	Only *Pixycephalus edulis* is consumed, but infrequently and not as a staple diet. This was not observed during this study. The use of amphibians as a hunting tool are not practised in South Africa.
	Genetic resources	Biomass contribution, drivers of genetic variation in terrestrial and aquatic food webs.	Amphibians contribute to terrestrial and aquatic food webs in the floodplain and play a key role as prey for waterfowl of importance to the conservation status of this region.
	Biochemical/ natural medicines	Use in scientific study of medicines, Use in traditional medicines.	Amphibian contribution to scientific study of western medicines has provided many breakthroughs on a global scale, and in this case is considered an ongoing global contribution, not limited to this region. The use of amphibians in local traditional medicine is documented.
Regulatory services	Pollination	Amphibians also have the potential to affect pollination trough predation on both invertebrate pollinators and other invertebrate predators. Although rare, some frogs are also known to consume fruit and disperse the seeds. The extent to which amphibians contribute to these areas as a component of overall ecosystem functioning have not yet been tested.	These impacts by amphibians have not yet been quantified and their contribution in this sense was considered with high uncertainty.
	Biodiversi ty	From an ecological perspective amphibian contributions to biodiversity lie in their key role in aquatic and terrestrial food webs and sensitivity to ecological and habitat change.	More than 25% of the amphibian diversity in Southern Africa is found in the Phongolo River floodlpain which speaks to the importance of the floodlpain habitats in maintaining amphibian biodiversity.
	Pest and disease regulation	Amphibians alter disease transmission through control of vectors including midges, flies, and mosquitoes, through predation on both larval and adult stages. Their role in this regard is amplified in ephemeral wetlands where other vector control agents such as fish are not always supported. Mosquito larvae can also be considered competition for filter feeding tadpole stages of some amphibian species. Amphibians can also control invertebrate pests contributing to crop protection in this manner.	This is an important aspect for malaria control in the Phongolo floodplain as the area contains many ephemeral pans as potential breeding ground for mosquito vectors with amphibians acting as main vector control agents in that regard. The efficacy of amphibians for crop protection in subsistence farming on floodplain pans has not yet been assessed.

Table 6.1 (continued)

Ecosystem Service		Amphibian contribution	Relevance in the Phongolo River floodplain
Cultural Services	Spiritual and religious values	Amphibians form part of religion, cultural legends and superstitions on a regional basis. This is more common in Asia and South America.	Local communities often consult traditional healers for religious activity who may use or refer to amphibians in rituals. Amphibians form part of local legends, but the true extent of amphibian importance in local religious practise has not been assessed.
	Education and inspiration	Amphibian dissections have long formed part of secondary and tertiary level education on vertebrate anatomy and physiology. Amphibians also form the inspiration for many video games and advertising campaigns, but the inspirational origins are often linked to cultural links to amphibians through legend.	Locally amphibians have not formally formed part of education to date. This is changing with the addition of local language translations of amphibian guides and increased amphibian ecotourism.
	Recreation and ecotourism	Amphibian contribution to biodiversity is increasing in value to humans through amphibian ecotourism, but is still considered a minor contribution in comparison to other ecotourism sectors.	Amphibian ecotourism is a future prospect in this region, which was initiatedat time of this study through guided amphibian tours in NGR.
	Cultural diversity and heritage	Many cultures have close links to amphibians as part of mythology and religion, often stemming from a strong association between amphibians and the supernatural.	Historical beliefs of the Zulu culture include, among others, that frogs can summon lightning. The strong association between amphibian emergence and rain often gives cultural significance to the sighting of frog species such as rain frogs (*Breviceps* sp.)
	Aesthetic values	Views on the aesthetic aspects of amphibians and their habitats vary greatly.	Amphibian presence is considered to improve the aesthetic value of local wetlands through its biodiversity contributions.
	Sense of place	Frog calling can be viewed by people as an annoyance or peaceful event depending on the species.	Frog calling is a natural regular aspect of floodplain life and contributes to the aesthetic aspects held by this region.

Table 6.1 (continued)

Ecosystem Service		Amphibian contribution	Relevance in the Phongolo River floodplain
Supporting Services	**Nutrient cycling**	Amphibians contribute to supporting services through alteration of ecosystem functions. This includes predation on both aquatic and terrestrial aspects of the food web as well as being preyed upon in both aquatic and terrestrial aspects of the food web. Different amphibian life stages (depending on species) have different feeding habits allowing nutrient regulation between aquatic and terrestrial ecosystems.	This is considered a globally applicable aspect of amphibian contribution to ecosystem functioning.

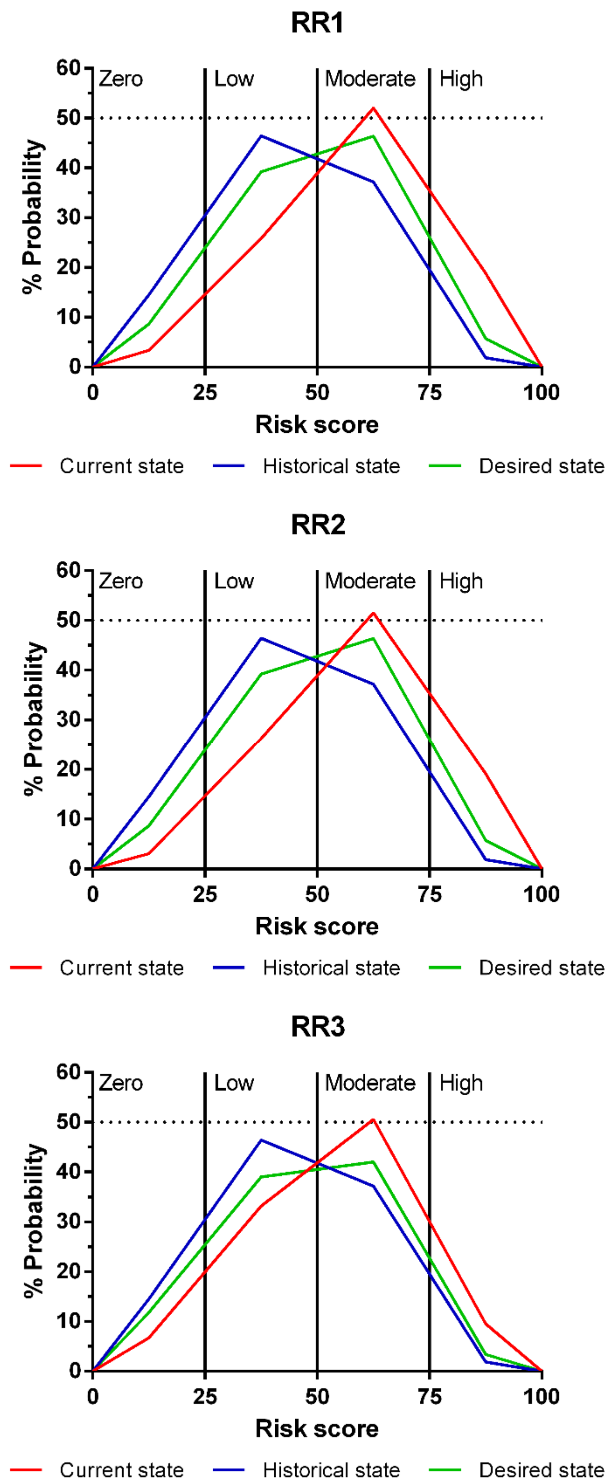

Figure 6.3 **Relative risk distributions for the amphibian well-being averaged across all eight habitats assessed. RR1 = Risk region 1, RR2 = Risk region 2, RR3 = Risk region 3**

Table 6.2 **The relative importance of assigned habitats for the provision of specific ecosystem services. Scoring is designated as: Zero –; Low +; Moderate ++; High +++. Scores were assigned based on expert opinion derived from literature, including Lankford et al. (2011), Maltby et al. (2017), and O'Brien et al. (2021).**

	Ecosystem Service	Urban	Cultivated land	Grassland	Woodland and forest	Low shrubland	Permanent pans	Temporary pans	River
Provisioning services	Food production	+	+++	++	+	++	++	+	+
	fibre, fuel and construction material	+	++	+	++	++	++	+	+
	Genetic resources	++	++	+++	+++	+++	+++	++	+++
	Biochemical/natural medicines	–	–	++	+++	++	+	+	+
	Ornamental resources	+	+	+	+	+	+	+	+
	Fresh water	++	++	+	+++	+++	+++	+++	+++
Regulatory services	Pollination	++	+++	+++	++	+++	++	++	+
	Biodiversity	–	+	++	+++	++	++	++	+
	Pest and disease regulation	++	+++	+	++	+	++	+	++
	Climate regulation	+++	+++	++	+++	++	+++	+	++
	Air quality regulation	+++	++	++	+++	++	+	+	++
	Flood attenuation/water regulation	++	++	++	++	++	+++	+	++
	Erosion regulation	+	++	++	++	+++	++	+	++
	Natural hazard regulation	+	++	++	++	+++	++	++	++
	Water purification/soil remediation/waste treatment	++	++	++	+++	+++	+++	+	++
Cultural Services	Spiritual and religious values	++	+	+	+	++	++	+	++
	Education and inspiration	+	+	++	++	++	+++	++	++
	Recreation and ecotourism	++	++	+++	+++	+++	+++	+++	+++
	Cultural diversity and heritage	+	++	++	+	++	++	+	++
	Aesthetic values	+++	+++	+++	+++	+++	+++	+++	+++
	Sense of place	+	+++	+++	++	+++	+++	++	+++
Supporting services	Primary production and photosynthesis	++	+++	+++	+++	++	+++	+	++
	Soil formation and retention	++	++	++	++	++	++	++	++
	Nutrient cycling	++	+++	++	++	++	++	++	++

Table 6.3 Ecosystem services of concern for each risk region based on the current state. White = negligible concern, light grey = low concern, dark grey = medium concern, black = high concern. No high concern levels were determined in the study area

Current state scenario	Risk region1								Risk region 2								Risk region 3							
Ecosystem Service	Urban	Cultivated land	Grassland	Woodland and forest	Low shrubland	Permanent pans	Temporary pans	River	Urban	Cultivated land	Grassland	Woodland and forest	Low shrubland	Permanent pans	Temporary pans	River	Urban	Cultivated land	Grassland	Woodland and forest	Low shrubland	Permanent pans	Temporary pans	River
Food production																								
Genetic resources																								
Biochemical/natural medicines																								
Fresh water																								
Pollination																								
Biodiversity																								
Pest and disease regulation																								
Climate regulation																								
Air quality regulation																								
Flood attenuation/water regulation																								
Erosion regulation																								
Natural hazard regulation																								
Water purification/soil remediation/waste treatment																								

Table 6.3 (continued)

Current state scenario	Risk region1								Risk region 2								Risk region 3							
Ecosystem Service	Urban	Cultivated land	Grassland	Woodland and forest	Low shrubland	Permanent pans	Temporary pans	River	Urban	Cultivated land	Grassland	Woodland and forest	Low shrubland	Permanent pans	Temporary pans	River	Urban	Cultivated land	Grassland	Woodland and forest	Low shrubland	Permanent pans	Temporary pans	River
Spiritual and religious values																								
Education and inspiration																								
Recreation and ecotourism																								
Cultural diversity and heritage																								
Aesthetic values																								
Sense of place																								
Primary production and photosynthesis																								
Soil formation and retention																								
Nutrient cycling																								

6.3.3 Habitat prioritisation

The risk to amphibian well-being (supplementary Figure D4-D6) indicated permanent pans, temporary pans, and rivers as being at the greatest risk. Factoring in the difference in risk scores between current state and desired state scenarios the habtats were prioritised as in Table 6.4 indicating permanent pans as the most important region to conserve for conservation of amphibian well-being in Risk region 1 whille temporary pans are considered most important in Risk regions 2 and 3. The risk score difference between habitats ranked 1 trough 6 was only 5 (on a scale of 0-100) indicating that the top 6 habitats are of almost equal importance to the conservation of amphibian well-being.

Based on the ecosystem service assessment risk ourcomes the aquatic habitats were again ranked 1 through 3 with all risk regions indicating river habitats as the most important habitat for conservation of ecosystem services in the floodplain. In Risk regions 2 and 3, permanent pans and woodland and forest were ranked equally at third. Risk score differences were ≈ 19 between the first and second ranking indicating riverine habitats as a clear priority for ecosystem service conservation.

Table 6.4 Priority habitat rankings based on the current state risk score and difference in score achievable through implementation of the desired state scenario. * = Habitats with equal risk scores were ranked as equal, but skipping the next rank in the process

		Urban	Cultivated land	Grassland	Woodland and forest	Low Shrubland	Permanent pans	Temporary pans	River
Amphibian Wellbeing	RR1	8	7	5	6	4	1	2	3
	RR2	8	7	5	6	4	2	1	3
	RR3	8	7	5	6	4	2	1	3
Ecosystem Services	RR1	8	7	6	4	5	3	2	1
	RR2	8	7	6	*3	5	*3	2	1
	RR3	8	7	6	*3	5	*3	2	1

6.4 Discussion

The application of RRM in water resources management in South Africa is increasing, as evidenced from the recent publication output in this field (e.g. Agboola et al. 2020; Vesi et al. 2020; Wade et al. 2021; O'Brien et al. 2021). In a recent publication on the evolution of RRM and BN-RRM, Landis (2021) indicated a South African study (O'Brien et al. 2018) as the only effective implementation of BN-RRM as a management tool (at time of publication). From the established importance of RRM in South Africa, the current study successfully built on the groundwork of O'Brien et al. (2021) and assessed risks to amphibian well-being and ecosystem services in the Phongolo River floodplain from chemical input and water availability to inform current management strategies in the floodlpain region and the NGR conservation area. The adapted RRM was applied to all three risk regions demarcated in the floodplain and habitats were ranked in terms of priority for risk management based on both amphibian well-being and ecosystem services separately. Three ecosystem state scenarios were assessed successfully to indicate the difference in ecological risk between a historical state, the current state and a desired state that can be achieved through ideal management practises. The aquatic habitats shoed the greatest risk with temporary pans as the highest priority habitat for conservation of amphibian well-being and river habitat ranked the most important habitat for conservation of ecosystem services.

6.4.1 Different scenario's based on land-use and management practices

The historical state indicated high sensitivity in the BN models resulting in a slight probability of moderate risk in most cases even though input stressor variables were mostly low or zero. A sensitive input in amphibian well-being was the potential for amphibians to occur in a habitat. This node contributed to 25% of the variation in the well-being output. As this is the case the historical state risk to amphibian well-being was largely between low to moderate risk, but this does not necessarily indicate amphibians are under threat from hazards in this regions, only that this region hosts the highest biodiversity of amphibians in Southern Africa. With 45 species, 26% of the total

amphibian diversity in Southern Africa is found in the Phongolo River floodplain (Acosta et al. 2020) and thus this region has a high potential for amphibian species loss. The high productivity (soil fertility, botanical diversity, historical water availability; Lankford et al. 2011) and subsistence based utilisation (as defined for the historical state) of the region resulted in the historical state showing negligible concern for all ecosystem services, which shifted the total risk toward a most probable outcome of low to zero risk.

The desired state scenario indicated that proper water use management and reduction in pesticide input has the potential to reduce the ecological risk to both amphibian well-being and ecosystem service provision in the floodlpain. In this case proper water use management is defined as the correct implementation of an artificial flood release program where timing (as suggested by Heeg and Breen 1982) and volume (as suggested by Brown et al. 2018) are considered for maximal ecological sustainability with minimal agricultural crop loss in the floodplain to sustain river flow for ecological integrity (O'Brien et al. 2021). Bouwman et al. (2019) called for urgent reduced DDT use in South Africa or implementation of application methods that further reduce risk to humans and aquatic ecosystems. This call is based on continued evidence of health risks to humans, birds, crocodiles, amphibians, and fish populations assessed in the Phongolo River floodplain and surrounding malaria risk region (Bouwman et al. 2019). The reduced pesticide use proposed for the historical scenario in the current study is based on a 50% reduction in DDT use as it was for O' Brien et al. (2021). Reduction to other agricultural pesticides were not taken into account in this regard as evidence of other pesticide residues in amphibian tissue could no be quanitified in Chapter 3 (Wolmarans et al. 2021). However, the interactions observed in mixture exposure studies from Chapter 4 and 5 indicate that if environmental levels can be quantified for other pesticides, such as pyrethroids, the influence on risk to amphibian well-being would be affected. If monitoring of agricultural pesticide inputs in this region is implemented it can improve the realistic output of the current amphibian well-being model in that regard. In Risk region 3 the desired state risk distribution resembled the historical state risk

distribution the closest. This indicates the potential of the conservation measures already in place in Risk region 3 to improve habitat quality when stressor effects are reduced. The desired state scenario reduced the most probable risk to between low and zero for all habitats assessed.

River flow inputs used to determine the current state scenario were derived from flows from the last five years. While an accurate description of the current state, it should be noted that this timeframe includes one of the worst prolonged droughts in the recorded history of the study region resulting in higher risk input in terms of water availability than that presented in Smit et al. (2016). Refined chemical input hazard determinations used in the current study along with the recent changes in water availability resulted in the current risk distribution for amphibian well-being shifting at least one risk level higher than the findings in Smit et al. (2016). The lower probability of high risk in Risk region 3 indicates the impact of current conservation efforts in protecting amphibian well-being in comparison to the other risk regions where no formal conservation occurs. This impact of a formal conservation region is justifiably limited by the fact that the stressors are transboundary issues and both water management and chemical input upstream can affect this region. The higher risk (relative to other habitats) to the river habitat for ecosystem services can be attributed to the large influence of water availability to ecosystem service provision for this habitat. Brown et al. (2018) noted that agricultural crop yields were better in years where dam overspill simulated natural flooding conditions and the optimal release conditions originally proposed by Heeg and Breen (1982), as opposed to artificial release strategies currently implemented. This indicates the importance of proper flood management in maintaining the floodplain ecosystem and reducing ecological risks.

6.4.2 Sustainability of ecosystem services, habitats, and amphibian well-being

The Cultural importance of the floodplain to the local communities is tied in with the provisioning services provided by the floodplain due to subsitance living practises in the region, with great importance placed on the pan habitats for fishing and fish trading (Heeg and Breen 1982; Coetzee

et al. 2015). In this assessment cultural ecosystem services were identified to be at highest risk. Recreation and ecotourism alongside aesthetic value and sense of place were indicated as the services most at risk throughout the whole floodplain, although this ranged from low to moderate concern only. The risk to amphibian wellbeing contributes to the risk to these ecosystem services through current pesticide levels having the potential to alter amphibian male calling (Hoffmann and Kloas 2016), which is directly connected to amphibian contributions to sense of place and aesthetics within the pan habitats specifically. The preservation of genetic resources, and subsequently biodiversity, were identified as provisional services of low to moderate concern in the study region. Amphibian biodiversity is taken into account in the well-being risk assessment through the potential of each habitat in hosting different species found in the region. While not a measure of risk to biodiversity this assessment does indicate the risk for health effects in amphibians within each habitat and the habitat's contribution to amphibian biodiversity as affected indirectly. Thus amphibian biodiversity contributions as an ecosystem service are of concern based on the outcomes of both the well-being and ecosystem service models. The differences between risk regions were not large enough to indicate major differences in ecosystem services of concern between regions. Although Risk region 3 is mainly situated in a conservation area, pressures on this system emanate from the two upstream risk regions (O'Brien et al. 2021) lowering the potential for service delivery. In this study the assessment indicated these pressures increase the risk (mostly to amphibians) within the conservation region. In Chapter 3 (Wolmarans et al. 2021) the impact of chemicals as a trans-boundary conservation issue was discussed and the increased pressure on NGR from pesticides was partly attributed to the relatively small size of the conservation area combined with high pressure on the borders of NGR due to rapid urbanisation in the region. This is an issue that can only be exacerbated in future as recent growth in commercial agriculture in the region immediately bordering NGR has benefitted the local economy and population growth in the nearby town of Ndumu (personal observation N. Wolmarans).

The habitat ranking shows a clear dependence on water availability as the main influencing stressor in this assessment. The tendency for amphibian species distribution to be higher toward water based habitats also contributed to river and pan habitats being identified as the habitats of concern given the large influence of the amphibian potential node on model B. The high biodiversity in the region is also driven largely by water (Brown et al. 2018) with the high diversity of fish, amphibians and waterfowl (Smit et al. 2016; Acosta et al. 2020). The woodland and forest habitat include riparian forest which is highly dependent on river ecosystem health. Woodland habitats were also identified as the main habitat for the production of biochemical resources and natural medicines as an ecosystem service based on the distributions of plant species important for ethnobotany (Hutchings et al. 1996; Van Wyk et al. 2009, cross referenced with habitats using SANBI 2018). Local communities in the floodplain make use of natural medicines to a large extent due to the subsistence living practises followed (Lankford et al. 2011; Smit et al. 2016).

Through the habitat prioritisation phase of the assessment of both amphibian well-being and ecosystem services showed great overlap in risk profiles. Aquatic habitats were prioritised (based on associated risk) over terrestrial habitats for amphibian well-being and for ecosystem services (except for woodland and forest). . The risk to the top three priority habitats (temoprary pans, permamnent pans, and river) in maintaining amphibian well-being can be viewed as overestimations of the risk to ecosystem services. This indicates amphibian well-being will likely be affected in these habitats prior to changes being observed in ecosystem service delivery. For the the ecosystem services of greatest concern, specifically in the river habitat, amphibians contribute as service providing units. Consequently these ecosystem services were correctly identified as sensitive endpoints in model C. Amphibian well-being can therefore be used as a more conservative risk estimate (compared to ecosystem services) to the aquatic habitats in the Phongolo River floodplain. This has implications for future monitoring as it qualifies amphibians as suitable indicators for aquatic ecosystem change in the floodplain. The higher risk to amphibians also means that changes observed in amphibian response to stressors will likely be

visible prior to those changes affecting delivery of ecosystem services from these habitats to an observable degree. the amphibian well-being model structure involves both chemical inputs and amphibian potential (as a measure of biodiversity). Regular amphibian biodiversity assessments can be implemented alongside periodic pesticide quantification (in amphibian tissue and the environment). This would allow changes to the system to be observed prior to cascading effects being observed in ecosystem service delivery. Such monitoring will offer a buffer for management authorities to implement strategy changes when needed, with minimal socio-ecological impact.

6.4.3 Shortcomings and improvements on the assessment model

The BN-RRM is an evolution of the RRM design used by Landis and Wiegers (1997) and improved on the original model through the incorporation of variance and uncertainty moving away from a three phase scoring system to the use of probabilities Landis (2021). In this study the ecosystem services assessment still made use of the three level scoring system (matrix method) and Monte Carlo permutation to simulate probability distribution in the risk to ecosystem services (Model C). This two-step method of deriving a probability distribution has the potential to introduce more uncertainty in the model when compared to the BN design. The simplified nature of relationships within the matrix method, however, simplifies ranking these relationships based on expert opinion in cases where objective quantification is limited (e.g. aesthetics and biodiversity).

In a critical review on wetland ecosystem service assessments, Xu et al. (2020) indicated that 67% of the reviewed studies on wetland ecosystem services made use of biophysical methods. They also found that this field was dominated by mixed approaches that included both ecological models and valuation methods. Economic valuation may in some cases be considered a more useful tool for communicating risk to governing authorities. However, valuation of aspects such as biodiversity can be complex and BN models have the advantage of being able to include aspects such as biodiversity that aren't easily quantified in terms of economic value, because quantification is based on the relationship between nodes (Landis 2021). The results from Xu et

al. (2020) indicate that there is no unified method (at least for wetland ecosystem services) being used in these assessments.

Biodiversity was identified as a unique and important aspect of the Phongolo floodplain in terms of ecological conservation and cannot be overlooked. Thus finding a balance between complexity and practicality, and the inclusion of biodiversity as a part of the ecosystem services assessment is essential. The method proposed by Maltby et al. (2017) was found to be effective for ES modelling in the current study. Ecosystem services are often the desired outcome for RRM (Landis 2021) as the results are immediately relatable to management goals and strategies. Outcomes such as amphibian well-being still require some translation into management strategies with implications to other sectors such as ecosystem services having to be taken into account. With this in mind the current study falls short of traditional RRM in two aspects. Firstly, ecosystem services were not selected as the sole endpoint, but rather analysed in parallel to amphibian-well-being for comparative purposes. Secondly, this study used both BNs and ranking methods where RRM usually follows one or the other with BN being the improved and preferred method (Landis 2021). While unorthodox, the use of both methods in the assessment allowed for comparisons to be made between the two outcomes.

Our justification for these deviations is that the main drive behind this study was to determine risks to amphibian well-being, and that the inclusion of ecosystem services was designed to place amphibian well-being in context with risks to other socio-ecological aspects of the floodplain ecosystem. The habitat prioritization approach was found to be the most efficient starting point for comparing risks to amphibian well-being and ecosystem services from a practical perspective. It allows for development of management strategies that are habitat focussed, addressing both concerns simultaneously.

In terms of model input it should be noted that the use of *X. laevis* as model species for the data generation phase of this assessment has its limitations. This species has various benefits as

discussed in Chapter 2 (Wolmarans et al. 2020). No other amphibian species from South Africa offers the same practical study opportunity in terms of available data, housing and husbandry, and being established as a standard test species. The limitation with the use of *X. laevis* is in its fully aquatic life cycle therefore only reflecting an aquatic route of exposure. This does however, offer better opportunities for behavioural analysis and standardised exposure testing, but terrestrial aspects of the amphibian life cycles cannot be accounted for with this species. A computational species sensitivity distribution approach was implemented to minimise the impact of this limitation on data outputs. However, a lack of toxicological data for other amphibian species limited the implementation of species sensitivity as decribed in Chapter 2 (Wolmarans et al. 2020).

6.4.4 The way forward

Currently the Phongolo River within the study region is classified as moderately protected based on the National Biodiversity Assessment of 2018 (Skowno et al. 2019; Van Deventer et al. 2019), meaning at least 50% of the ecosystem's biodiversity target is protected in conservation areas and is in natural or near-natural condition. Based on current data, such as increasing temporal OCP accumulation patterns in amphibians (Chapter 3; Wolmarans et al. 2021) and conclusions by Bouwman et al. (2019) on bird health risks, pressures on the Phongolo floodplain system (in terms of chemical exposure at least) are not currently on a downward trajectory. Regardless, the scenario analysis from the current study indicates the potential for lowering risks to river habitat exists, which could increase the protection status of the system. Based on the outcomes of this study the aquatic habitats that were prioritised need to be included in future specific protection goals for the region. These protection goals will have to be determined through close collaboration with the conservation management authority of the region (Ezemvelo KZN Wildlife), the human communities living in the region, and other relevant government departments at provincial and possibly national level. Water use management and drought alleviation plans are suggested as

the main concern that needs to be addressed by management authorities to reduce the ecological risk in this region with regard to ecosystem services, while benefitting amphibian well-being in the process. The generally low risk to ecosystem services and the similarity in top priority habitats derived from the two components (ecosystem services and amphibian well-being), will provide resource managers with some leeway for focus on preserving ecosystem services without conceding potential impacts to amphibian well-being. Conversely, focus on amphibian conservation efforts is also not likely to impede progress towards sustainable ecosystem service delivery. Recently Phaka et al. (2017; 2019) have successfully integrated indigenous knowledge of amphibians of the Zululand region with existing scientific taxonomy. This was an important step in relating the importance of amphibian conservation to the local and non-scientific community. The inclusion of indigenous knowledge in this regard has also increased the economic potential of amphibian conservation through enabling the possibility of local amphibian based ecotourism initiatives (personal communication. E. Netherlands). Bird diversity is already an important attraction for ecotourism in the region (Smit et al. 2019), and expansion initiatives focussing on other taxa such as amphibians can build on the existing value of the sector. Due to the importance of water availability as a stressor in this study it is critical that future research on this floodplain system continues to incorporate river flow monitoring alongside amphibian biodiversity monitoring. This is essential to derive a database from which more specific amphibian threshold flow rates can be ascertained to improve upon existing risk models.

O'Brien et al. (2021) identified that a continued trajectory of increased aquatic ecosystem pollution and reduced artificial flood releases from the Pongolapoort Dam would result in unsustainable ecosystem service losses in the region. Risk to aquatic habitats identified in the current study suggests that amphibian well-being will likely be affected earlier than ecosystem services in such a scenario. Continuous amphibian diversity and health monitoring can be a very useful tool in the long term management of the aquatic ecosystems in the floodplain. Implementation of such a monitoring plan alongside continued environmental education, citizen science, eco-tourism and

incorporation of local and indigenous knowledge could potentially thrive as a management practise in this region if implemented with a long term vision of improving sustainable interaction between humans and the natural ecosystem.

6.5 Conclusion

This study successfully assessed ecosystem services and amphibian well-being as major outcomes in an ecological risk assessment of the Phongolo River floodplain. The risk to each aspect was determined in three risk regions demarcated within the floodplain and each was assessed in a current state, historical state, and desired state scenario. The desired state outcome closely resembled that of the historical state for both ecosystem services and amphibian well-being indicating that reduction in chemical input and effective water use management can lower the risk to the entire floodplain. The aquatic habitats were identified as priority habitats for conservation management based on both the risks to amphibian well-being and ecosystem services. The river habitat was identified as the habitat most at risk for ecosystem service delivery, which included biodiversity and genetic resources to which amphibians contribute as an essential aspect of linking the aquatic and terrestrial habitat food webs. The cultural and aesthetic values of the pans and river in the Phongolo floodlpain are considered to be of moderate concern. Risk to amphibian well-being was higher than eccosytem services with overlap in priority risk habitats. This provides evidence for the usefulness of amphibians as an early warning monitoring tool in the floodlpain and their potential use toward conservation of ecosystem service delivery in the Phongolo River floodlpain. This study provides useful information for conservation- and water resources management authorities in the study region and will allow for the setting of viable protection goals for the identified priority habitats through community involvement.

CHAPTER 7 DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

7.1 Introduction

This research study followed a tiered risk assessment approach (Figure 1.1) in order to determine the risk to amphibian well-being within the Phongolo River floodplain in South Africa. This tiered assessment approach entailed building on a previous risk assessment of the region by Smit et al. (2016) and O'Brien et al. (2021) and building focussed testable hypotheses to enhance confidence and data quality related to amphibian well-being within the floodplain. During initial literature review specific focus was drawn to the influence of malaria vector control pesticides based on preliminary field surveys by Viljoen et al. (2016) and later Wolmarans et al. (2018) indicating the accumulation of DDT in amphibians from malaria risk regions in South Africa (Chapter 2; Wolmarans et al. 2020). The second step of the assessment (literature review being the first) entailed a more comprehensive biomonitoring assessment on the accumulation of legacy and current use pesticides in amphibians from the Phongolo River floodplain and the possible threats these pesticides pose to amphibian health. This step prompted a comparative study between amphibians from the conservation area in the floodplain Ndumo Game Reserve (NGR) and Kruger National park (KNP), the largest and most prominent conservation area in South Africa that also falls within the malaria risk region (Wolmarans et al. 2021). The study in Chapter 3 (Wolmarans et al. 2021) indicated amphibians from the Phongolo River floodplain were actively accumulating DDT which was attributed to both the limited size of the protected area in comparison to KNP (i.e. distance from sites to sources) and possibly the active use of DDT inside NGR for indoor residual spraying (IRS), but illegal use could not be disregarded as a possible source. The main concern arising from this comparative study was that trans-boundary-contamination of pesticides is an active hazard to amphibians in NGR.

7.2 Data generation for amphibian well-being risk assessment

A shortcoming in available literature data was identified in Chapter 2 (Wolmarans et al. 2020) with regard to the health effects of MCV pesticides. Not only concerning DDT, but also other pesticide groups including pyrethroids and carbamates. Due to a lack of available toxicological data, the health risks posed by field measured concentrations could not be accurately derived. This echoed the shortcomings identified in the regional assessment by Smit et al. (2016) in terms of risk to amphibians. Following a tiered approach methodology as outlined by Aagaard et al. (2013) and Arts et al. (2014), the lack of applicable data prompted for laboratory based investigations on the effects of these pesticides on amphibians. This step was worked out mainly in Chapter 4, but several collaborative studies (South et al. 2019; 2020) also informed the ultimate data needs at this tier level. *Xenopus laevis*, an established fully aquatic amphibian model organism (Wolmarans et al. 2020) was used as test species for these experiments. In Chapter 4 we successfully linked laboratory based exposure concentrations for DDT and deltamethrin (a representative pyrethroid MVC pesticide) to accumulation levels in a laboratory setting. Initial behavioural changes were observed in frogs exposed to 1% of the $LC50_{FETAX}$ (embryo toxicity based on the Frog Embryo Teratogenesis Assay – *Xenopus*; FETAX) concentration for both DDT and deltamethrin with behavioural changes persisting after 96 h in a mixture exposure of the two pesticides. Furthermore, evidence of metabolomic changes was found in the liver of frogs exposed at these sub-lethal concentrations relating to changes in energetics. We concluded that this observation was a possible indication of general exposure stress as compound specific differences were not as prominent as exposed vs. non-exposed metabolomic differences. The studies by South et al. (2019; 2020) assessed the interaction between frogs and mosquito larvae as natural food source, with South et al. (2020) establishing a baseline of the functional response and behavioural interaction and South et al. (2019) assessing how the behaviours of both predator and prey are affected by the introduction of DDT as chemical stressor. These studies indicated

the possibility of change in the behavioural interaction between aquatic frogs and their prey with the possibility of ecological shifts predicted in a real-world sub-lethal DDT exposure scenario.

Due to the generality of metabolomic responses observed in Chapter 4 there was uncertainty as to how these results would translate into a field based setting in more variable and complex systems. To assess the environmental applicability of the effects observed in Chapter 4 and the possibility of ecological shifts based on predator prey-interactions, simulated field exposure experiments were then conducted (Chapter 5). The simulated field exposures in Chapter 5 were successfully used to assess the environmental applicability to laboratory based exposure results. The increased variability in complex systems led to a shift in metabolomic response indicating the sensitivity of biochemical reactions to environmental conditions. It was concluded that metabolomic effects could not be confirmed in a simulated field environment. Effects on predator prey-interaction were, however, evident and even exacerbated by a mixture exposure of deltamethrin and DDT. A mixture exposure at 1% of the $LC50_{FETAX}$ resulted in significant shifts in the aquatic macro-invertebrate community structure with loss of taxa diversity. Interestingly, the presence of *X. laevis* as predators resulted in a general increase in evenness in invertebrate community structure mainly through significant predation on dominant taxa regardless of the chemical stressor influence.

Through identification of data requirements in Chapter 2 the first aim of this study, i.e. To assess the specific data needs for the implementation of a risk assessment of amphibians in the Phongolo River floodplain with regard to MVC pesticides, was successfully met. Through data generation in Chapters 3 4 and 5, the second aim, i.e. To generate the necessary toxicological data for amphibians and MVC pesticides required as identified through the first aim, was successfully met.

7.3 Risk assessment

The data generated through the tiered approach (Chapters 2 to 5) were implemented in the ecological risk assessment models by informing interactions between pesticides and amphibians.

These data were utilised to derive risk ranks of zero, low, moderate, and high risk to amphibian well-being due to DDT and other MVC pesticide exposure. Rankings for current inputs were derived based on the field accumulation measurements from Chapter 3. In Chapter 6 we successfully used a relative risk model to assess the risks to amphibian well-being in the Phongolo River floodplain based on chemical input and water availability as main stressors to habitat quality. This was combined with an ecosystem service assessment of the floodplain in order to place the results in context with the greater socio-ecological importance of the floodplain system. Through the combination of these assessments we were able to identify four habitats within the floodplain as priority habitats for both amphibian well-being and ecosystem service delivery. The risks were fairly uniform to these habitats in terms of habitat prioritisation with minimal compromise between amphibian well-being and broader ecosystem services. Habitats were prioritised as rivers > temporary wetlands > permanent wetlands > woodland and forests based on the current ecological risk and achievable protection of amphibian well-being and ecosystem services through lowering the current risk state.

With successful implementation of the regional risk assessment in Chapter 6 and optimal habitat prioritisation for both ecosystem services and amphibian well-being protection, the third and fourth aims of this study as set in Chapter 1 were met. Thus the data obtained support the general hypothesis that MVC pesticide use in the Phongolo River floodplain contributes as the major risk factor to amphibian well-being in the floodplain. The data also support the hypothesis that there is a strong correspondence between the risks to amphibian well-being and ecosystem services in the floodplain. Based on the importance of maintaining habitat structure, water requirements for the floodplain are considered a more significant risk factor than pesticide exposure to the whole ecosystem and ecosystem services in particular. However, pesticide exposure remains the major factor influencing amphibian well-being. The close relationship that exists between ecosystem services and amphibian well-being in the habitats of concern, suggests that amphibians are good

indicators of ecosystem functioning in the Phongolo floodplain, especially with regard to aquatic habitats.

7.4 Practical implications of study outcome

Through the tiered process of data generation and implementation of the risk assessment a few practical implications of the study results were conceived. Firstly, by promoting research into standard test species we expect the probability of study recommendations resulting in data generation from future studies to be higher than if we proposed research on obscure species with narrow distribution ranges. An extensive database on one species can more easily be expanded upon to other species once the optimal methods, crucial aspects to investigate, and limitations involved are well known. This is important for amphibian ecotoxicology, especially in Africa where data scarcity continues to be a major issue in communicating risks to stakeholders and shifting from the assessment phase into the management phase of dealing with risks to amphibian well-being. In our opinion this study forms an excellent basis and provides useful guidance from which data can be generated in future studies.

In terms of risk assessment, an important finding of this study was that amphibians have the potential to serve as good indicators of not only ecosystem health, but to a large extent also of socio-ecological functioning. While their importance as environmental indicators is well known, supporting evidence from this risk assessment provides more substantive basis for the use of amphibian monitoring in management practises. This is currently only demonstrated for the Phongolo River floodplain study region, but could have widespread implications if similar results are found in other regions. From the overlapping habitat prioritisation between ecosystem services and amphibian well-being found in this study, floodplain ecosystems are a very good starting point for testing the extent of amphibian use as indicators. Because the risk to amphibian well-being is higher, but the same habitats are considered most at risk, the amphibian contribution to ecosystem services in the region is a sensitive endpoint. For instance, the low concentration

used for DDT experimental exposures in Chapters 4 and 5 of this study, is higher than the concentration that demonstrated disturbance in male frog calling patterns (Hoffman and Kloas 2016). The current field accumulation patterns (Wolmarans et al. 2021) are in line with the high DDT exposure concentration used in Chapter 5. It then stands to reason that it is very likely that male frog calling patterns can be altered by lower than current field-measured DDT levels in the Phongolo floodlpain. This has implications for not only frog breeding in itself, but also for the sense of place and aesthetic value contribution that frogs make to the floodlpain. Amphibian calling is commonly used in biodiversity assessments and offers long term monitoring benefits (Acevedo and Villanueva-Rivera 2010). Utilising long term frog call monitoring in the floodplain will essentially serve as a marker for initiation of changes to ecosystem services in the aquatic habitats of the floodlpain as the data suggest amphibians as service providing units to be affected earlier than broader ecosystem services as a whole.

An ecosystem aspect not assessed in this study is its adaptive plasticity. Adaptive plasitcity refers to the ability of environmental change an ecosystem can absorb, adapted to, or recover from if the change is acute in nature (Lundsgaard-Hansen et al. 2014). For example, the invertebrate communities of the Phongolo River floodlpain have shown resilience to supra-seasonal drought which resulted in hypersalinity in the Nyamithi Pan. Rapid recovery was observed post-drought, driven by invertebrates with aerial dispersal mechanisms and desiccation resistant life stages. The adaptation of indigenous species to deal with wet-dry cycles was also evidenced by the slow recovery of the alien invasive snail *Tarebia granifera* in comparison (De Necker et al. 2021). The risk to chemical exposure and water availability assessed in the current study does not sufficiently factor in the currently unknown pasticity of amphibian communities in the floodplain toward these threats. Some amphibian species such as bullfrogs (*Pixycephalus* sp.) make use of estivation to survive seasonal droughts underground (Du Preez and Carruthers 2017), and this ability would impact the resilience of those species in an extended drought compared to species that do not have these adaptations. The effects of chronic exposure on amphibian genetics in the region is

currently unknown. The development of genetic resistance is possible in non-target organisms just as it is a reality in target vectors. This is one of the theorised reasons why current pesticide levels in the Phongolo River floodplain are not actively resulting in observed ecological shifts and invertebrate community collapse as seen in the mixture exposure in a simulated field environment (Chapter 5). In this case, long term exposure may well have led to genetic adaptation in amphibian and invertebrate species in the floodplain and the current floodplain aquatic community structure may well represent a pesticide resistant community. This remains speculative reasoning as no evidence of such adaptations has been observed or assessed other than the lack of food web shift expected with mixed pesticide exposure.

In terms of management strategies, this study has successfully identified the priority habitats within the floodplain. Monitoring programs in these key habitats can limit the resources spent while optimising the value of data gained. Effective interventions for optimised conservation of amphibians and sustainability of ecosystem services will need to focus specific aspects of each habitat. For example, lowering the rate of deforestation is important for conserving ecosystem services such as the provision of natural medicines and biodiversity in the woodland and forest habitats. Deforestation is however a consequence of increased food demand in the region. Thus food provision in this habitat is in active competition with other provisioning services in the woodland and forest habitat in particular. This will require production of alternative fuel sources for the local community, education on the effects of deforestation, and sustainable use of resources. Indigenous tree planting initiatives can be of use in this regard. Close working relations with the community and governing stakeholders are key in making such programs successful. A balance between land use for agriculture and protection of natural habitats is also important in this region, but with a growing population this will be far more difficult to achieve. River flow and quality monitoring also needs to be upgraded in order for data to be used for drought and flood management in future. Such data will benefit both the natural ecosystem, conservation

management in NGR and subsequently amphibian well-being, but also agriculture and food production on the floodplain pans.

7.5 Future research recommendations

The continued generation of baseline toxicological data for amphibians with regard to vector control and agricultural pesticides is strongly recommended for future research based on the immense knowledge gaps identified in this study. Species sensitivity based not only on embryonic and tadpole data, but also including adult responses would be a useful dataset for refining toxicological criteria for amphibians. This is especially necessary for African species as these have received the least experimental attention of amphibians globally.

Uncertainty in metabolomic responses of amphibians to pesticides require further investigation with the addition of different analytical techniques to ensure the most relevant metabolomic data are collected. The incorporation of gene expression analyses and enzymatic responses would refine observed effects based on an adverse outcome pathway approach.

Field simulations for amphibian exposure to pesticides need to be improved and performed at different scales in future studies in order to assess how pond size variability changes the predator-prey interaction effects observed.

One aspect lacking in the practical use and implementation of amphibian health research, compared to that of other aquatic organisms, is the use of standardised field monitoring indices. The fish health assessment index (HAI) originally proposed by Adams et al. (1993) is an example of such an organism and necropsy based index that can be routinely used in fish evaluations. The resulting comparable datasets make it easy to track trends in fish health over time and can be correlated with chemical measurements to better form an understanding of threshold environmental levels that affect the health of individuals within a population. Amphibian health has not, to our knowledge, been sufficiently explored in this field. Results from the present study

indicated sub-lethal metabolomic shifts could be occurring in amphibians from the Phongolo River floodplain at current pesticide exposure concentrations. Research into the formulation of an easily applicable organism based health assessment index could very well be the assessment tool required to effectively split adaptive and adverse effects at organism level. This would require progress on the previous recommendations in order to have practical value.

Collaborative workshops with conservation management authorities, local and provincial government departments and the floodplain community is the next step required for incorporating the prioritised habitats into realistic achievable management strategies with specific protection goals. The relative risk assessment performed in this study identified water availability as a major stressor in the Phongolo River floodplain. A comprehensive detailed climate change prediction assessment of the study area will help inform management strategies in this regard. Future monitoring of amphibian communities in the floodplain is also necessary to track any biodiversity changes and chemical exposure changes in the future and can serve as an indicator tool for the efficacy of applied management strategies in specific sections of the floodplain. Local community involvement will be critical for this type of monitoring and it could be implemented on the basis of amphibian ecotourism providing benefits to both the local and scientific community, and continued integration of indigenous knowledge into amphibian conservation.

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APPENDIX A CHAPTER 3 SUPPLEMENTARY MATERIAL

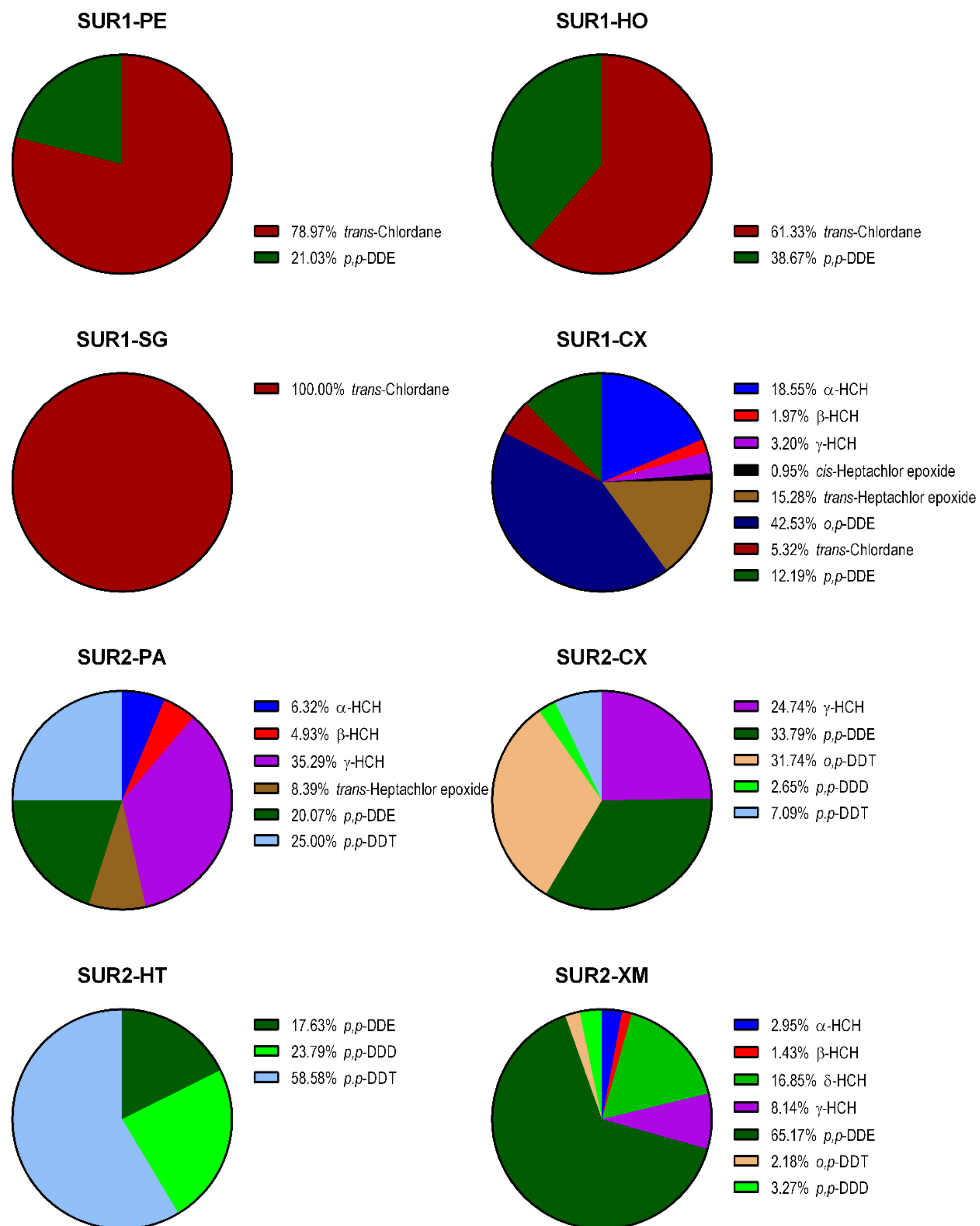


Figure A1 **Relative contribution of organochlorine pesticides (OCPs) in terms of total OCPs measured in frogs from Kruger National Park (KNP) during survey one (SUR1) and from Ndumo game reserve (NGR) during survey two (SUR2). PE = *P. edulis*; HO = *H. ornata*; SG = *S. garmani*; CX = *C. xerampelina*; PA = *P. anchietae*; XM = *X. muelleri*; HT = *H. tuberlinguis***

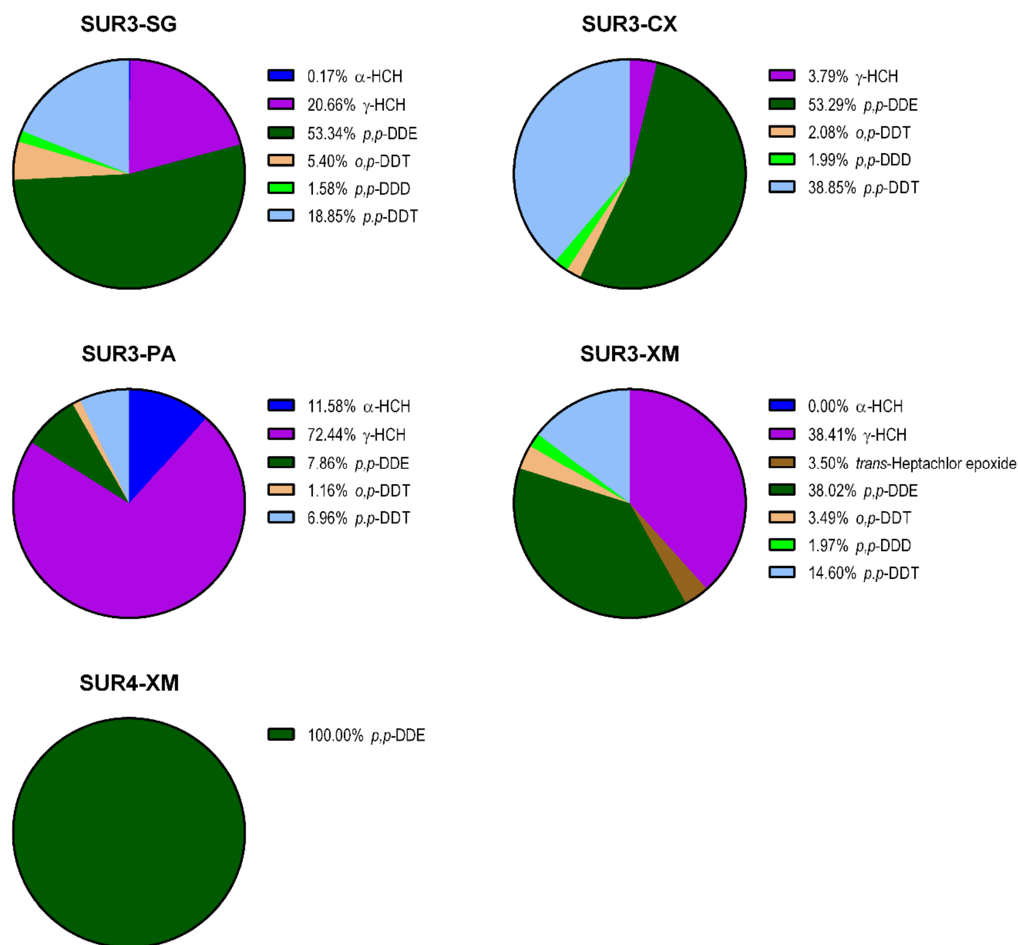


Figure A2 **Relative contribution of organochlorine pesticides (OCPs) in terms of total OCPs measured in frogs from Ndumo game reserve (NGR) during survey three (SUR3) and survey four (SUR4). SG = *S. garmani*; CX = *C. xerampelina*; PA = *P. anchietae*; XM = *X. muelleri***

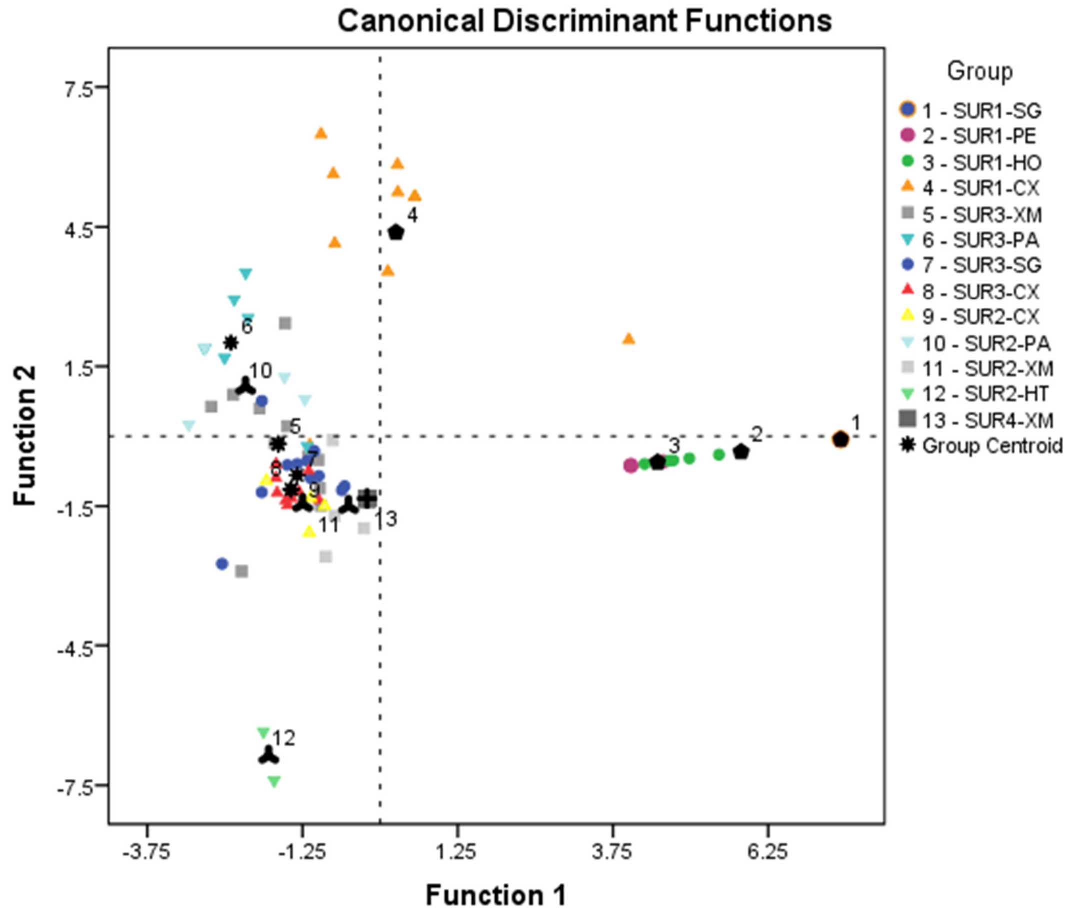


Figure A3 Biplot of the first two functions from a discriminant function analysis performed on the composition factor (CF) data for OCP accumulation in frogs from Kruger National Park (KNP and Ndumo Game Reserve (NGR). Sample groups were based on the combination of species and survey. Group centroid symbols relate to survey with: pentagon = survey 1; tripod = survey 2, flower = survey 3; plus = survey 4. Individual symbols indicate habit/habitat categories as selected for this study with: square = aquatic; downward triangle = semi-aquatic; circle = semi-terrestrial; upward triangle = tree dwelling. Colours relate to species as per legend. Legend description: SUR = survey; SG = *S. garmani*; PE = *P. edulis*; HO = *H. ornata*; CX = *C. xerampelina*; XM = *X. muelleri*; PA = *P. anchietae*; HT = *H. tuberlinguis*

Table A1 **Structure matrix for Discriminant function analysis showing OCP contribution factor (CF) correlation to the first five functions with significant contribution to explained variance (α <0.001 based on Wilks' Lambda). The cumulative explained variance across the first five functions was 96%. Largest absolute correlation between each variable and any discriminant function indicated with * (only where absolute correlation > 0.5)**

	Function				
	1	2	3	4	5
trans-chlordane	.882*	-.033	.252	-.353	.071
p,p-DDE	-.077	-.269	.017	.696*	-.193
p,p-DDD	-.122	-.417	-.500	-.532*	.333
γ-HCH	-.267	.154	.457	-.353	.597*
p,p-DDT	-.180	-.215	-.045	-.378	-.540*
δ-HCH	-.011	-.053	-.057	.195	.471
o,p-DDT	-.064	-.067	.039	.036	-.019
β-HCH	-.020	.081	-.052	-.008	-.006
α-HCH	-.030	.201	-.098	-.046	.104
trans-heptachlor epoxide	-.002	.142	-.144	.004	-.034
o,p-DDE	.010	.289	-.311	.023	-.092
cis-heptachlor epoxide	.004	.101	-.109	.008	-.032

Table A2 **Predicted group membership percentages for discriminant function analysis based on OCP composition. Overall 61.7% of original groups were correctly classified. Grey shading indicates correct group prediction. Group descriptions: SUR = survey; SG = *S. garmani*; PE = *P. edulis*; HO = *H. ornata*; CX = *C. xerampelina*; XM = *X. muelleri*; PA = *P. anchietae*; HT = *H. tuberlinguis***

	Predicted group membership												
	SUR1-SG	SUR1-PE	SUR1-HO	SUR1-CX	SUR3-XM	SUR3-PA	SUR3-SG	SUR3-CX	SUR2-CX	SUR2-PA	SUR2-XM	SUR2-HT	SUR4-XM
SUR1-SG	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SUR1-PE	50.0	0.0	50.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SUR1-HO	14.3	14.3	57.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	14.3
SUR1-CX	0.0	0.0	9.1	81.8	9.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SUR3-XM	0.0	0.0	0.0	0.0	27.3	18.2	27.3	0.0	18.2	9.1	0.0	0.0	0.0
SUR3-PA	0.0	0.0	0.0	0.0	0.0	77.8	11.1	0.0	0.0	11.1	0.0	0.0	0.0
SUR3-SG	0.0	0.0	0.0	0.0	0.0	9.1	36.4	27.3	9.1	0.0	0.0	0.0	18.2
SUR3-CX	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
SUR2-CX	0.0	0.0	0.0	0.0	0.0	0.0	25.0	0.0	50.0	0.0	25.0	0.0	0.0
SUR2-PA	0.0	0.0	0.0	0.0	25.0	25.0	0.0	25.0	0.0	25.0	0.0	0.0	0.0
SUR2-XM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	16.7	0.0	50.0	0.0	33.3
SUR2-HT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0
SUR4-XM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0

Table A3 **Compounds analysed for in pesticide screening using Primoris GC-MS/MS method GMSO_01_A. RL = reporting limit as per accreditation; (A) = BELAC (Belgian accreditation body) accredited**

Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL
1,4-dimethylnaphthalene (A) 0.01 mg/kg	2-phenylphenol (ortho-) (A) 0.02 mg/kg	acetochlor 0.01 mg/kg	aclonifen (A) 0.01 mg/kg	fenvalerate (A) 0.01 mg/kg	fenvalerate (sum of SS,RR,SR and RS) (A) 0.01 mg/kg	fipronil 0.01 mg/kg	fipronil (sum fipronil + sulfone metabolite (MB46136) expressed as fipronil) 0.01 mg/kg
1,4-dimethylnaphthalene (A) 0.01 mg/kg	2-phenylphenol (ortho-) (A) 0.02 mg/kg	acetochlor 0.01 mg/kg	aclonifen (A) 0.01 mg/kg	fenvalerate (A) 0.01 mg/kg	fenvalerate (sum of SS,RR,SR and RS) (A) 0.01 mg/kg	fipronil 0.01 mg/kg	fipronil (sum fipronil + sulfone metabolite (MB46136) expressed as fipronil) 0.01 mg/kg
acrinathrin (A) 0.01 mg/kg	alachlor (A) 0.01 mg/kg	aldrin (A) 0.01 mg/kg	aldrin and dieldrin (aldrin and dieldrin combined expressed as dieldrin) 0.02 mg/kg	fipronil-desulfinyl 0.01 mg/kg	fipronil-sulfone 0.01 mg/kg	flucythrinate (flucythrinate including other mixtures of constituent isomers (sum of isomers)) (A) 0.01 mg/kg	fludioxonil (A) 0.01 mg/kg
anthraquinone (A) 0.01 mg/kg	benalaxyl including other mixtures of constituent isomers including benalaxyl-M (sum of isomers) (A) 0.01 mg/kg	benfluralin (A) 0.01 mg/kg	benzoylprop-ethyl (A) 0.01 mg/kg	flumetralin (A) 0.01 mg/kg	formothion (A) 0.01 mg/kg	HCH (alpha-) (A) 0.01 mg/kg	HCH (beta-) 0.01 mg/kg

Table A3 (continued)

Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL
bifenazate 0.05 mg/kg	bifenox (A) 0.01 mg/kg	bifenthrin (A) 0.01 mg/kg	biphenyl (A) 0.05 mg/kg	HCH (delta-) (A) 0.01 mg/kg	HCH (epsilon-) 0.01 mg/kg	heptachlor (A) 0.01 mg/kg	heptachlor (sum of heptachlor and heptachlor epoxide expressed as heptachlor) (A) 0.01 mg/kg
bromofos (bromofos methyl) (A) 0.01 mg/kg	bromophos -ethyl (A) 0.01 mg/kg	bromopropylate (A) 0.01 mg/kg	butachlor (A) 0.01 mg/kg	heptachlor epoxyde (A) 0.01 mg/kg	heptenophos (A) 0.01 mg/kg	hexachlorbenzene (HCB) (A) 0.01 mg/kg	hexachlorocyclohexane (HCH), sum of isomers, except the gamma isomer 0.01 mg/kg
butafenacil (A) 0.01 mg/kg	butralin (A) 0.01 mg/kg	butylate (A) 0.01 mg/kg	cadusafos (A) 0.01 mg/kg	iodofenfos (A) 0.01 mg/kg	ipconazole (A) 0.02 mg/kg	isocarbophos (A) 0.01 mg/kg	isofenphos (-ethyl) (A) 0.01 mg/kg
carbophenothion (A) 0.01 mg/kg	chinomethionat (A) 0.02 mg/kg	chloorbufam (A) 0.01 mg/kg	chlorbenside (A) 0.01 mg/kg	isofenphos-methyl (A) 0.01 mg/kg	isoprocarb (A) 0.01 mg/kg	isopropalin (A) 0.01 mg/kg	lindane (Gamma-isomer of hexachlorocyclohexane (HCH)) (A) 0.01 mg/kg
chlordane (sum of cis and trans-chlordane) (A) 0.01 mg/kg	chlorfenapyr (A) 0.01 mg/kg	chlorfenson (A) 0.01 mg/kg	chlormephos (A) 0.01 mg/kg	malaoxon (A) 0.01 mg/kg	malathion (A) 0.01 mg/kg	malathion (sum of malathion and malaoxon expressed as malathion) (A) 0.01 mg/kg	mecarbam (A) 0.05 mg/kg
chlorobenzilate (A) 0.01 mg/kg	chloroneb (A) 0.01 mg/kg	chlorothalonil (A) 0.01 mg/kg	chlorpropham (A) 0.01 mg/kg	mepronil (A) 0.01 mg/kg	methacrifos (A) 0.01 mg/kg	methidathion (A) 0.01 mg/kg	methoprene (A) 0.02 mg/kg
chlorpyrifos (-ethyl) (A) 0.01 mg/kg	chlorpyrifos -methyl (A) 0.01 mg/kg	chlorthal-dimethyl (DCPA) (A) 0.01 mg/kg	chlozolinate (A) 0.01 mg/kg	methoxychlor (A) 0.01 mg/kg	metrafenone (A) 0.01 mg/kg	metribuzin 0.01 mg/kg	mevinphos (sum of E and Z-isomers) (A) 0.01 mg/kg
coumaphos (A) 0.01 mg/kg	crimidine (A) 0.01 mg/kg	cyanofenphos (A) 0.01 mg/kg	cycloate 0.01 mg/kg	mirex (A) 0.01 mg/kg	nitrofen (A) 0.01 mg/kg	nitrothal-isopropyl (A) 0.01 mg/kg	oxadiargyl (A) 0.01 mg/kg

Table A3 (continued)

Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL
cyflufenamid: sum of cyflufenamid (Z-isomer) and its E-isomer (A) 0.01 mg/kg	cyfluthrin (cyfluthrin including other mixtures of constituent isomers (sum of isomers)) (A) 0.01 mg/kg	cyhalofop-butyl (A) 0.01 mg/kg	cyhalothrin (sum of gamma and lambda) (A) 0.01 mg/kg	oxadiazon (A) 0.01 mg/kg	oxychlordane 0.01 mg/kg	oxyfluorfen (A) 0.01 mg/kg	paraoxon-methyl (A) 0.01 mg/kg
cypermethrin (cypermethrin including other mixtures of constituent isomers (sum of isomers)) (A) 0.01 mg/kg	DBCP (A) 0.01 mg/kg	DDD (o,p'-) (A) 0.01 mg/kg	DDD (p,p'-) = TDE (A) 0.01 mg/kg	parathion (-ethyl) (A) 0.01 mg/kg	parathion-methyl (A) 0.01 mg/kg	parathion-methyl (sum of parathion-methyl and paraoxon-methyl expressed as parathion methyl) (A) 0.01 mg/kg	pebulate (A) 0.01 mg/kg
DDE (o,p') (A) 0.01 mg/kg	DDE (p,p') (A) 0.01 mg/kg	DDT (o,p') (A) 0.01 mg/kg	DDT (p,p') (A) 0.01 mg/kg	pendimethalin (A) 0.01 mg/kg	pentachloroaniline (A) 0.01 mg/kg	pentachloroanisol (A) 0.01 mg/kg	penthiopyrad (A) 0.01 mg/kg
DDT (sum of p,p´-DDT, o,p´-DDT, p-p´-DDE and p,p´-TDE (DDD) expressed as DDT) (F) (A) 0.01 mg/kg	DEET (N.N-diethyl-M toluamide) 0.01 mg/kg	deltamethrin (cis deltamethrin) (A) 0.01 mg/kg	desmetryn (A) 0.01 mg/kg	permethrin (sum of isomers) (A) 0.02 mg/kg	phenothrin (phenothrin including other mixtures of constituent isomers (sum of isomers)) (A) 0.05 mg/kg	phorate (A) 0.01 mg/kg	phosalone (A) 0.01 mg/kg
diazinon (A) 0.01 mg/kg	dichlobenil (A) 0.01 mg/kg	dichlofenthion (A) 0.01 mg/kg	dichlofluanid (A) 0.01 mg/kg	phosmet 0.01 mg/kg	phosmet (phosmet and phosmet oxon expressed as phosmet) 0.01 mg/kg	phosmet-oxon 0.05 mg/kg	piperonyl butoxide (A) 0.01 mg/kg
dichlormid (A) 0.01 mg/kg	dichlorvos (A) 0.01 mg/kg	diclofop-methyl (A) 0.01 mg/kg	diclofop-methyl - TOTAL (A) 0.01 mg/kg	pirimiphos-ethyl (A) 0.01 mg/kg	pirimiphos-methyl (A) 0.01 mg/kg	pretilachlor (A) 0.01 mg/kg	procymidone (A) 0.01 mg/kg

Table A3 (continued)

Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL
dicloran (A) 0.01 mg/kg	dicofol (o,p') (A) 0.02 mg/kg	dicofol (p,p') (A) 0.02 mg/kg	dicofol (sum of p, p´ and o,p´ isomers) (A) 0.02 mg/kg	profluralin (A) 0.01 mg/kg	prometryn (A) 0.01 mg/kg	propargite (A) 0.02 mg/kg	prothiofos (A) 0.01 mg/kg
dieldrin 0.02 mg/kg	dimethachlor (A) 0.01 mg/kg	diphenamid (A) 0.01 mg/kg	diphenylamine (A) 0.02 mg/kg	pyrazophos (A) 0.01 mg/kg	pyridaben (A) 0.01 mg/kg	pyriproxyfen (A) 0.01 mg/kg	quinalphos (A) 0.01 mg/kg
ditalimfos (A) 0.01 mg/kg	DMSA (A) 0.01 mg/kg	DMST (A) 0.05 mg/kg	endosulfan (alpha-) 0.01 mg/kg	quintozene (A) 0.01 mg/kg	quintozene (sum of quintozene and pentachloro-aniline expressed as quintozene) (A) 0.01 mg/kg	silafluofen (A) 0.01 mg/kg	silthiofam (A) 0.01 mg/kg
endosulfan (beta-) 0.01 mg/kg	endosulfan (sulphate-) 0.01 mg/kg	endosulfan (sum of alpha- and beta- isomers and endosulfan-sulphate expresses as endosulfan) 0.01 mg/kg	endrin (A) 0.02 mg/kg	spirodiclofen (A) 0.01 mg/kg	spiromesifen (A) 0.01 mg/kg	sulfotep (A) 0.01 mg/kg	sulprofos (A) 0.01 mg/kg
EPN (A) 0.01 mg/kg	EPTC (ethyl dipropylthiocarbamate) (A) 0.01 mg/kg	esfenvalerate (A) 0.01 mg/kg	ethalfluralin (A) 0.01 mg/kg	tau-fluvalinate (A) 0.01 mg/kg	tecnazene (A) 0.01 mg/kg	tefluthrin (A) 0.01 mg/kg	terbacil (A) 0.01 mg/kg

Table A3 (continued)

Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL
ethopropho s (A) 0.01 mg/kg	etofenprox (A) 0.01 mg/kg	etridiazole 0.01 mg/kg	etrimfos (A) 0.01 mg/kg	tiocarbazil 0.05 mg/kg	tolclofos-methyl (A) 0.01 mg/kg	tolfenpyrad (A) 0.01 mg/kg	tolylfluanid (sum of tolylfluanid and dimethylam inosulfotolui di de expressed as tolylfluanid) (R) (A) 0.01 mg/kg
famoxadon e (A) 0.01 mg/kg	fenchlorpho s (A) 0.01 mg/kg	Fenchlorph os (sum of fenchlorpho s and fenchlorpho s oxon expressed as fenchlorpho s) (A) 0.01 mg/kg	fenchlorpho s-oxon (A) 0.01 mg/kg	tolylfluanide (A) 0.01 mg/kg	transfluthrin (A) 0.01 mg/kg	tri-allate (A) 0.01 mg/kg	trifluralin (A) 0.01 mg/kg
fenitrothion (A) 0.01 mg/kg	fenpropathr in (A) 0.01 mg/kg	fenpropimo rph (A) 0.01 mg/kg	fenson (A) 0.01 mg/kg	vinclozolin (A) 0.01 mg/kg	vinclozolin - TOTAL (A) 0.01 mg/kg		

Table A4 **Compounds analysed for in pesticide screening using Primoris LC-MS/MS method LMSO_01_A. RL = reporting limit as per accreditation; (A) = BELAC (Belgian accreditation body) accredited**

Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL
1-Naphthylacetamide (A) 0.01 mg/kg	6-benzyladenine (A) 0.01 mg/kg	acephate (A) 0.01 mg/kg	acetamiprid (A) 0.01 mg/kg	fenarimol (A) 0.01 mg/kg	fenazaquin (A) 0.01 mg/kg	fenbuconazole (A) 0.01 mg/kg	fenhexamid (A) 0.01 mg/kg
acibenzolar- S- methyl (sum of acibenzolar- S methyl and acibenzolar acid (free and conjugated), expressed as acibenzolar- S methyl) 0.01 mg/kg	acibenzolar-acid 0.05 mg/kg	acibenzolar-S-methyl 0.01 mg/kg	aldicarb (A) 0.01 mg/kg	fenobucarb (A) 0.01 mg/kg	fenoxaprop-P (A) 0.01 mg/kg	fenoxaprop-P-ethyl (A) 0.01 mg/kg	fenoxycarb (A) 0.01 mg/kg
aldicarb (sum of aldicarb, its sulfoxide and its sulfone, expressed as aldicarb) (A) 0.01 mg/kg	aldicarb-sulfone (A) 0.01 mg/kg	aldicarb-sulfoxide (A) 0.01 mg/kg	allethrin (A) 0.02 mg/kg	fenpiclonil (A) 0.01 mg/kg	fenpropidin (sum of fenpropidin and its salts, expressed as fenpropidin) (A) 0.01 mg/kg	fenpyrazamine (A) 0.01 mg/kg	fenpyroximate (A) 0.01 mg/kg
ametoctradin (A) 0.01 mg/kg	ametryn (A) 0.01 mg/kg	amidosulfuron (A) 0.01 mg/kg	amisulbrom 0.01 mg/kg	fensulfothion (A) 0.01 mg/kg	fensulfothion-oxon (A) 0.01 mg/kg	fensulfothion-oxon sulfone (A) 0.01 mg/kg	fensulfothion-sulfone (A) 0.01 mg/kg
atrazine (A) 0.01 mg/kg	azadirachtin 0.01 mg/kg	azamethiphos (A) 0.01 mg/kg	azimsulfuron (A) 0.01 mg/kg	fenthion (A) 0.02 mg/kg	Fenthion (fenthion and its oxigen analogue, their sulfoxides and sulfone expressed as parent) (A) 0.02 mg/kg	fenthion-oxon (A) 0.02 mg/kg	fenthion-oxon-sulfone (A) 0.02 mg/kg
azinphos-ethyl 0.01 mg/kg	azinphos-methyl 0.01 mg/kg	azoxystrobin (A) 0.01 mg/kg	beflubutamid (A) 0.01 mg/kg	fenthion-oxon-sulfoxid (A) 0.02 mg/kg	fenthion-sulfone (A) 0.02 mg/kg	fenthion-sulfoxide (A) 0.02 mg/kg	fenuron (A) 0.01 mg/kg

Table A4 (continued)

Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL
bendiocarb (A) 0.01 mg/kg	bensulfuron-methyl (A) 0.02 mg/kg	benthiavalicarb-isopropyl (A) 0.01 mg/kg	bispyribac-sodium (A) 0.01 mg/kg	flazasulfuron (A) 0.01 mg/kg	flonicamid (A) 0.01 mg/kg	florasulam (A) 0.01 mg/kg	fluazifop-P (A) 0.01 mg/kg
bitertanol (A) 0.01 mg/kg	bixafen (A) 0.01 mg/kg	boscalid (A) 0.01 mg/kg	bromacil (A) 0.01 mg/kg	fluazifop-P-butyl (A) 0.01 mg/kg	fluazifop-P-butyl (fluazifop acid (free)) (A) 0.01 mg/kg	fluazinam (A) 0.01 mg/kg	flubendiamide (A) 0.01 mg/kg
bromuconazole (sum of diasteroisomers) (A) 0.01 mg/kg	bupirimate (A) 0.01 mg/kg	buprofezin (A) 0.01 mg/kg	carbaryl (A) 0.01 mg/kg	flufenacet (A) 0.01 mg/kg	flufenoxuron (A) 0.01 mg/kg	fluometuron 0.02 mg/kg	fluopicolide (A) 0.01 mg/kg
carbendazim and benomyl (sum of benomyl and carbendazim expressed as carbendazim) (A) 0.01 mg/kg	carbetamide (A) 0.01 mg/kg	carbofuran (A) 0.01 mg/kg	carbofuran (3-OH-) (A) 0.01 mg/kg	fluopyram (A) 0.01 mg/kg	fluoxastrobin (A) 0.01 mg/kg	flupyradifurone (A) 0.01 mg/kg	flupyrsulfuron-methyl sodium (A) 0.01 mg/kg
carbofuran (sum of carbofuran (including any carbofuran generated from carbosulfan, benfuracarb or furathiocarb) and 3-OH carbofuran expressed as carb (A) 0.01 mg/kg	carbosulfan 0.05 mg/kg	carboxin (A) 0.01 mg/kg	carfentrazone-ethyl (determined as carfentrazone and expressed as carfentrazone-ethyl) (A) 0.01 mg/kg	fluquinconazole (A) 0.01 mg/kg	flurochloridone (A) 0.01 mg/kg	fluroxypyr 0.02 mg/kg	flurtamone (A) 0.01 mg/kg
chlorantraniliprole (DPX E-2Y45) (A) 0.01 mg/kg	chlorbromuron 0.01 mg/kg	chlorfenvinphos (A) 0.01 mg/kg	chlorfluazuron (A) 0.01 mg/kg	flusilazole (A) 0.01 mg/kg	flutolanil (A) 0.01 mg/kg	flutriafol (A) 0.01 mg/kg	fluxapyroxad (A) 0.01 mg/kg
chloridazon (A) 0.01 mg/kg	chlorotoluron (A) 0.01 mg/kg	chloroxuron (A) 0.01 mg/kg	chlorsulfuron (A) 0.01 mg/kg	FM-6 (A) 0.01 mg/kg	fonofos (A) 0.02 mg/kg	foramsulfuron (A) 0.01 mg/kg	forchlorfenuron (A) 0.01 mg/kg

Table A4 (continued)

Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL
cinerin I 0.01 mg/kg	cinerin II 0.01 mg/kg	clethodim 0.02 mg/kg	clethodim (sum of sethoxydim and clethodim including degradation products calculated as sethoxydim) 0.01 mg/kg	fosthiazate (A) 0.01 mg/kg	fuberidazole (A) 0.01 mg/kg	furalaxyl (A) 0.01 mg/kg	furathiocarb (A) 0.01 mg/kg
clodinafop (A) 0.01 mg/kg	clodinafop and its S isomers and their salts, expressed as clodinafop (A) 0.01 mg/kg	clodinafop-propargyl (A) 0.01 mg/kg	clofentezine (A) 0.01 mg/kg	haloxyfop including haloxyfop-R (Haloxyfop R methyl ester and haloxyfop-R expressed as haloxyfop-R) (A) 0.01 mg/kg	haloxyfop-methyl (A) 0.01 mg/kg	haloxyfop-R (A) 0.01 mg/kg	hexaconazole (A) 0.01 mg/kg
clomazone (A) 0.01 mg/kg	clothianidin (A) 0.01 mg/kg	cyantraniliprole (A) 0.01 mg/kg	cyazofamid (A) 0.01 mg/kg	hexazinone (A) 0.01 mg/kg	hexythiazox (A) 0.01 mg/kg	imazalil (A) 0.01 mg/kg	imazamox (A) 0.01 mg/kg
cycloxydim 0.02 mg/kg	cyflumetofen (A) 0.01 mg/kg	cymiazole (A) 0.01 mg/kg	cymoxanil (A) 0.01 mg/kg	imazapyr (A) 0.01 mg/kg	imazaquin (A) 0.01 mg/kg	imazosulfuron (A) 0.01 mg/kg	imidacloprid (A) 0.01 mg/kg
cyproconazole (A) 0.01 mg/kg	cyprodinil (A) 0.01 mg/kg	demeton-s-methyl (A) 0.01 mg/kg	demeton-S-methyl-sulfon (A) 0.01 mg/kg	indoxacarb (sum of indoxacarb and its R enantiomer) (A) 0.01 mg/kg	iodosulfuron-methyl (iodosulfuron-methyl including salts, expressed as iodosulfuron-methyl) (A) 0.01 mg/kg	iprobenfos (A) 0.01 mg/kg	iprodione (A) 0.01 mg/kg
desmethylpirimicarb (A) 0.01 mg/kg	dicrotophos (A) 0.01 mg/kg	diethofencarb (A) 0.01 mg/kg	difenoconazole (A) 0.01 mg/kg	iprovalicarb (A) 0.01 mg/kg	isoprothiolane (A) 0.01 mg/kg	isoproturon (A) 0.01 mg/kg	isopyrazam (A) 0.01 mg/kg

Table A4 (continued)

Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL
diflubenzuron (A) 0.01 mg/kg	diflufenican (A) 0.01 mg/kg	dimefox (A) 0.01 mg/kg	dimethenamid including other mixtures of constituent isomers including dimethenamid P (sum of isomers) (A) 0.01 mg/kg	isoxaben (A) 0.01 mg/kg	kresoxim-methyl (A) 0.01 mg/kg	lenacil (A) 0.01 mg/kg	linuron (A) 0.01 mg/kg
dimethoate (A) 0.01 mg/kg	dimethoate (sum of dimethoate and omethoate expressed as dimethoate) (A) 0.01 mg/kg	dimethomorph (sum of isomers) (A) 0.01 mg/kg	dimoxystrobin (A) 0.01 mg/kg	lufenuron (A) 0.01 mg/kg	mandipropamid (A) 0.01 mg/kg	mepanipyrim (A) 0.01 mg/kg	mesosulfuron-methyl (A) 0.01 mg/kg
diniconazole (A) 0.01 mg/kg	dinotefuran (A) 0.01 mg/kg	disulfoton 0.01 mg/kg	disulfoton (sum of disulfoton, disulfoton sulfoxide and disulfoton sulfone expressed as disulfoton) 0.01 mg/kg	metaflumizone (sum of E and Z-isomers) (A) 0.01 mg/kg	metalaxyl and metalaxyl M (metalaxyl including other mixtures of constituent isomers including metalaxyl-M (sum of isomers)) (A) 0.01 mg/kg	metamitron (A) 0.01 mg/kg	metazachlor (A) 0.01 mg/kg
disulfoton-sulfone 0.01 mg/kg	disulfoton-sulfoxide 0.01 mg/kg	diuron 0.02 mg/kg	dodemorph 0.01 mg/kg	metconazole (sum of isomers) (A) 0.01 mg/kg	methabenzthiazuron (A) 0.01 mg/kg	methamidophos (A) 0.02 mg/kg	methiocarb (A) 0.01 mg/kg

Table A4 (continued)

Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL
dodine (A) 0.02 mg/kg	epoxiconazole (A) 0.01 mg/kg	ethametsulfuron-methyl (A) 0.01 mg/kg	ethiofencarb (A) 0.01 mg/kg	methiocarb (sum of methiocarb and methiocarb sulfoxide and sulfone, expressed as methiocarb) (A) 0.01 mg/kg	methiocarb-sulfone (A) 0.01 mg/kg	methiocarb-sulfoxide (A) 0.01 mg/kg	metholachlor and metholachlor-S (metholachlor including other mixtures of constituent isomers including S-metholachlor (sum of isomers)) (A) 0.01 mg/kg
ethirimol (A) 0.01 mg/kg	ethoxysulfuron (A) 0.01 mg/kg	etoxazole (A) 0.01 mg/kg	fenamidone (A) 0.01 mg/kg	methomyl (A) 0.01 mg/kg	methomyl and thiodicarb (sum of methomyl and thiodicarb expressed as methomyl) (A) 0.01 mg/kg	methoxyfenozide (A) 0.01 mg/kg	metobromuron (A) 0.01 mg/kg
fenamiphos (A) 0.01 mg/kg	fenamiphos (sum of fenamiphos and its sulphoxide and sulphone expressed as fenamiphos) (A) 0.01 mg/kg	fenamiphos-sulfone (A) 0.01 mg/kg	fenamiphos-sulfoxide (A) 0.01 mg/kg	metosulam (A) 0.01 mg/kg	metoxuron (A) 0.01 mg/kg	metsulfuron-methyl (A) 0.01 mg/kg	molinate (A) 0.01 mg/kg
monocrotophos (A) 0.01 mg/kg	monolinuron (A) 0.01 mg/kg	myclobutanil (A) 0.01 mg/kg	napropamide (A) 0.01 mg/kg	nicosulfuron (A) 0.01 mg/kg	nitenpyram (A) 0.01 mg/kg	novaluron 0.01 mg/kg	nuarimol (A) 0.01 mg/kg

Table A4 (continued)

Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL
ofurace (A) 0.01 mg/kg	omethoate (A) 0.01 mg/kg	oxadixyl (A) 0.01 mg/kg	oxamyl (A) 0.01 mg/kg	oxycarboxin (A) 0.01 mg/kg	oxydemeton-methyl (A) 0.01 mg/kg	oxydemeton-methyl (sum of oxydemeton-methyl and demeton-S methylsulfone expressed as oxydemeton-methyl) (A) 0.01 mg/kg	paclobutrazol (A) 0.01 mg/kg
penconazole (A) 0.01 mg/kg	pencycuron (A) 0.01 mg/kg	penoxsulam (A) 0.01 mg/kg	pethoxamid (A) 0.01 mg/kg	phenmedipham (A) 0.01 mg/kg	phenthoate (A) 0.01 mg/kg	phosphamidon (A) 0.01 mg/kg	phoxim (A) 0.01 mg/kg
picolinafen 0.01 mg/kg	picoxystrobin (A) 0.01 mg/kg	pinoxaden (A) 0.01 mg/kg	pirimicarb (A) 0.01 mg/kg	prochloraz (A) 0.01 mg/kg	profenofos (A) 0.01 mg/kg	promecarb (A) 0.01 mg/kg	propamocarb (sum of propamocarb and its salt expressed as propamocarb) (A) 0.01 mg/kg
propanil (A) 0.01 mg/kg	propaquizafop (A) 0.01 mg/kg	propazin (A) 0.01 mg/kg	propham (IPC) (A) 0.01 mg/kg	propiconazole (sum of isomers) (A) 0.01 mg/kg	propoxur (A) 0.01 mg/kg	propyzamide (A) 0.01 mg/kg	proquinazid (A) 0.01 mg/kg
prosulfocarb (A) 0.01 mg/kg	prosulfuron (A) 0.01 mg/kg	prothioconazool: prothioconazool-dethio (som van isomeren) (A) 0.01 mg/kg	pymetrozine (A) 0.01 mg/kg	pyraclostrobin (A) 0.01 mg/kg	pyraflufen-ethyl (A) 0.01 mg/kg	pyrethrin I 0.01 mg/kg	pyrethrin II 0.01 mg/kg

Table A4 (continued)

Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL
pyrethrins 0.01 mg/kg	pyridafol 0.05 mg/kg	pyridalyl (A) 0.01 mg/kg	pyridaphenthion (A) 0.01 mg/kg	pyridate 0.01 mg/kg	pyridate (sum of pyridate. its hydrolysis product CL 9673 (6-chloro-4-hydroxy-3-phenylpyridazin) and hydrolysable conjugates of CL 9673 expressed as p 0.01 mg/kg	pyrifenox 0.01 mg/kg	pyrimethanil (A) 0.01 mg/kg
pyriofenone (A) 0.01 mg/kg	quinclorac (A) 0.01 mg/kg	quinmerac (A) 0.01 mg/kg	quinoxyfen (A) 0.01 mg/kg	quizalofop, incl. quizalofop-P (A) 0.01 mg/kg	quizalofop-ethyl (A) 0.01 mg/kg	rimsulfuron 0.01 mg/kg	rotenone (A) 0.01 mg/kg
sethoxydim 0.01 mg/kg	simazine (A) 0.01 mg/kg	spinetoram (A) 0.01 mg/kg	spinetoram I (A) 0.01 mg/kg	spinetoram II (A) 0.01 mg/kg	spinosad: sum of spinosyn A and spinosyn D, expressed as spinosad (A) 0.01 mg/kg	spinosyn A (A) 0.01 mg/kg	spinosyn D (A) 0.01 mg/kg
spirotetramat (A) 0.01 mg/kg	spirotetramat and its 4 metabolites BYI08330-enol, BYI08330-ketohydroxy, BYI08330-monohydroxy, and BYI08330 enol-glucoside, expressed as spirotetramat (A) 0.01 mg/kg	spirotetramat-enol (A) 0.01 mg/kg	spirotetramat-enol glucoside (A) 0.01 mg/kg	spirotetramat-keto-hydrox (A) 0.01 mg/kg	spirotetramat-mono hydrox (A) 0.01 mg/kg	spiroxamine (sum of isomers) (A) 0.01 mg/kg	sulfosulfuron (A) 0.01 mg/kg
sulfoxaflor (A) 0.01 mg/kg	tebuconazole (A) 0.01 mg/kg	tebufenozide (A) 0.01 mg/kg	tebufenpyrad (A) 0.01 mg/kg	tepraloxydim (A) 0.01 mg/kg	terbufos (A) 0.01 mg/kg	terbufos-sulfon (A) 0.01 mg/kg	terbufos-sulfoxide (A) 0.01 mg/kg

Table A4 (continued)

Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL	Compound (A) RL
tetraconazole (A) 0.01 mg/kg	tetramethrin (A) 0.01 mg/kg	thiabendazole (A) 0.01 mg/kg	thiacloprid (A) 0.01 mg/kg	thiametoxam (A) 0.01 mg/kg	thifensulfuron-methyl (A) 0.01 mg/kg	thiobencarb (A) 0.01 mg/kg	thiodicarb (A) 0.01 mg/kg
thionazin (A) 0.01 mg/kg	thiophanate-methyl (A) 0.01 mg/kg	triadimefon (A) 0.01 mg/kg	triadimefon and triadimenol (sum of triadimefon and triadimenol) (A) 0.01 mg/kg	triadimenol (A) 0.01 mg/kg	triasulfuron (A) 0.01 mg/kg	triazophos (A) 0.01 mg/kg	trichlorfon (A) 0.01 mg/kg
tricyclazole (A) 0.01 mg/kg	tridemorph 0.01 mg/kg	trifloxystrobin (A) 0.01 mg/kg	triflumizole (A) 0.01 mg/kg	triflumizole: Triflumizole and metabolite FM-6-1 (N-(4-chloro-2-trifluoromethylphenyl)-n propoxyacetamidine), expressed as Triflumizole (A) 0.01 mg/kg	triflumuron (A) 0.01 mg/kg	triforine (A) 0.01 mg/kg	trinexapac (sum of trinexapac (acid) and its salts, expressed as trinexapac) (A) 0.05 mg/kg
triticonazole (A) 0.01 mg/kg	valifenalate (A) 0.01 mg/kg	vamidothion (A) 0.01 mg/kg	zoxamide (A) 0.01 mg/kg				

Table A5 The lipid mass converted organochlorine pesticide (OCP) concentrations measured in different anuran species, given as mean (and range) of detected measurements (ng/g lipid weight) per chemical group. Concentrations were measured in frog carcasses from Ndumo Game Reserve (NGR) and Kruger National Park (KNP) across three surveys (SUR1-3). Survey 4 is excluded as the analytical results from Primoris did not include lipid concentrations.

	α-HCH	**β-HCH**	**δ-HCH**	**γ-HCH**	***cis*-Hep-epox**	***trans*-Hep-epox**	**o,p-DDE**	***trans*-chlordane**	***p,p*-DDE**	***o,p*-DDT**	***p,p*-DDD**	***p,p*-DDT**
SUR1-SG	ND	ND	ND	ND	ND	ND	ND	49.4 (ND-248)	ND	ND	ND	ND
SUR1-PE	ND	ND	ND	ND	ND	ND	ND	63.3 (ND-304)	19.6 (ND-176)	ND	ND	ND
SUR1 HO	ND	ND	ND	ND	ND	ND	ND	287 (ND-1112)	109 (ND-344)	ND	ND	ND
SUR1-CX	29.4 (ND-431)	4.8 (ND-81.9)	ND	6.2 (ND-123)	1.6 (ND-41.3)	19.8 (ND-397)	32.6 (ND-355)	18.6 (ND-536)	19.3 (ND-227)	ND	ND	ND
SUR2-CX	ND	ND	ND	34.4 (ND-53.8)	ND	ND	ND	ND	183 (ND-518)	148 (ND-227)	15.8 (ND-50.2)	39.8 (ND-122)
SUR2-PA	11 (ND-29.1)	8.7 (ND-22.1)	ND	34.4 (ND-53.8)	ND	13 (ND-38.6)	ND	ND	30 (ND-89.8)	ND	ND	ND
SUR2-HT	ND	ND	ND	ND	ND	ND	ND	ND	13.7 (11.4-16)	ND	18.4 (15.7-21)	46.4 (34.3-58.6)
SUR2-XM	13.8 (ND-72.6)	3.57 (ND-9.9)	18.3 (ND-76.4)	29.6 (ND-85.6)	ND	ND	ND	ND	128 (22.8-238)	8.24 (ND-48.6)	9.78 (ND-28.2)	ND
SUR3-XM	ND	ND	ND	329 (16.1-2534)	ND	25.7 (ND-103)	ND	ND	282 (ND-1358)	119 (ND-1101)	17.7 ND-80.4)	44.1 (ND-152)
SUR3-PA	5 (ND-11.9)	ND	ND	44.5 (8.7-180)	ND	ND	ND	ND	3.6 (ND-2.1)	0.7 (ND-2.2)	ND	4.4 (ND-32.8)

Table A5 (continued)

	α-HCH	*β*-HCH	*δ*-HCH	*γ*-HCH	*cis*-Hep-epox	*trans*-Hep-epox	o,p-DDE	*trans*-chlordane	*p,p*-DDE	*o,p*-DDT	*p,p*-DDD	*p,p*-DDT
SUR3-SG	ND	ND	ND	13.1 (ND-33.8)	ND	ND	ND	ND	41.1 (0.82-159)	3.4 (ND-17.3)	1.5 (ND-12.89)	22.9 (ND-123)
SUR3-CX	ND	ND	ND	6.9 (1.4-16.2)	ND	ND	ND	ND	134 (51-318)	5 (ND-14.3)	5.4 (ND-20.1)	92.9 (48-271)

APPENDIX B CHAPTER 4 SUPPLEMENTARY MATERIAL

Table B1 Body mass (BM; g), Hepatosomatic index (HSI), lipid content (as percentage of BM) and detection rate of pesticides in frog tissue for each exposure treatment (percentage of samples with detectable values >LOQ)

Treatment	n	BM	HSI	Lipid content	*o,p*-DDE	*p,p*-DDE	*o,p*-DDD	*p,p*-DDD	*o,p*-DDT	*p,p*-DDT	DTM
Control	8	3.76 ± 0.46	0.022 ± 0.005	3.49 ± 1.56	0	0	0	0	0	0	0
DDT 0.1%	8	4.17 ± 1.02	0.018 ± 0.003	4.59 ± 1.9	12.5	12.5	87.5	100	50	50	0
DDT 1%	8	3.77 ± 1.17	0.025 ± 0.006	5.97 ± 3.37	0	100	100	87.5	0	87.5	0
DTM 0.1%	8	3.68 ± 1.35	0.024 ± 0.003	4.13 ± 2.45	0	0	0	0	0	0	0
DTM 1%	8	5.96 ± 1.82	0.029 ± 0.006	5.15 ± 2.83	0	0	0	0	0	0	0
Mix 0.1%	8	4.22 ± 1.24	0.025 ± 0.005	4.63 ± 1.16	0	12.5	12.5	75	87.5	100	0
Mix 1%	8	4.26 ± 0.81	0.029 ± 0.006	4.7 ± 2.24	0	100	100	100	100	100	0

Table B2 **Metabolome data filtered to only show spectral hits with statistical significance (indicated with an asterisk*) in comparison to control for each exposure group with up- (↑) and down-regulation (↓) indicated based on fold change > 2, and/or effect size (Cohen's-d value) > 0.8. All hits only present (p) or absent (a) in the exposure treatment compared to control are also indicated. RT = retention time**

Spectral hits		DDT 0.1%	DDT 1%	DTM 0.1%	DTM 1%	Mix 0.1%	Mix 1%
Statistically significant changes	Up-regulated	14	8	6	7	1	3
	Down-regulated	4	5	11	3	2	7
	Total	18	13	17	10	3	10
Adenine				↑*	↑	↑	↑
Oleic acid		↑*	↑		↑	↑*	
Arabinopyranose		↑*					
Analyte 104(RT:835.97)			↓	↓*	↓	↓*	↓*
Analyte 112(RT:855.54)				↑p			
Analyte 123(RT:827.46)		↓	↓a	↓a		↓a	↓a
Analyte 149(RT:658.61)						↑p	
Analyte 213(RT:1098.94)			↓	↓		↓	↓*
Analyte 28(RT:615.61)		↑	↑*	↑*	↑*	↑	↑*
Analyte 333(RT:1245.48)		↓*				↓	
Analyte 349(RT:1322.6)		↑*	↑		↑		
Analyte 353(RT:1712.99)				↓*		↓	↓
Analyte 387(RT:2051.93)		↓	↓*	↓*		↓	↓
Analyte 393(RT:1523.4)			↓a	↓		↓a	↓a
Analyte 394(RT:1500.65)			↑*	↑	↑*		↑
Analyte 417(RT:1349.48)				↓a		↓	↓
Analyte 430(RT:1549.71)		↓	↓a	↓		↓	↓
Analyte 433(RT:1080.14)		↓	↓a	↓	↓	↓	↓a
Analyte 446(RT:1540.58)		↓*	↑				
Analyte 458(RT:1376.42)			↓	↓*		↓	↓a
Analyte 518(RT:1850.73)		↑*	↑	↑	↑	↑	↑
Analyte 524(RT:2033.04)		↑*			↑		
Analyte 530(RT:1515.53)			↑p	↑p	↑p*		
Analyte 541(RT:1828.53)			↓*	↓*	↓		↓
Analyte 571(RT:1395.71)			↓a*	↓a*	↓	↓a	↓
Analyte 572(RT:1955.04)			↑p		↑p		
Analyte 587(RT:1657.65)			↑p		↑p		
Analyte 631(RT:1546.84)		↓					↓a
Analyte 636(RT:1904.8)			↑	↑	↑*	↑	↑
Analyte 638(RT:1647.02)		↓		↓	↓	↓	↓*
Analyte 664(RT:2106.15)				↓*		↓a*	↓*
Analyte 665(RT:1940.46)		↓*			↓		

Table B2 (continued)

Spectral hits	DDT 0.1%	DDT 1%	DTM 0.1%	DTM 1%	Mix 0.1%	Mix 1%
Analyte 668(RT:2053.71)	↓a	↓a	↓a	↓	↓a	↓**a***
Analyte 673(RT:1752.06)	↑*			↑		
Analyte 676(RT:1697.68)			↑		↓a	↑
Analyte 681(RT:2068.24)	↑*					
Analyte 7(RT:376.31)			↑*			
Analyte 706(RT:1804.92)	↑*	↑		↑	↑	
Analyte 712(RT:1931.19)	↑**p***			↑p		
Analyte 729(RT:2197.61)	↑*					
Analyte 92(RT:648.11)			↓*		↑	↓
2-Hydroxybutyric acid		↓	↓*	↓		↓
Cadaverine		↓	↓a		↓	↓a
Gluconolactone	↑*			↑		
L-Sorbitol		↓**a***	↓**a***	↓**a***	↓	↓a
D-Mannose-6-phosphate		↑				
Myoinositol	↑*	↑	↑	↑	↑	
Tryptophan	↓	↓	↓		↓	↓*
D-Glucuronic acid 1-phosphate		↑*				
Malic-Acid		↑*				
Stearic acid	↓*	↓*	↓*	↓*	↓	↓*
Dodecamethylpentasiloxane		↑*	↑	↑*	↑	↑
Glyceraldehyde-3-phosphate	↑*	↑*	↑*	↑*	↑	↑
3-Phosphoglyceric acid			↑*			
Ethanolamine	↓	↓	↓a			
Phosphoric acid	↑	↑*	↑*	↑	↑	↑*
Boric acid				↑		↑*

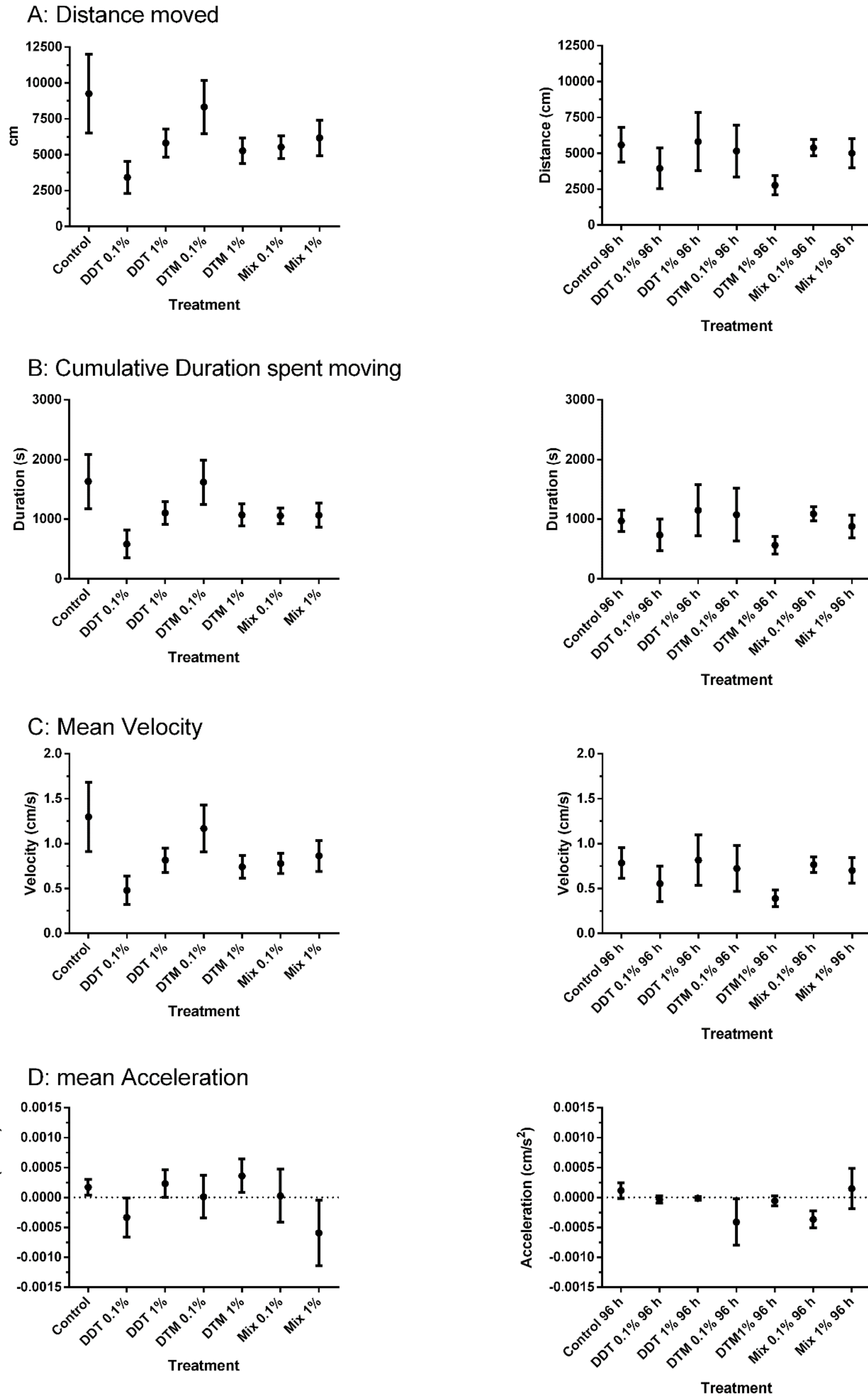


Figure B1 (A-E) Movement behaviour metrics for all treatments. (*see* full caption on next page)

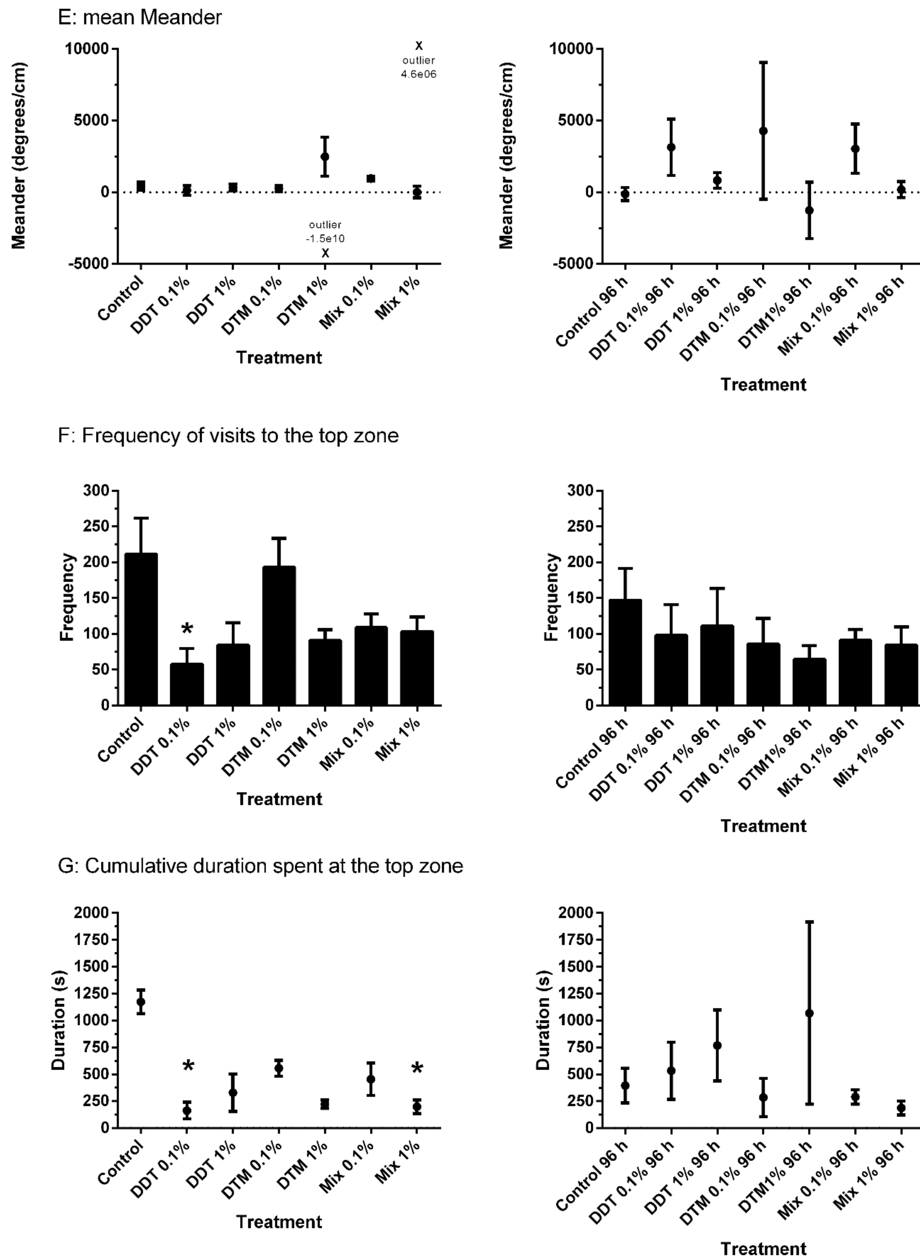
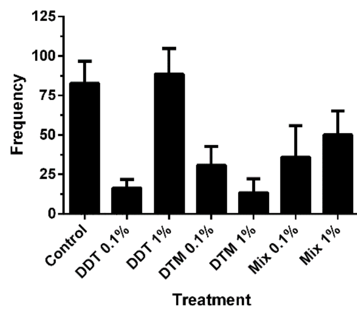
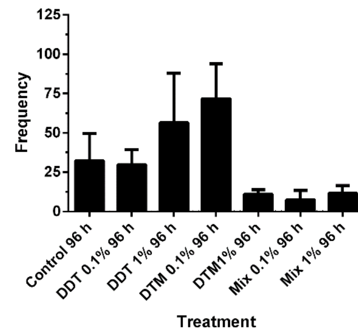


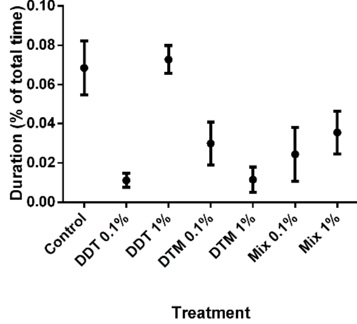
Figure B1 (continued, E-G)): Movement behaviour metrics for all treatments separated into 0 h (left) and 96 h (right) on equal scaling. Data represented as means (± standard error) from the four replicates recorded in each treatment. Individual specimen data are means / totals from 2 h recordings, collected as 1min time bins. A = Total distance moved; B = Cumulative duration spent moving; C = Mean velocity; D = Mean acceleration; E = Mean meander; F = Frequency of visits to the top zone; G = Cumulative duration spent in the top zone. X = outliers; * = $p < 0.05$.

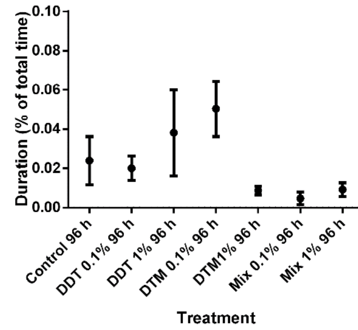
A: Frequency of being in a highly mobile state



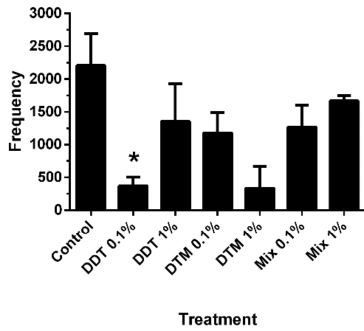


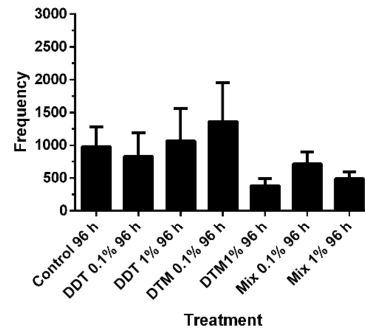
B: Duration spent in a highly mobile state



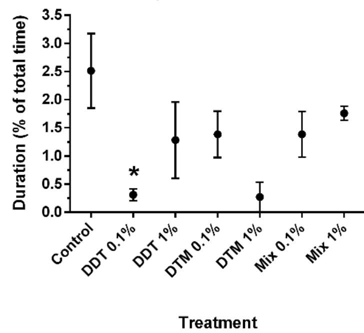


C: Frequency of being in a mobile state





D: Duration spent in a mobile state



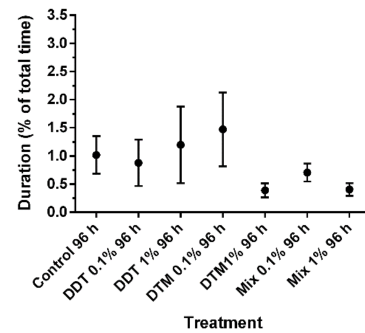
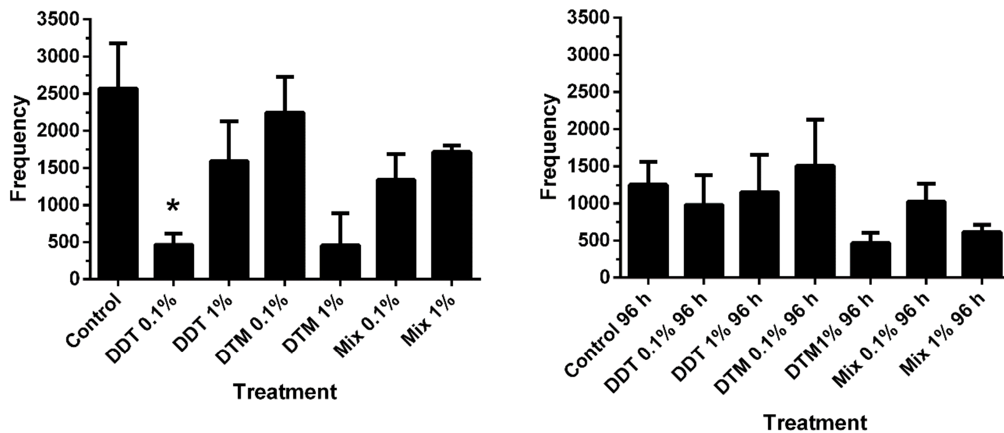


Figure B2 (A-D) Mobility state metrics for all treatments. (*see* full caption on next page)

E: Frequency of being in an immobile state



F: Duration spent in an immobile state

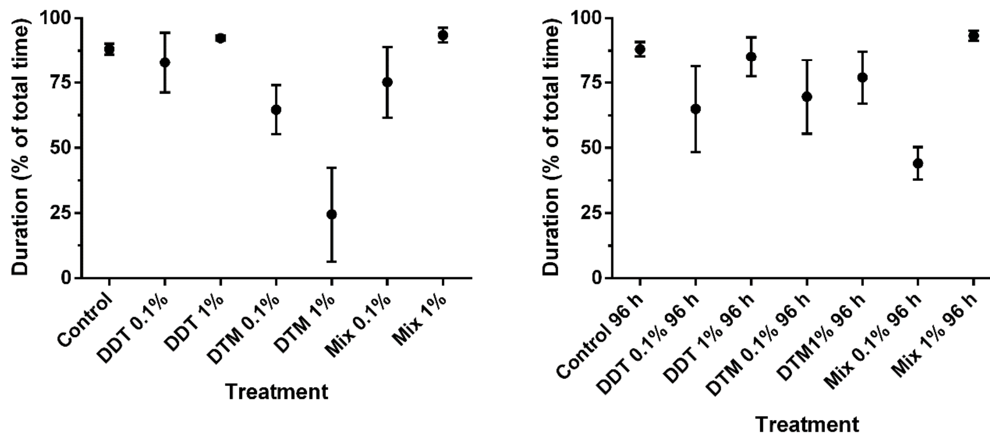


Figure B2 (continued, E-F) Mobility state metrics for all treatments separated into 0 h (left) and 96 h (right) on equal scaling. Data represented as means (± standard error) from the four replicates recorded in each treatment. Individual specimen data are means / totals from 2 h recordings, collected as 1min time bins. A = Frequency of being in a highly mobile state; B = Cumulative duration spent in a highly mobile state; C = Frequency of being in a mobile state; D = Cumulative duration spent in a mobile state; E = Frequency of being in an immobile state; F = Cumulative duration spent in an immobile state. X = outliers; * = $p < 0.05$

APPENDIX C CHAPTER 5 SUPPLEMENTARY MATERIAL

Table C1 **Physico-chemical water quality and nutrient variables measured in the outdoor microcosms over the duration of the experiment**

	Ctrl		DDT0.1		DDT1		DTM0.1		DTM1		Mix1	
	96h	28d	96h	28d	96h	28d	96h	28d	96h	28d	96h	28d
pH	8.46 ± 0.06	7.93 ± 0.16	8.17 ± 0.03	8.32 ± 0.03	8.4 ± 0.05	7.87 ± 0.24	8.28 ± 0.1	8.13 ± 0.02	8.43 ± 0.05	7.88 ± 0.13	8.3 ± 0.03	8.26 ± 0.1
Total dissolved solids (ppm)	844 ± 42	870.7 ± 22.1	791 ± 11	794 ± 11	830 ± 31	868.3 ± 12.5	812 ± 10	828.3 ± 28.8	824 ± 29	849.7 ± 33.6	790 ± 16	764.3 ± 95.2
Salinity (ppm)	592 ± 29	688.3 ± 73.05	552 ± 10	554.7 ± 7.4	579 ± 22	610 ± 11.43	567 ± 4	576.7 ± 22.5	579 ± 22	631.7 ± 70.5	551 ± 9	572.3 ± 111.9
Electrical conductivity (µS)	1210 ± 58	1203.3 ± 67.5	1134 ± 13	1138.7 ± 15.4	1187 ± 46	1240 ± 14.24	1164 ± 22	1186.3 ± 40	1177 ± 42	1191.7 ± 29.3	1130 ± 18	1094.7 ± 133.7
Oxygen (mg/L)	6.71 ± 0.21	5.94 ± 0.21	5.5 ± 0.13	6.91 ± 0.34	6.04 ± 0.45	5.85 ± 0.422	5.81 ± 0.32	6.37 ± 0.44	6.21 ± 0.35	6.16 ± 0.1	5.88 ± 0.14	6.86 ± 0.21
Oxygen (%)	73.1 ± 2.42	67.03 ± 2.18	60.37 ± 0.48	75.4 ± 4.4	65.17 ± 4.34	66.13 ± 4.37	63.13 ± 2.82	71.53 ± 1.15	68.83 ± 3.64	69.9 ± 0.9	63.73 ± 1.97	75.7 ± 1.47
Temperature (°C)	19.27 ± 0.17	22.03 ± 0.21	18.67 ± 0.05	20.97 ± 0.05	18.83 ± 0.09	21.23 ± 0.33	18.97 ± 0.05	22.93 ± 2.17	19.27 ± 0.17	21.7 ± 0.2	18.93 ± 0.19	20.67 ± 0.25
SO_4 (mg/L)	301 ± 1.4	304.7 ± 9.6	264.7 ± 4.5	250.3 ± 16.1	301.7 ± 2.4	291.7 ± 11.8	276.3 ± 48.3	288 ± 11.2	309.7 ± 11.8	281.3 ± 26.4	263.3 ± 29.2	272.7 ± 15.8
NO_3 (mg/L)	1.67 ± 0.68	3.53 ± 0.39	2.67 ± 0.45	2.9 ± 0.28	3.55 ± 0.25	3.2 ± 0.43	3.6 ± 0.41	3.37 ± 0.34	3.23 ± 2.42	3.83 ± 0.13	3.03 ± 0.29	2.6 ± 0.14
NO_2 (mg/L)	0.03 ± 0.008	0.047 ± 0.009	0.02 ± 0	0.04 ± 0.008	0.02 ± 0	0.04 ± 0.008	0.02 ± 0	0.033 ± 0.005	0.027 ± 0.009	0.043 ± 0.005	0.027 ± 0.005	0.037 ± 0.005

Table C1 (continued)

	Ctrl		DDT0.1		DDT1		DTM0.1		DTM1		Mix1	
	28d	**96h**	**28d**	**96h**	**28d**	**96h**	**28d**	**96h**	**28d**	**96h**	**28d**	
PO_4 (mg/L)	3.2 ± 1.82	0.27 ± 0.13	4.93 ± 0.1	0.37 ± 0.09	3.6 ± 1.98	0.44 ± 0.26	2.23 ± 1.55	0.19 ± 0.005	2.38 ± 1.88	0.52 ± 0.27	3.31 ± 1.31	0.55 ± 0.14
NH_4 (mg/L)	0.11 ± 0.02	0.05 ± 0.01	0.07 ± 0.01	0.14 ± 0.02	0.2 ± 0.14	0.09 ± 0.04	0.14 ± 0.09	0.09 ± 0.01	0.12 ± 0.06	0.3 ± 0	0.26 ± 0.24	0.14 ± 0.02

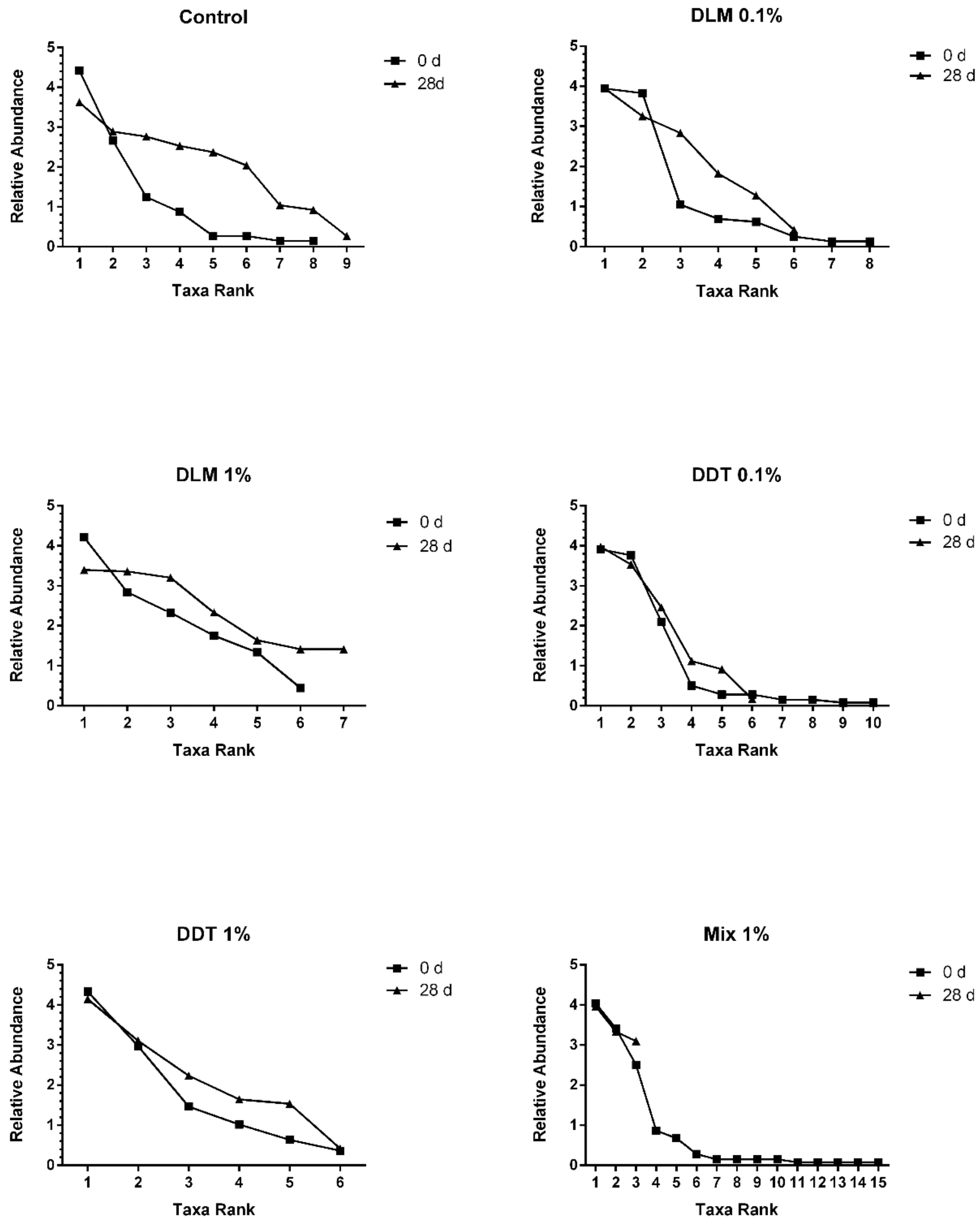


Figure C1 **Rank Abundance graphs of aquatic macro-invertebrate taxa (family level) from all exposure treatments comparing pre- (0 d) and post-exposure (28d) diversity and abundance. Relative abundance = Ln(count+1) using the average count from the three replicate microcosms per treatment**

APPENDIX D CHAPTER 6 SUPPLEMENTARY MATERIAL

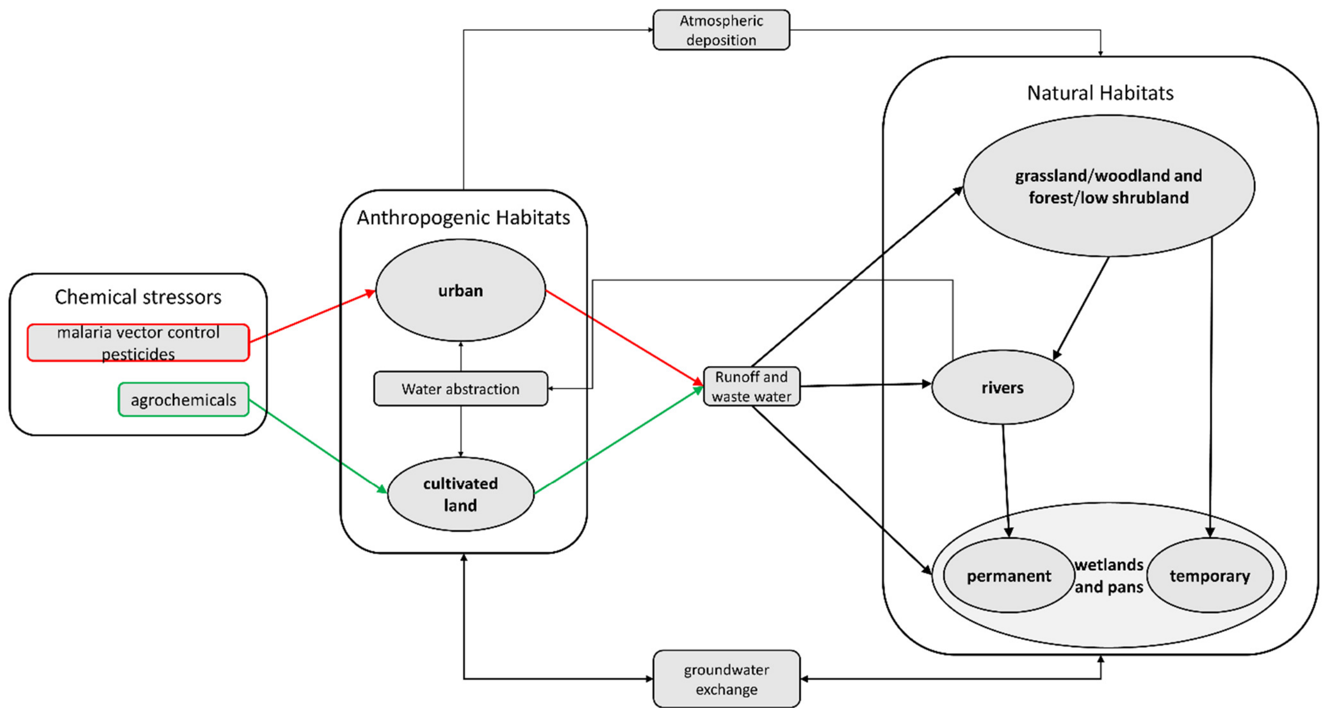


Figure D1 Conceptual model used for discerning relationships between chemical stressors and habitats in used in Model A of the assessment. Arrow thickness indicates the severity of influence. Black arrows indicate a mixture of stressor types. Pharmaceutical influence was not assessed in the analytical model

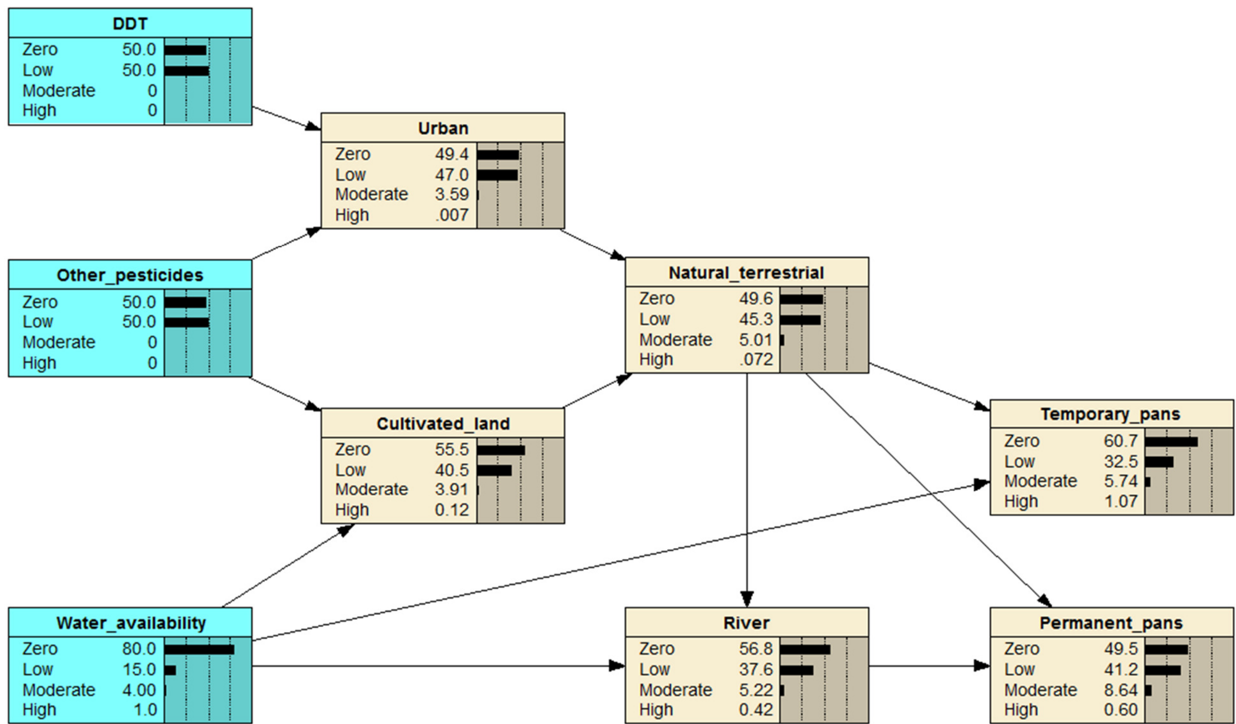


Figure D2 **The Bayesian Network outline used to quantify relationships between stressors and habitats in Model A. Input variables are in light blue and output variables in grey**

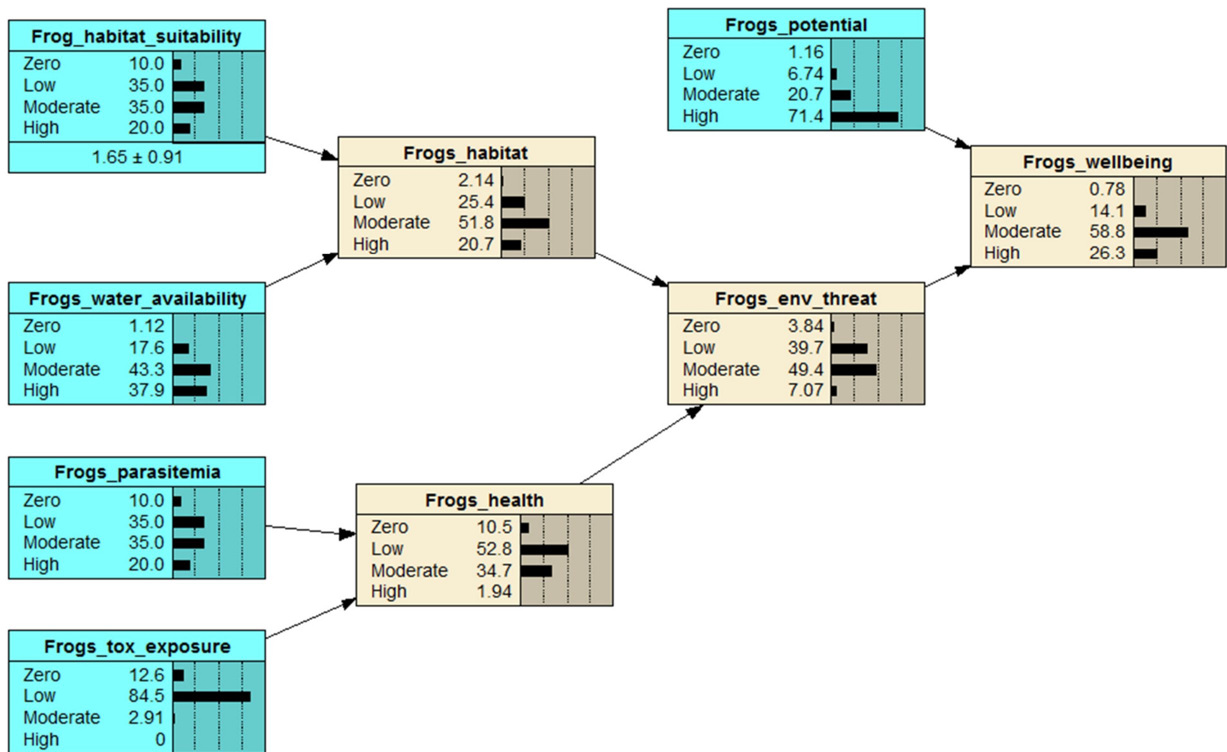
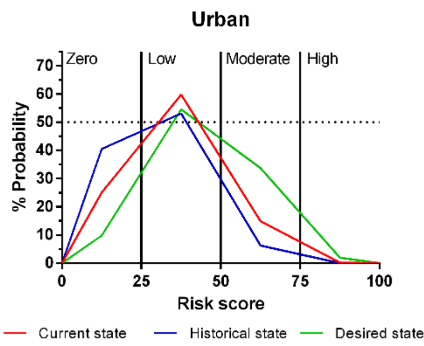
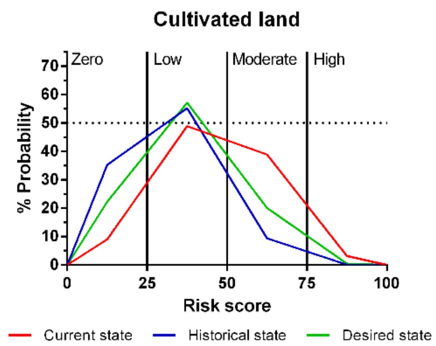
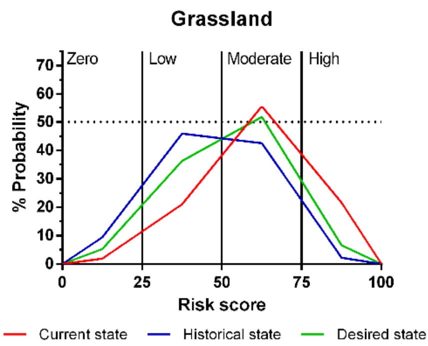
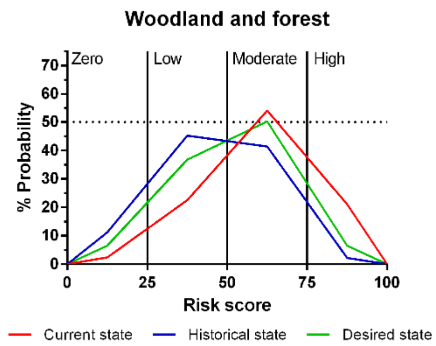


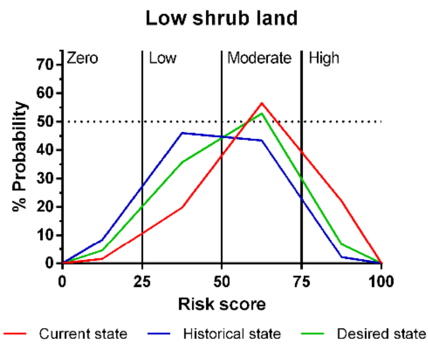
Figure D3 **The Bayesian Network outline used to quantify relationships between stressors, habitats and amphibian well-being in Model B. Input variables are in light blue and output variables in grey**

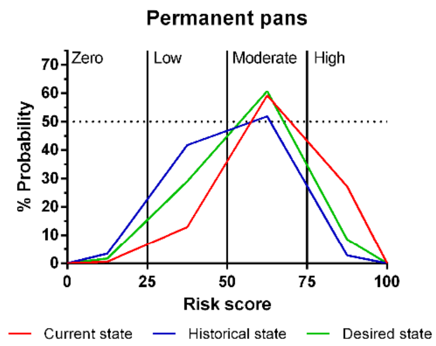


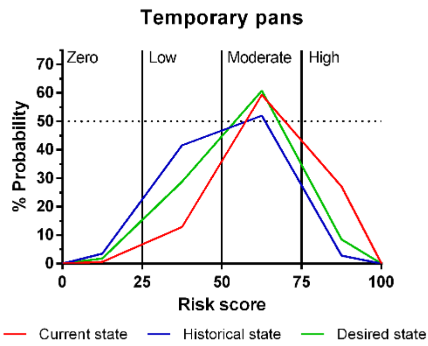












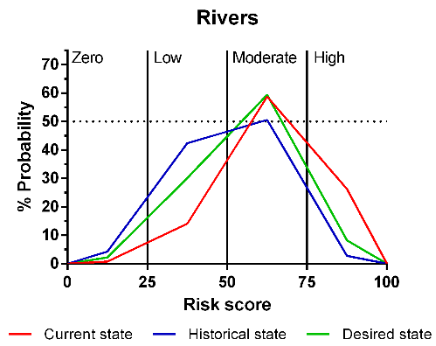
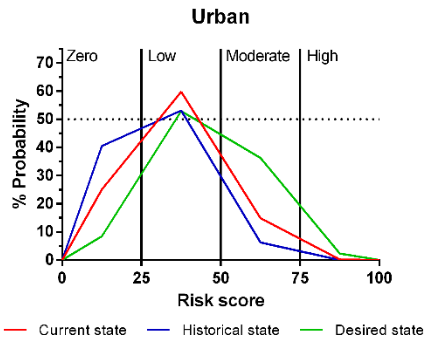
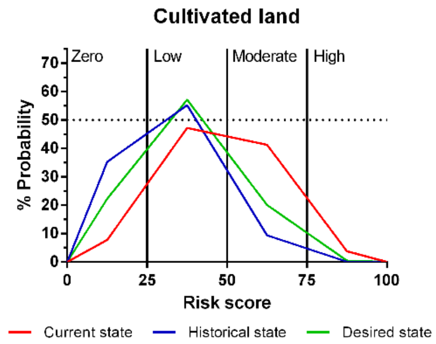
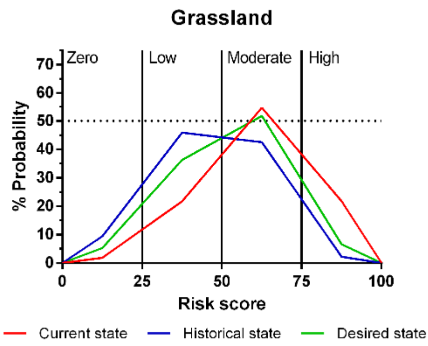
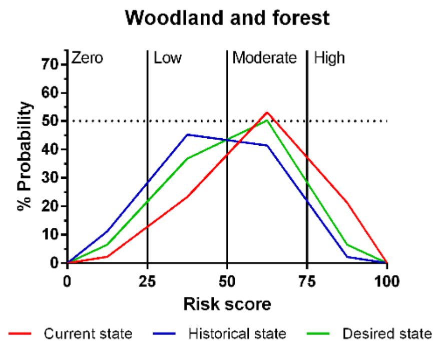


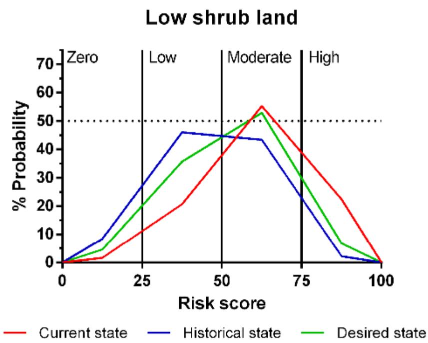
Figure D4 Amphibian well-being risk distributions for each habitat in Risk region 1

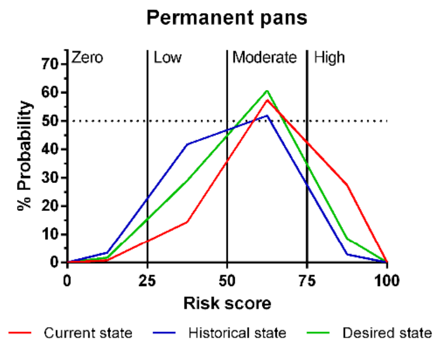


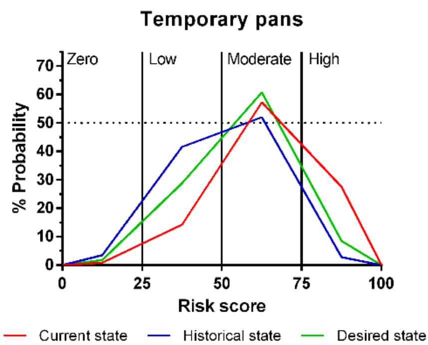












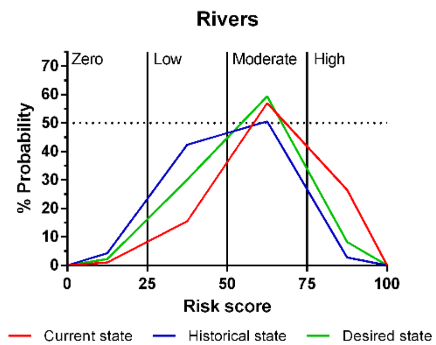
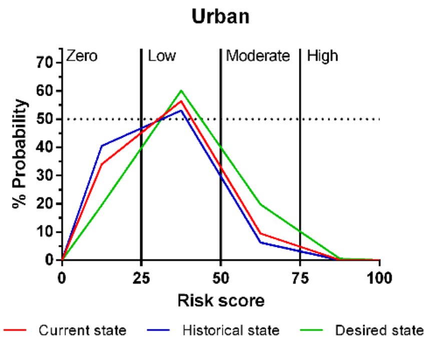
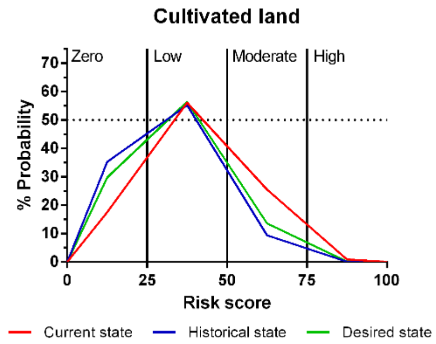
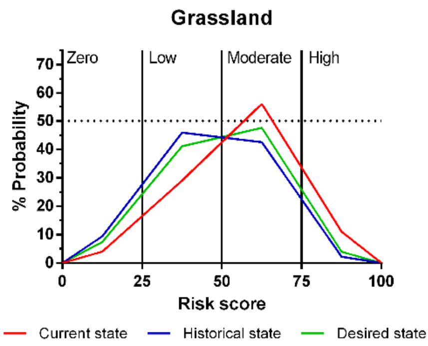
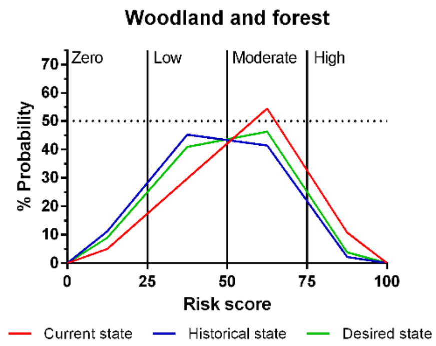


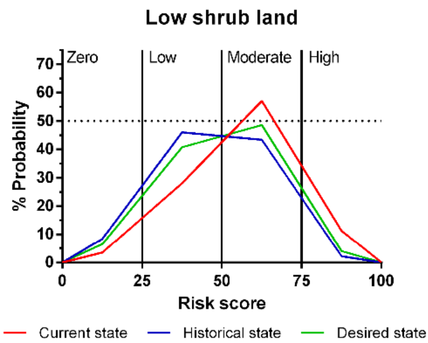
Figure D5 Amphibian well-being risk distributions for each habitat in Risk region 2

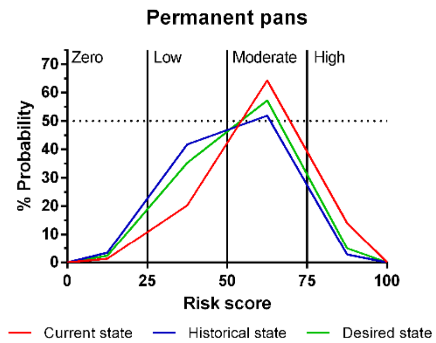


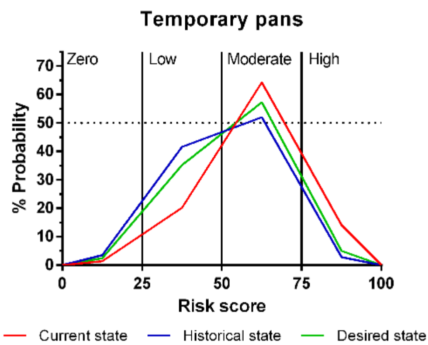












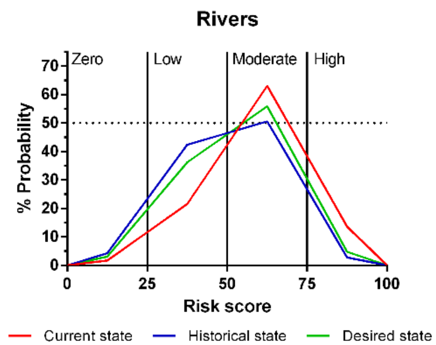


Figure D6 Amphibian well-being risk distributions for each habitat in Risk region 3

Table D1 Justifications for risk ranks used in Model A

Parent nodes description (node name)	Rank	Score	Narrative criteria for measure	Numerical criteria (units)	Justification	References
DDT input in terms of use (DDT)	Zero	25	no effects	<0.001 (µg/L)	Environmental levels were associated with environmental and human health effects. US regulations based on water concentrations were used as basis. Moderate risk was extrapolated from high risk values due to lack of data	ATSDR 2002; Chapter 4; Chapter 5
	Low	50	Chronic effects to freshwater systems	0.001 – 0.5 (µg/L)		
	Moderate	75	50% of limit for acute effects to freshwater systems	0.5 – 1.1 (µg/L)		
	High	100	limit for acute effects to freshwater systems	<1.1 (µg/L)		
Input of other vector control and agricultural pesticides (Other_pesticides)	Zero	25	no effects	<1.9 (µg/L)	Environmental levels were associated with human health and environmental effects. US regulations based on water concentrations were used. Low rank limit was based on short term behavioural effects observed in amphibians from the current study due to lack of data	ATSDR 2003; Chapter 4; Chapter 5
	Low	50	behavioural changes observed in aquatic biota	1.9 – 20		
	Moderate	75	chronic exposure drinking water limit based on human health risk	20 –180 (µg/L)		
	High	100	limit for acute effects to freshwater systems	180 (µg/L)		

Table D1 (continued)

Parent nodes description (node name)	Rank	Score	Narrative criteria for measure	Numerical criteria (units)	Justification	References
River flow requirements for functionality of the floodplain environment (Water_availa bility)	Zero	25	100% of pans inundated	>142 (m^3/s)	The measure selected to represent the pan inundation variable is average flow (m3/s). The extent of inundation and duration in the floodplain were considered in this section. No data are available on the duration of inundation of pans. Information that describes the cumulative inundation of pans in the floodplain is available. The duration of the inundation of the pans located in close proximity to the river is considered to be associated with the extent of the inundation. This information was used as the measure for this assessment. Available cumulative percentage inundation data of the pans in the floodplain are linked to the average flow released from the Pongolapoort Dam into the floodplain. Natural breaks (Jenks) were used to divide the data into zero, low, moderate and high categories due to limited data.	Smit et al. 2016
	Low	50	75% of pans inundated	85-142 (m^3/s)		
	Moderate	75	50% of pans inundated	72-85 (m^3/s)		
	High	100	25% of pans inundated	<72 (m^3/s)		

Table D2 **Justifications for CPT ranks used in Model B. This table was modified from the original assessment by Smit et al. (2016) to align with the current study model**

Parent nodes description (node name)	Rank	Score	Narrative criteria for measure	Numerical criteria (units)	Justification	References
Suitability of habitat for amphibians (Frog_habitat_suitability)	Zero	25	Well vegetated, grass dominated pans, unmodified banks	>95% vegetation cover	This variable considers the availability of riparian vegetation for frogs within different habitats. Well vegetated, grass dominated pans and riparian forest with high vegetation cover are required to maintain communities. The extent of vegetation loss and bank modification was selected as the measure for this variable.	This study; Smit et al. 2016
	Low	50	Moderate change to vegetation cover no isolated bank modification	50-95% vegetation cover		
	Moderate	75	Extensive bank modification	5-50% vegetation cover		
	High	100	No vegetation available	<5% vegetation cover		

Table D2 (continued)

Parent nodes description (node name)	Rank	Score	Narrative criteria for measure	Numerical criteria (units)	Justification	References
Suitability of floods required to inundate pans for frogs (Frogs_water_availability)	Zero	25	100% of pans inundated	>142 m^3/s	The measure selected to represent the pan inundation variable is average flow (m3/s). The extent of inundation and duration in the floodplain were considered in this section. Frogs that are mobile have a requirement for pans to be inundated for at least 30 days to complete important life cycle events. No data are available on the duration of inundation of pans. Information that describes the cumulative inundation of pans in the floodplain is available. The duration of the inundation of the pans located in close proximity to the river is considered to be associated with the extent of the inundation. This information was used as the measure for this assessment. Available cumulative percentage inundation data of the pans in the floodplain are linked to the average flow released from the Pongolapoort Dam into the floodplain.	Lankford et al. 2011; Smit et al. 2016
	Low	50	75% of pans inundated	85-142 m^3/s		
	Moderate	75	50% of pans inundated	72-85 m^3/s		
	High	100	25% of pans inundated	<72 m^3/s		

Table D2 (continued)

Parent nodes description (node name)	Rank	Score	Narrative criteria for measure	Numerical criteria (units)	Justification	References
Threat of prevalence and intensity of parasitemia of frogs (Frogs_parasitemia)	Zero	25	<20% blood cell infection rates	No to low parasitemia	Parasitemia of frogs was selected as an indicator measure of the parasite threats. The measure selected for this variable is blood cell infection rates.	Smit et al. 2016
	Low	50	20-80% blood cell infection rates	Minimal parasitemia no changes to communities		
	Moderate	75	81-97% blood cell infection rates	Parasitemia changes frog behaviour		
	High	100	>98%	Parasitemia causes mortality		
Threat of frogs being exposed to toxicants (Frogs_tox_exposure)	Zero	25	None	0 µg/g (wet weight)	This variable considers the threat of pesticides to frogs in the study area related to DDT accumulation in the frogs when exposed in mixture with other pesticides. The risk levels were calculated based on available international toxicity data combined with data collected in this study relating exposure concentrations to effects. Here the threat levels to *Xenopus laevis* generated through experimental work from this study were applied and adjusted according to the sensitivity distribution data available for frog species with regard to vector control pesticides.	This study; Chapter 4; Chapter 5; Wolmarans et al. 2020
	Low	50	No observable effects / sublethal cellular effects may occur.	>0 – 0.05µg/g (wet weight)		
	Moderate	75	Behavioural changes / Developmental changes	0.05 – 5.5 µg/g (wet weight)		
	High	100	Morphological changes / Mortality	>5.5 µg/g (wet weight)		

Table D2 (continued)

Parent nodes description (node name)	Rank	Score	Narrative criteria for measure	Numerical criteria (units)	Justification	References
Potential for frog diversities to occur in the study area (Frogs_potential)	Zero	25	Low potential for high diversities of frogs to occur	Potential for 5% of species to occur	The potential for frogs to occur in a specific habitat was included in the assessment as an effect variable. The potential percentage of known species to occur in the region considered was used.	This study; Acosta et al. 2020 Smit et al. 2016
	Low	50	Moderate potential for high diversities of frogs to occur	Potential for 6-50% of species to occur		
	Moderate	75	High potential for high diversities of frogs to occur	Potential for 51-94% of species to occur		
	High	100	Excellent potential for high diversities of frogs to occur	Potential for 95% of species to occur		

Table D3 matrix used for the determination of concern levels for ecosystem services based on the importance of the habitat for providing the specific service combined with the potential impact on that service derived from the risk distributions to each habitat

		Potential impact			
		zero	Low	moderate	high
Importance of Ecosystem service	low	Negligible concern	Negligible concern	Negligible concern	Low concern
	medium	Negligible concern	Negligible concern	Low concern	Moderate concern
	high	Negligible concern	Low concern	Moderate concern	High concern

Appendix D references

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