

The relationship between organochlorine pesticide exposure and biomarker responses of amphibians in the lower Phongolo River floodplain

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Abstract

Amphibians are regarded as sensitive indicators of environmental change and are therefore excellent subjects for use in ecotoxicology. The Phongolo River floodplain is South Africa's most diverse natural floodplain system and hosts more than 40 frog species. It is also a malaria endemic region and is subjected to active spraying with Dichlorodiphenyltrichloroethane (DDT) through means of indoor residual spraying over the summer months. The upper Phongolo River runs through agricultural landscape and is subjected to runoff from forest plantations, orchards and sugar cane plantations. In this study residue levels of 22 different organochlorine pesticides (OCPs) were analysed in selected amphibian species from in and around the Ndumo Nature Reserve coupled with 12 different biomarker response assays to determine environmental exposure levels and possible sub-lethal effects in amphibians from the lower Phongolo River floodplain. Seasonal change, direct influence of anthropogenic activity and the influence of species' aquatic preference in habitat selection were all factors considered during this assessment. Stable Isotope analyses were performed on 11 different food web components in order to determine the food web structure pertaining to *Xenopus muelleri* (Müller's platanna). Samples were collected during both high and low flow seasons from inside and outside Ndumo Nature Reserve. Organochlorine pesticide bioaccumulation was analysed in whole frog samples using a GC- μ ECD. Results indicated significant seasonal variation in OCP levels and exposure composition. Significant differences between inside and outside sites were also noted. Dichlorodiphenyltrichloroethane in its different isomer forms and their metabolites along with the hexachlorocyclohexane (HCH) isomers was the two main contributing OCP groups detected. Total OCP levels from all sample sets ranged between 8.71 ng/g lipid and 21,399.03 ng/g lipid. An increase in OCP accumulation was observed for *X. muelleri* over a period of one year. Organochlorine pesticides are known to have neurotoxic effects causing imbalances in Na⁺, K⁺, and Ca⁺ ion exchange. Hyperactivity has been reported in *Rana temporaria* (European Common frog) tadpoles exposed to *p,p*-DDT concentrations above 110,000 ng/g lipid. Despite OCP levels measured in frogs from this study being lower than reported toxic levels, the biomarker response assays indicated definite oxidative stress responses correlating to OCP bioaccumulation, with other minor responses shown. Cellular energy allocation showed a shift in the main energy source type from proteins to lipids correlating to increased OCP bioaccumulation. A slight inhibition response was noted in the hepato-somatic index correlating to γ -HCH bioaccumulation. Stable isotope analyses indicated food web structure differences between inside and outside the reserve, with outside showing less clear distinction between trophic groups and nitrogen enrichment of primary producers.

Key words: Amphibian ecology, Aquatic ecotoxicology, Bioaccumulation, Biomarker responses, DDTs, HCHs, OCPs, Phongolo River floodplain, Pollution, Stable isotope analysis

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Solemn Declaration

I, the undersigned, hereby declare that the content of this document is my own work.

A handwritten signature in black ink, appearing to be 'Nico Wolmarans', written over a horizontal line.

Nico Wolmarans

List of Abbreviations

μECD – micro Electron Capture Detector

ACh – Acetylcholine

AChE – Acetylcholine esterase

ASE – Accelerated Solvent Extraction

ATSDR – Agency for Toxic Substances and Disease Registry

ATP – Adenosine triphosphate

BSS – Buffered substrate solution

CAT – Catalase

CCME – Canadian Council of Ministers of the Environment

CEA – Cellular energy allocation

CYP450 – Cytochrome P450

DAFF – Department of Agriculture, Forestry and Fisheries

DDT – Dichlorodiphenyltrichloroethane

DDD – Dichlorodiphenyldichloroethane

DDE – Dichlorodiphenyldichloroethylene

DWA – Department of Water Affairs

Ea– Energy availability

Ec– Energy consumption

EDTA – Ethylene-diamine-tetraacetic acid

ETS – Electron transport system

FETAX – Frog Embryo Teratogenesis Assay – *Xenopus*

GABA – *Gamma*-aminobutyric acid

GC – Gas Chromatography

GPC – Gel Permeation Chromatography

GST – Glutathione-S-transferase

HCH – Hexachlorocyclohexane

HSI – Hepato-somatic index

IC₅₀ – Half maximal inhibitory concentration

IPCS – International Program for Chemical Safety

LC₅₀ – Half maximal lethal concentration

LP – Lipid peroxidation

MDA – Malondialdehyde

NADPH – Nicotinamide adenine dinucleotide phosphate

ND – Not detected

NOEC – No observed effects concentration

OCP – Organochlorine pesticides

OP – Organophosphate

PAH – Polycyclic aromatic hydrocarbons

PC – Protein carbonyls

PCB – Polychlorinated biphenyl

PCDD/F – Polychlorinated dibenzo-dioxin / -furan

PFOS – Perfluorooctanesulfonic acid

POP – Persistent organic pollutant

PPB – Potassium phosphate buffer

RDA – Redundancy analysis

RNA – Ribonucleic acid

SEM – Standard error of the mean

SIA – Stable isotope analysis

SOD – Superoxide dismutase

TP – Trophic position

U.S. EPA – United States Environmental Protection Agency

UNEP – United Nations Environment Programme

WHO – World Health Organization

WRC – Water Research Commission of South Africa

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1. Introduction

1.1 General introduction

Water is in many ways the most essential resource on earth. Without water life cannot be sustained as can be seen through the vast array of interactions water has with the ecosystem, from some of the most fundamental biochemical actions that sustain life (Garret & Grisham, 2010), through to hosting life as a habitat itself (Schmidt-Nielsen, 2004; Begon *et al.*, 2006). Water resource management is therefore extremely important. Efforts to conserve biological diversity are futile if water sources aren't managed correctly. The South African National Water Act, Act 36 of 1998 (DWA, 1998) acknowledges that there is a need for the implementation of monitoring programs to assess the health of aquatic ecosystems. When assessing aquatic ecosystem health, there is an important distinction to be made between pollutants and contaminants. Walker *et al.* (2006) describes pollutants as influences that cause measurable adverse effects on the environment and can be quantified as concentrations of certain chemicals exceeding the threshold levels that the specific environment can sustain. Contaminants are, however, described as xenobiotics or anthropogenic materials that enter an ecosystem (Van der Oost *et al.*, 2003).

Chemical pollution of water resources has become a great concern over the last few decades due to an increase in anthropogenic activities such as urban development, agriculture and industrial activity (Van der Oost *et al.*, 2003; Leticia & Gerardo, 2008; Cazenave *et al.*, 2009; Ghedira *et al.*, 2009). Although research has led to a better understanding of the effects of the chemicals being released, and in some cases provided better substitutes, there are still many hurdles to overcome before a balance can be found between anthropogenic progress and conservation of natural resources.

One such hurdle is the chemical control of mosquitoes in order to prevent the spread of malaria. Malaria is an illness caused by single celled parasites from the genus *Plasmodium* (see Guerrant *et al.*, 2011). The World Health Organisation (WHO) reports 72 deaths attributed to malaria in South Africa in 2012 (WHO, 2013). This is extremely low compared to neighbouring country Mozambique with 2818 deaths due to malaria in the same year (WHO, 2013). This can partly be due to South Africa's effective implementation of a malaria vector control programme. The programme allows for the use of dichlorodiphenyltrichloroethane (DDT) as chemical control of mosquitoes and in effect prevents the transfer of the *Plasmodium* parasite to humans (WHO, 2013).

Dichlorodiphenyltrichloroethane, an organochlorine pesticide (OCP) is a highly persistent chemical with a half-life of ± 5 years in soil (Addendum Table A5) and is also classified as a persistent organic pollutant (POPs) pesticide (Ritter *et al.*, 1995; ATSDR, 2002a). Its production and use was banned during the Stockholm Convention in 2001, with certain exceptions such as South Africa's case due to its effectiveness against mosquitoes (Ritter *et al.*, 1995). Although DDT is an insecticide it can also be harmful to other organisms in high enough doses (ATSDR, 2002a). The problem with a persistent pollutant such as DDT is that it is washed away by rain and ends up in the sediments in water bodies and accumulates rather than breaking down (Stegeman & Hahn, 1994) increasing the effective dosage that aquatic organisms are exposed to (Van der Oost *et al.*, 2003).

Being amphibian creatures, most frog species spend part of their life outside of water and part inside (Duellman & Trueb, 1994). Even though they do not have constant interaction with the water they do have very permeable skin (Duellman & Trueb, 1994; Du Preez & Carruthers, 2009) making them extremely vulnerable to pollutants in the water. Most frogs also have fully aquatic larval stages that are more susceptible to the effects of pollutants (Blaustein *et al.*, 1994; Carey and Bryant, 1995; Venturino *et al.*, 2003).

Amphibian populations around the world are currently experiencing the most rapid decline of any vertebrate species, partly due to habitat loss, but pollution and disease have also been reported as major contributing factors (Blaustein *et al.*, 1994; Berger *et al.*, 1998; Stuart *et al.*, 2004). Frogs are often habitat specific and are sometimes found in very small distribution areas (Du Preez & Carruthers, 2009). If the water resources in those areas are under pressure from pollution those frog populations might show even greater decline with possible species loss. It is thus important to assess the situation in such unique habitats as to prevent the irreversible loss of biodiversity.

The acute toxicity of POP's pesticides is not the only concern. Low level exposures can still affect organisms on a biochemical level (Van der Oost *et al.*, 2003). The key to determining why frogs are so sensitive to changes in their environment might just lie in the biochemical equilibrium responsible for their normal functioning. Very few studies have ever been conducted on the bioaccumulation of pesticides such as DDT in amphibians from Africa (Pauli *et al.*, 2000), and literature available on toxicity tests mostly originate from before 1980 (Addendum Table A6).

This study focuses on certain biochemical changes known to be induced or affected by pollutant exposure referred to as biomarker responses. The connection between pollutant exposure in a DDT sprayed area, the trophic level of the organisms as well as these biomarker responses is investigated with frogs as the indicator organism. If low level exposures are found to induce biomarker responses the actual environmental threshold for pollutants might be much lower than conventionally calculated levels indicate.

1.1.1 Amphibians as indicator species

Amphibians are good indicators of overall ecosystem health (Hilty & Merenlender, 2000). Their presence or absence alone is an excellent indicator as some species only inhabit very specific habitats and are very sensitive towards change within that habitat (Du Preez & Carruthers, 2009). The use of frogs as indicator organism also allows for the determination of pollutant exposure and effects of the animals inhabiting the river/riparian habitat. Frog habitats range from fully aquatic through to fully terrestrial (Du Preez & Carruthers, 2009) and testing for biomarkers in frogs across this range would then indicate the effects of contaminants in the aquatic system versus the terrestrial environment.

As previously mentioned of the factors contributing to amphibians' sensitivity towards pollution is the fact that they are the only vertebrates with a free-larval stage (Blaustein *et al.*, 1994; Duellman & Trueb, 1994; Beltz, 2009). The embryonic development of amphibians is the basis for one of the most widely used toxicity assays, the Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX) developed by Dumont (1983). This assay focuses on developmental abnormalities of *Xenopus laevis* (Common platanna) in terms of morphology, and rate of development (Dumont, 1983) based on the 46 stages in amphibian development as set out by Gosner (1960). Frog tadpoles hatch as free swimming larvae with gills, but most gaseous exchange occurs through the very permeable skin (Duellman & Trueb, 1994; Beltz, 2009).

During early development (Gosner stage 1-25) chemical uptake from the surrounding environment is of great concern as tadpoles do not yet appear to have the required enzyme activity to metabolize xenobiotics (Cooke *et al.*, 1970; Venturino *et al.*, 2003). After Gosner stage 42 tadpoles go into a fast and do not eat again until metamorphosis is complete (Beltz, 2009; Saha & Gupta, 2011). This leads to loss of body mass between tadpole and fully metamorphosed small adults (Saha & Gupta, 2011). Organochlorine pesticides accumulated in the body are stored in lipids (Hascheck *et al.*, 2013). Based on this information, the assumption can be made that early larval stage accumulation of OCPs in lipids that are used as energy source during the late stages of development can result in an increased re-release of OCPs in the body during this period, thus increasing the chance of toxic effects manifesting in this time.

During development vast arrays of morphological and physiological changes occur that are regulated by hormones (Gosner, 1960; Saha & Gupta, 2011). Endocrine disrupting compounds cause changes in the hormonal regulation, most often mimicking sex-hormones such as oestrogens and androgens and binding to receptors of these hormones activating secondary hormonal changes (Newman, 2010; Hascheck *et al.*, 2013). Amphibians are thus most susceptible to endocrine disruption during the early stages of development (Hayes, 2006).

Water loss is of great concern to frogs, with major loss and reabsorption occurring through the skin (Duellman & Trueb, 1986). This high level of water exchange can lead to high transfer rates of water soluble contaminants. Organochlorine pesticides are however mostly insoluble in water (Addendum Table A5) and are considered lipophilic (ATDSR, 1994; ATDSR, 2002a; ATDSR, 2002b; ATDSR, 2005; ATDSR, 2007; Newman, 2010; Yohannes *et al.*, 2013). This means that they bind more readily to organic compounds such as lipids, which is also where most long term storage of OCPs in the body takes place (Hascheck *et al.*, 2013). The enzyme activity in the liver where most xenobiotics are metabolised (Newman, 2010; Hascheck *et al.*, 2013), along with the dynamics of lipid storage in the body can therefore be considered to have greatest physiological influences on the toxicity of OCPs accumulated in amphibians. The link between these changes and toxicity is still quite a mystery, and although some literature on toxicity is available (Addendum Table A6), no proper guidelines (nationally or internationally) are available for OCPs in amphibians (CCME, 1999). Thus far research has only focussed on observable teratogenic effects such as morphological changes in tadpole development, or mortality in adults (Addendum Table A6; Pauli *et al.*, 2000).

Very few studies have ever been conducted that combine physiological changes such as biomarker responses and sub-lethal OCP exposure (Pauli *et al.*, 2000). The lack of literature in this capacity supports the necessity of research that incorporates biomarker responses in amphibians, especially ecological risk assessment type studies (Den Besten & Munawar, 2005).

1.1.2 Bioaccumulation analysis

Uptake of chemicals can take place through several different pathways and bioaccumulate in different tissues or organs inside the organism depending on metabolic variables (Newman, 2010). Uptake can occur through food consumption and simple diffusion through the intestinal tract (Fagotti *et al.*, 2005). Some chemicals can also be metabolised during the digestion process (Matsumura, 1987; Kitamura *et al.*, 2002). Contaminants accumulate in the food web (Kidd *et al.*, 2001) and can sometimes be transferred to the next trophic level through biomagnification (Van der Oost *et al.*, 2003). Another means of contaminant uptake is through skin absorption. For organisms such as frogs with highly permeable skin (Du Preez & Carruthers, 2009) this method is of greater concern than most other organisms. Contaminants can diffuse through skin simply through physical contact (Fagotti *et al.*, 2005). If the contaminant is present in the sediment and the organism lives in the mud in and around the aquatic environment, and has very permeable skin the accumulation level (uptake/contaminant ratio) can increase drastically.

1.1.3 Organochlorine pesticide toxicity

Uptake is however not the only concern when xenobiotics enter the aquatic environment. Many anthropogenic chemicals have toxic effects towards aquatic biota (Hascheck *et al.*, 2013). Discerning the concentrations of accumulation at which these toxic effects are expressed is a challenging, but necessary field of research in order to determine the health of an aquatic ecosystem.

Toxicity of organic compounds often stems from the ability of the contaminants to mimic certain naturally occurring chemicals in the body, combined with the inability of the body to excrete, metabolize, or sometimes even identify the contaminants as xenobiotic compounds as can be observed for most organic compounds described by Den Besten & Munawar (2005), Newman (2010), and Hascheck *et al.* (2013). The mode of action of OCPs such as DDTs is of neurotoxic origin and pertains to regulation of the Na⁺, K⁺, and Ca⁺ ion exchange between nerve endings. Dichlorodiphenyltrichloroethanes specifically inhibit the transmission of Na⁺ and K⁺ at the axon, while hexachlorocyclohexanes (HCHs) act by binding to *gamma*-aminobutyric acid (GABA) receptors inhibiting Ca⁺ flow (ATSDR, 2002a; Hascheck *et al.*, 2013). These modes of action lead to constant firing of nerve endings and can cause symptoms such as apprehension, tremors, facial paraesthesia, and seizures from DDT intoxication, or confusion, dizziness, agitation, nausea, as well as seizures from hexachlorocyclohexane intoxication (Hascheck *et al.*, 2013).

There are however other pathways through which OCPs can exhibit toxic effects. The *o,p*-isomer of DDT has been reported to have minor oestrogen-like characteristics, while *para*-, *para*-dichlorodiphenyldichloroethylene (*p,p*-DDE) and to a lesser extent *p,p*-DDT have been reported to act as anti-androgens (Hascheck *et al.*, 2013). Other OCPs have also been shown to have minor endocrine disrupting properties (IPCS, 2006; Hascheck *et al.*, 2013). In terms of carcinogenic effects, DDT and other OCPs have been reported to promote hepatic neoplasia in rats (ATSDR, 2002a; IPCS, 2006; Hascheck *et al.*, 2013). These toxic effects are the main reason why OCPs are introduced into the environment, as they have exceptional insecticidal properties (IPCS, 1979; ATSDR, 2002a; IPCS, 2006; Hascheck, *et al.*, 2013)

1.1.4 Biomarker responses

When an organism absorbs a hazardous chemical or toxicant there are certain biochemical responses that occur. Usually this response is either the inhibition or promotion of the production of certain enzymes or molecules in the body (Ellman *et al.*, 1961; Cohen *et al.*, 1970; Greenwald, 1989; De Coen & Janssen, 2003; Van der Oost *et al.*, 2003; Parves & Riasuddin, 2005; Üner *et al.*, 2005). These enzymes or molecules that are affected are referred to as biomarkers because they give an indication of the amount of hazardous chemicals that have been taken up (Van der Oost *et al.*, 2003). This is the first form of reaction the body has towards phenomenon such as chemical exposure, infection and disease and therefore serves as early indication of exposures that could cause more prominent issues in future (Bayne *et al.*, 1985; Van der Oost *et al.*, 2003).

Also if the level of exposure is very low, the effects may never become visible at higher levels even though the organism is constantly under stress, which could affect its overall fitness and ability to forage or escape danger (Bayne *et al.*, 1985). Through biomarker analysis this stress can be quantified (Van der Oost *et al.*, 2003).

There are mainly three biomarker response classes. Biomarkers of exposure are biomarkers that indicate direct exposure to toxicants in that these toxicants when taken up directly inhibit certain enzymes or promote detoxification enzyme production (Van der Oost *et al.*, 2003). Biomarkers of exposure utilised in this study are Acetylcholine esterase (AChE), and the Cytochrome P450 (CYP450) demethylating group. Biomarkers of effect are molecules or enzymes of which the levels in the body are affected because of physiological changes that occur when the body is under stress (Van der Oost *et al.*, 2003). The stressors in this case do not cause the change in itself, but the body's reaction towards these stressors then causes certain measurable changes (Van der Oost *et al.*, 2003). In this study a group of biomarker responses that indicate oxidative stress and the cellular energy allocation (CEA) biomarkers were used as biomarkers of effect. These are the two most common biomarker classes.

There is a third class, biomarkers of susceptibility that indicates the ability of an organism to respond to xenobiotic exposures and genetic factors that influence these responses (Van der Oost *et al.*, 2003). No biomarkers from this class were used in this study. Biomarkers have recently been incorporated in many environmental risk assessments, mainly with fish as target organism (Van der Oost *et al.*, 2003; Den Besten & Munawar, 2005). Studies done on frogs tend to focus on specific exposures (Peltzer *et al.*, 2013) or on fully aquatic *Xenopus* species in order to assess aquatic ecosystems (Burýšková *et al.*, 2006).

Acetylcholinesterase

This enzyme is responsible for the hydrolysis of acetylcholine (ACh), a critical molecule in the modulation of multiple neurological functions of the central nervous system, including functions associated with learning such as short term memory and attention span regulation (Van der Oost *et al.*, 2003; Garret & Grisham, 2010). Acetylcholine also plays an important role in the neuromuscular system as it binds to ACh-receptors at the axon in order to open sodium channels in the cellular membrane allowing ion exchange to take place, which in turn initiates muscle contraction (Garret & Grisham, 2010). The hydrolysis of ACh (facilitated by AChE) resulting in the formation of an acetate group and choline is crucial in terms of deactivating the ACh and ion transfer (Garret & Grisham, 2010). This makes AChE activity very important in preventing constant neuron firings.

A variety of chemicals can inhibit AChE activity. Organophosphates are known for their inhibiting effect on this enzyme in particular (Van der Oost *et al.*, 2003; Hannam *et al.*, 2008). Other inhibitors include various pesticides, antibiotics and nerve gases (Connell *et al.*, 1999; Pfeifer *et al.*, 2005; Wepener *et al.*, 2005; Tu *et al.*, 2009). AChE levels naturally differ between species as their habitat and food sources differ (Pfeifer *et al.*, 2005).

Cytochrome P450

Cytochrome P450 (CYP450) is a monooxygenase enzyme group which facilitates phase I type biotransformation of xenobiotics in the body as a means of detoxification (Garret & Grisham, 2010). Phase I biotransformation includes reduction, oxidation, or hydrolysis reactions which produce more water-soluble metabolites required for excretion (Hascheck *et al.*, 2013). The CYP450 monooxygenase system is made up of different isoforms that act on specific substrates giving this system the ability to influence a wide array of substrates making it an efficient detoxification mechanism (Newman, 2010). This study focuses on the demethylating CYP450 group, which includes the CYP450 3A4, 2B4 and 2D6 isoforms, as polychlorinated biphenyls (PCBs), DDT, and other OCPs are known CYP2 inducers while CYP3 gene induction is facilitated by steroid-like drugs (Newman, 2010). Amphibian specific CYP genes are not well known. *Xenopus tropicalis* does not possess CYP2 or CYP3 genes (Newman, 2010), but *X. laevis* is known to have both CYP2 and CYP3 genes (Xenbase, 2015). Induction of other CYP450 isoforms with demethylating activity by OCPs in amphibians is likely as research has shown that frogs do have a means of metabolising pesticides such as DDT (Cooke, 1970; De Solla *et al.*, 2002). The CYP4 genes can be induced by hepatocarcinogens (Newman, 2010), which would make induction by DDT a possibility.

Oxidative stress biomarkers

Superoxide dismutase (SOD) and catalase (CAT) are enzymes with consecutive roles in the cellular antioxidant system. Superoxide dismutase is responsible for catalysing the superoxide radical detoxification through the formation of hydrogen peroxide (Pandey *et al.*, 2003), which is a far less oxidative species, whilst CAT breaks down hydrogen peroxide into water and oxygen (Lionetto *et al.*, 2003; Garret & Grisham, 2010). SOD activity has been reported to be affected by temperature change (Parihar *et al.*, 1996), and the presence of metals, but the effects vary with different metals (Lushchak *et al.*, 2009; Vieira *et al.*, 2009).

Increased SOD activity, indicating higher superoxide levels, can affect the activity of actonitase, an enzyme found in the citric acid cycle (Ferreira *et al.*, 2007). Hydrogen peroxide is a crucial defence against infection on a cellular level (Pandey *et al.*, 2003; Garret & Grisham, 2010).

Although hydrogen peroxide is necessary, high concentrations can still cause oxidative damage to the cell itself and therefore levels have to be regulated through CAT activity (Garret & Grisham, 2010). Increase in CAT activity would indicate higher hydrogen peroxide levels being produced that need to be broken down (Parihar *et al.*, 1996). Inhibition of CAT by metals and cyanide groups is reported by Lionetto *et al.* (2003). In these cases CAT activity was low, but other effects of oxidative stress such as protein carbonyl (PC) formation was still be visible.

There are two indicators of oxidative damage used in this study, malondialdehyde (MDA) and PC. Malondialdehyde is produced through the process of lipid peroxidation (LP), the breakdown of phospholipids in cell membranes due to oxidative stress (Parihar *et al.*, 1996; De Almeida *et al.*, 2007; Lushchak *et al.*, 2009). According to Maria *et al.* (2008), MDA plays an important role in DNA damage caused by oxidative stress. MDA content is used to quantify LP, which can disrupt cell membrane functionality (Garret & Grisham, 2010). The lipid chain length and saturation status affects membrane permeability (Parihar *et al.*, 1996). These can in turn be affected by external factors such as temperature (Parihar *et al.*, 1996). This biomarker is of importance due to the severity of the adverse effects caused by high LP levels (Parihar *et al.*, 1996). Protein carbonyls are formed when amino acids undergo oxidation indicating oxidative stress effects on proteins (Garret & Grisham, 2010). Elevated levels are caused by exposure to pesticides, specifically deltamethrin, endosulfan and paraquat (Parvez & Riasuddin, 2005), and could lead to cellular damage. Protein carbonyl formation is irreversible and results in a decrease of enzyme catalyst activity (Ferreira *et al.*, 2007). Protein damage through oxidation takes longer to recover than that of other systems (Ferreira *et al.*, 2007).

Cellular energy allocation

The CEA is a summary of the energy production, storage and use that takes place within the muscle tissue of the target organism (De Coen & Janssen, 1997). De Coen & Janssen (1997) developed the short term CEA assay through which energy reserve and energy consumption changes are assessed and an energy budget is set up by subtracting the consumed energy (E_c) results from the available energy (E_a) results (De Coen & Janssen, 1997). Available energy reserves are calculated through the sum of three main energy reserves namely, carbohydrates, proteins, and lipids. The content for each of these are determined separately and converted to energetic equivalent values. Energy consumption is based on electron transport system (ETS) activity that essentially converts energy reserves into adenosine triphosphate (ATP) as required by the body. The resulting energy budget indicates dietary or nutritional stresses the organism is experiencing (De Coen & Janssen, 1997).

Reduction in the CEA can be caused by various external factors such as food availability and seasonal change (Gourley & Kennedy, 2009), but also by factors such as the organism's ability to hunt, which in itself can be affected by various toxicant exposures (Gourley & Kennedy, 2009).

1.1.4 Stable isotope analysis

The combination of $\delta^{13}\text{C}$ & $\delta^{15}\text{N}$ ratios can be used to determine the trophic levels of organisms as these ratios either change or don't change as material (atoms) are transferred through the food web (Peterson & Fry, 1987; Fry, 1991; Abend & Smith, 1997). Different isotopes of the same chemical element refer to a difference in neutrons found in the nucleus of the same type of atom (Kotz *et al.*, 2009). Thus the atomic number is the same, but the atomic mass differs. Different isotopes of elements occur naturally, but some are only found in a very small percentage in the environment (Kotz *et al.*, 2009). Stable isotope ratios make use to these percentages and how they differ between organisms and environments (Peterson & Fry, 1987).

The food web structure of an aquatic environment can be displayed by plotting the $\delta^{15}\text{N}$ ratios against the $\delta^{13}\text{C}$ ratios for all the different food web components of that habitat (Fry, 1991). The $\delta^{13}\text{C}$ ratios indicate carbon pathways through which energy is transferred in the food web, with the $\delta^{15}\text{N}$ ratios providing information on the trophic levels of the different food web components (Peterson & Fry, 1987; Abend & Smith, 1997). The separation of components in such a plot provides information on the trophic groups into which these components fall such as primary producers, primary consumers and predators, as well as providing pathway information on the different food chains within the food web (Peterson & Fry, 1987; Fry, 1991; Abend & Smith, 1997). Stable isotope analysis (SIA) is widely used to study ecosystem functioning (Peterson & Fry, 1987; Fry, 1991) as it provides researchers with integrated data on the dietary dynamics of target organisms (Davis *et al.*, 2012). The information received from SIA is collective long term data on feeding habits, rather than the short term, but more precise information provided through stomach content analysis (Abend & Smith, 1997; Layman, 2007; Davis *et al.*, 2012).

1.1.5 Study area

The research area selected for this study lies in the lower Phongolo River floodplain situated in the eastern part of South Africa. The Phongolo floodplain is regarded as the most diverse floodplain in South Africa, as well as being one of the largest (Mallory, 2002; Lankford *et al.*, 2010). Research sites are mainly focused around Ndumo Game Reserve and surrounding villages. The area is categorised by the WHO (WHO, 2013) as a medium-high risk malaria area and therefore a malaria vector control programme is implemented in this area. The area also has a history of dramatic ecological changes, most originating due to the building of the Pongolapoort Dam in the early 1970s, which had many foreseen social and ecological impacts in the immediate environment, but also many unforeseen consequences in the floodplain situated downstream of the dam (Van Vuuren, 2009). The natural floodplain consists of many wetlands and pans that are dependent on seasonal floods to regulate the water levels and water flow (Lankford *et al.*, 2010). These seasonal floods no longer occurred once the dam was built which had immense effects on local agriculture and the natural ecosystem (Van Vuuren, 2009). After extensive research an artificial flooding program was introduced in the late 1970's to rectify this issue (Van Vuuren, 2009). However, political and economic influences over the years led to ineffective use of the artificial flooding, leaving the floodplain potentially damaged (Van Vuuren, 2009). A more detailed description of the research area is given in section 2.1.

1.1.6 Rationale

The ecological history of the study area and its importance as a unique ecosystem substantiates that the use of persistent pesticides in the area is a great cause for concern (Mallory, 2002; Van Vuuren, 2009; Lankford *et al.*, 2010). The potential effects of this and other threats call for an assessment of current ecosystem functioning and possible implementation of management strategies in order to protect the aquatic resources of the region (Lankford *et al.*, 2010). An essential step in this process is to gain a holistic view of the current situation. This is done through an assessment of the current ecosystem health (Lackey, 2001). This study forms part of the ecotoxicological aspect involved in this assessment. In itself the study should provide valuable data in terms of amphibian ecotoxicology, but in combination with other current and future studies on the lower Phongolo River floodplain it may be used to aid in decision making on ecosystem management options in the area.

1.2 Hypotheses

- Amphibians from the lower Phongolo River floodplain area are exposed to potentially harmful levels of DDT, its derivatives and other organochlorine pesticides (OCPs).
- Amphibians from this region exhibit biochemical (biomarker) responses towards changes in environmental conditions
- These biomarker responses indicate biochemical stress to amphibians due to exposure to environmental contaminants.

1.3 Aims and Objectives

1.3.1 Project aims

This study aims to determine whether, and to what extent, organic pollutants in the lower Phongolo River floodplain system are affecting the health of amphibians in the area. The responses to OCP bioaccumulation will be determined through the use of the following ecotoxicological biomarker responses: Acetylcholine esterase (AChE), catalase (CAT), superoxide dismutase (SOD), malondyaldehyde (MDA), protein carbonyls (PC) and cellular energy allocation (CEA) in liver and muscle tissue samples in four indicator frog species (*Amietophrynus garmani*, *Chiromantis xerampelina*, *Ptychadaena anchietae* & *Xenopus muelleri*). Through the use of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ stable isotope analyses this study aims to determine the trophic level of selected frogs in this region and also to what degree biomagnification of chemical exposure takes place.

Through the habitat selection of the target species this study also aims to determine whether there is a relationship between the chemical bioaccumulation and biomarker responses and the degree of water association of the frog species. It is further aimed to determine whether the chemical bioaccumulation and biomarker responses in frogs differ on a spatial (within the Ndumo Game Reserve and outside the reserve) and temporal (high and low flow periods) scale.

1.3.2 Research objectives

In order to achieve the stipulated aims certain objectives were set:

- To determine OCP levels in frog tissue on a spatial and temporal scale to determine the influence of seasonal variation (i.e. different flow periods) and human activity on bioaccumulation levels.
- To measure biomarker responses of frogs to OCP bioaccumulation on a spatial and temporal scale to determine the influence of seasonal variation (i.e. different flow periods) and human activity.
- To determine if any relationships exist between chemical bioaccumulation and biomarker responses on a spatial and temporal scale.
- To determine if there is a relationship between the frog species' water dependence and chemical bioaccumulation and concomitant biomarker responses.
- To collect stable isotope data for different food web components involved in the diet of *X. muelleri* to determine the trophic interactions of those components, stability of the food web structure, and the possible connection between food web structure and OCP bioaccumulation.

2. Materials & Methods

2.1 Site selection

2.1.1 Research location

Reaching across 7000 km², the Phongolo River catchment passes between the Lebombo and Ubombo Mountains through a narrow gorge in which the Pongolapoort Dam (also known as Jozini Dam) was built in 1972 (Lankford *et al.*, 2010). The area downstream from the dam is known as the lower Phongolo River floodplain (Figure 2.1). As previously stated the area hosts a wide biological diversity, including over 40 fish species and more than 400 bird species (Mallory, 2002).

Ndumo Game Reserve is situated inside the lower Phongolo River floodplain. To the north the reserve is bordered by the Usuthu River, which is also the border between South Africa and Mozambique. The Phongolo River flows through the reserve from south to north where it joins into the Usuthu River. The river essentially forms the eastern border to the accessible part of the reserve. Swaziland is situated less than 15 km from the reserve border towards the west. The reserve plays host to a wide variety of habitats including extensive wetlands and pans creating excellent habitats for frogs. Plant growth is characterised by fever trees (*Vachellia xanthophloea*), acacia savannah, sand forest, and reed beds. This sub-tropical area experiences average temperatures of 14 °C – 23 °C (min) & 26 °C – 31 °C (max) through the year (Mallory, 2002; Jaganyi *et al.*, 2008). The mean rainfall in the area is over 600 mm per year with heaviest rainfall usually occurring between November and January (Jaganyi *et al.*, 2008; Lankford *et al.*, 2011). Three sites were identified inside the reserve on the basis of their connection to the Phongolo River during high flow conditions. Samples for OCP bioaccumulation, biomarker responses and SIA were collected from these sites (Figure 2.1).

Clusters of rural settlements dominate the area around the reserve where subsistence farming (mostly maize, cattle, goat, and poultry) is practised. Four sites were selected outside the reserve, one of which was used for OCP analysis due to high anthropogenic activity observed at the site and also its direct connection to the Phongolo River during the high flow periods (Figure 2.1). The three other sites were used for the collection of food web components for SIA and were selected on the basis of their proximity to local settlements.

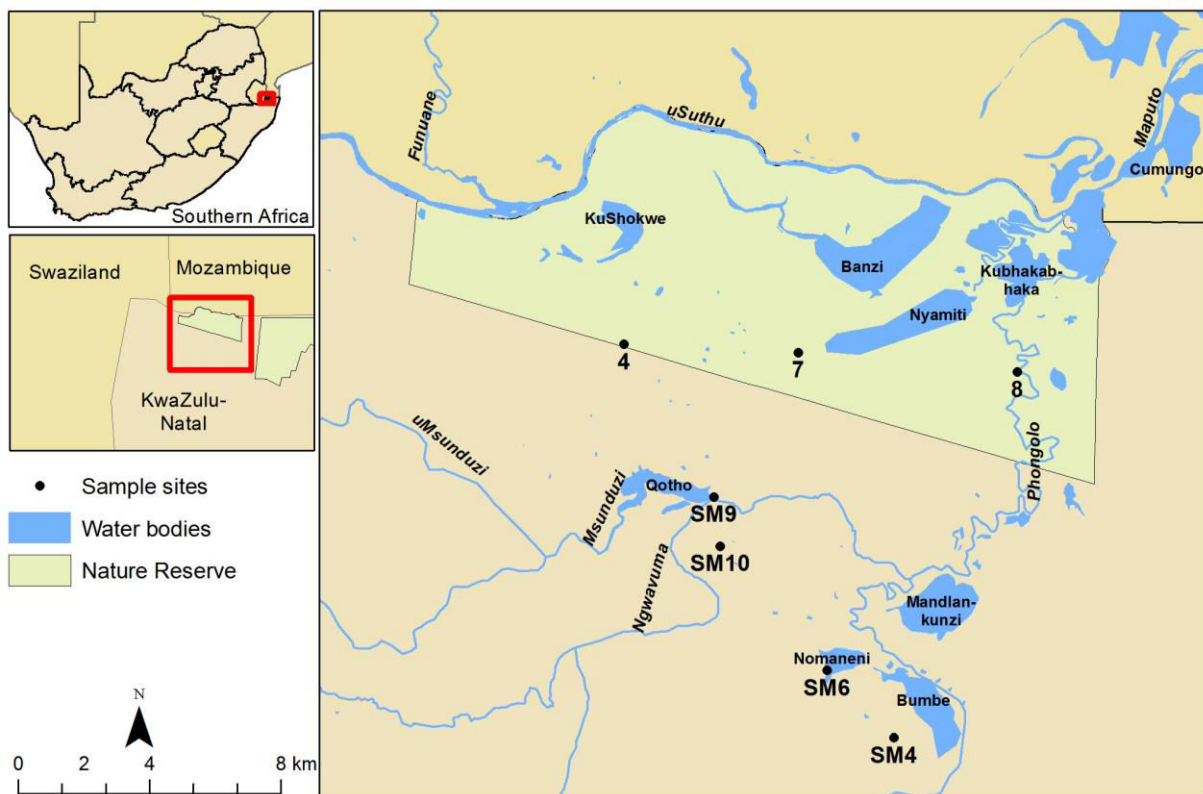


Figure 2.1: Map of the research area indicating large water bodies, Ndumo Game Reserve, and sampling sites. Sites 4, 7 and 8 represent inside sampling for organochlorine pesticide bioaccumulation, biomarker responses and stable isotope analysis. Biomarker response and organochlorine pesticide bioaccumulation samples from outside the reserve were collected from site M6, and food web component samples for stable isotope analysis were collected from sites SM4, SM9, and SM10. Map rendered in ARCGIS by Natasha Vogt

2.1.2 Non-site-specific selection

Initial research design included collecting frogs per site within the reserve. This proved difficult as abundances of frog species differed greatly at the selected sites. After careful consideration of the river catchment and water flow dynamics of the area, it was decided to collect frogs in a non-site-specific manner as to compensate for the low abundances at some sites, and shift focus towards the holistic assessment of pesticide exposure and effect inside the reserve. For this purpose sites 4, 7, and 8 representing different habitat sites were sampled inside Ndumo Game Reserve while sites SM4, SM6, SM9, and SM10 were sampled outside the reserve (Figure 2.1).

2.2 Target species

2.2.1 Species selection criteria

Target species were selected based on two main factors. Firstly, the abundance of the species in the research area was considered. Secondly, the species' direct contact with water through their habitat selection and general behaviour was taken into account. Four species were selected to represent fully terrestrial (*Amietophrynus garmani*), semi-terrestrial (*Chiromantis xerampelina*), semi-aquatic (*Ptychadaena anchietae*), and fully aquatic (*Xenopus muelleri*) species (Figure 2.2a-d).

2.2.2 *Amietophrynus garmani*

Amietophrynus garmani (Eastern olive toad) (Figure 2.2a) is a typical toad from the Bufonidae that inhabits marshes and pans in high rainfall areas of the bushveld savanna (Du Preez & Carruthers, 2009). During this study it was commonly found near the riverbank and pan edges and seemed to prefer areas with moderate shade and thick leaf litter. In South Africa its distribution stretches from the Gauteng province through Limpopo and down the eastern parts of Mpumalanga through to northern KwaZulu-Natal. It has a thickset body with short legs, feeds on invertebrates (Beltz, 2009) and grows to a maximum size of 115 mm (Du Preez & Carruthers, 2009).

2.2.3 *Chiromantis xerampelina*

Chiromantis xerampelina (Southern Foam Nest Frog) (Figure 2.2b) belongs to the Rhacophoridae and is a quite unique species in South Africa. It is found around open water bodies, both permanent and temporary (Du Preez & Carruthers, 2009), and prefers branches and trees hanging over the water. It is also found in the bushveld savannah biome with its distribution in South Africa similar to that of *A. garmani*. It has terminal disks on its toes and fingers with its toes also being extensively webbed (Du Preez & Carruthers, 2009). It has a typical tree frog build, but with horizontally elongated pupils. It is also large compared to tree frogs from South Africa with a maximum size of 85 mm (Du Preez & Carruthers, 2009). Colour varies from dark grey to whitish, but colour change within this

range is possible depending on factors such as surroundings, temperature and disturbance (Du Preez & Carruthers, 2009).

Its eggs are laid in white foam nests that can be easily spotted hanging from branches (Beltz, 2009; Du Preez & Carruthers, 2009). In order to produce this nest the females absorb a large amount of water from the water body before and during the spawning process (Taylor, 1971; Du Preez & Carruthers, 2009). This behaviour may cause higher levels of exposure to aquatic pollutants during the mating season (October to February).

2.2.4 *Ptychadaena anchietae*

Ptychadaena anchietae (Plain Grass Frog) (Figure 2.2c) belongs to the Ptychadenidae. With their powerful hind legs grass frogs are known for their long-jumping ability. The Plain Grass Frog is widely distributed in the same areas as *C. xerampelina*. It tends to shelter in vegetation around a breeding site, but are sometimes found in the open alongside riverbanks or pan edges (Du Preez & Carruthers, 2009). This species tend to have more regular contact with water than *C. xerampelina* (this might however differ during the breeding season) and are therefore considered semi-aquatic for the purposes of this study.

Their hind legs are much larger than their front legs, and they tend to have an upright posture with hind legs folded beneath the body ready to jump and front legs almost extended below the body. Adults may reach a maximum body size of 62 mm (Du Preez & Carruthers, 2009).

2.2.5 *Xenopus muelleri*

Xenopus muelleri (Müller's Platanna) (Figure 2.2d) as it is commonly known, belongs to the Pipidae and is an aquatic clawed frog found in South Africa only along the most eastern parts of Limpopo, Mpumalanga and north-eastern KwaZulu-Natal. It is, however, commonly found in these areas and inhabit mostly still and slow-flowing water bodies. They spend their entire lives in water and will only leave the aquatic environment during migration events (Beltz, 2009; Du Preez & Carruthers, 2009). They are well adapted to their environment with large webbed hind feet and a streamline body growing to a maximum size of 90 mm (Du Preez & Carruthers, 2009). They stay submerged and only break the surface to breathe (Du Preez & Carruthers, 2009). They have a fish-like lateral line organ with which they can sense vibrations in water (Beltz, 2005).

Xenopus tadpoles are filter feeders, while adults feed on invertebrates and small fish as well as scavenging from dead organisms (Beltz, 2009). *Xenopus muelleri* is easily distinguishable from other species of the same genus in South Africa through its prominent sub-ocular tentacles that are at least half as long as the diameter of its eye (Du Preez & Carruthers, 2009).

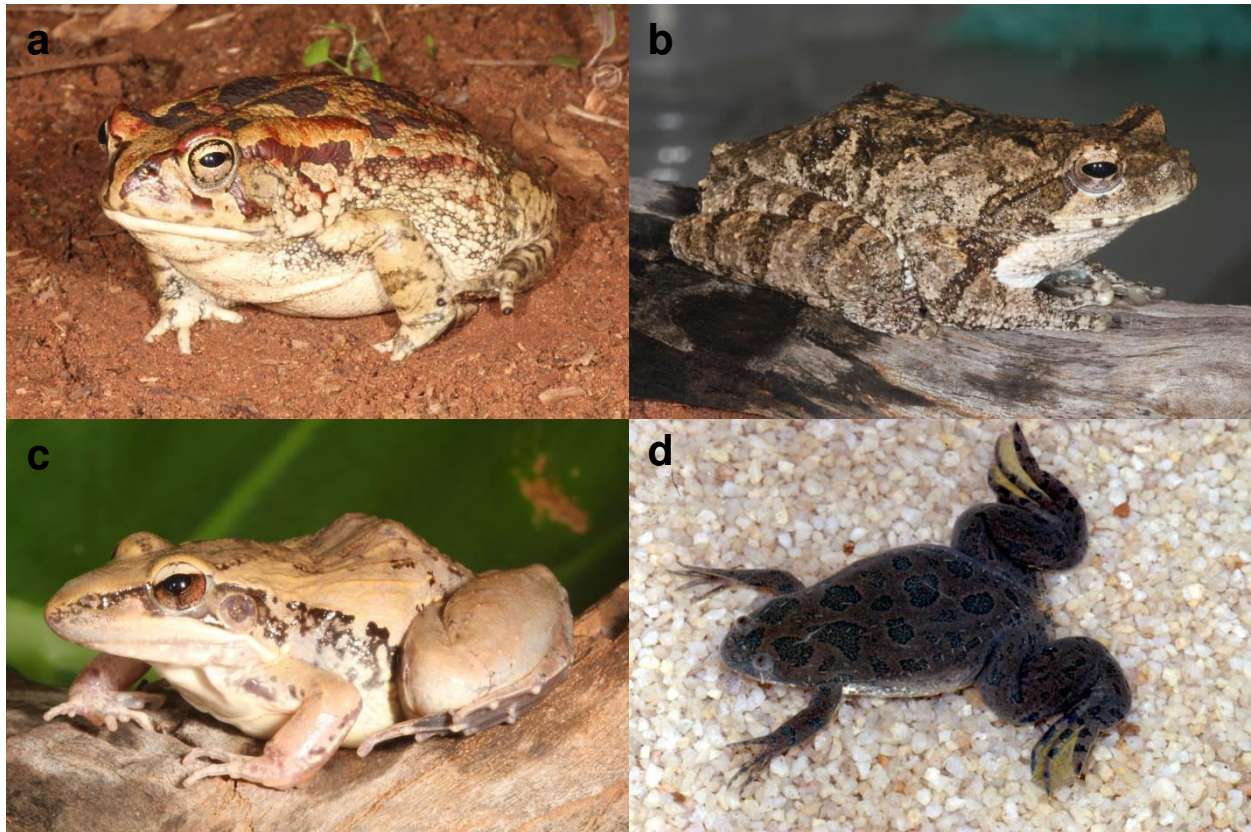


Figure 2.2: The frog species selected as target organisms for this study as described above being (a) *Amietophrynus garmani*, (b) *Chiromantis xerampelina*, (c) *Ptychadaena anchietae*, (d) *Xenopus muelleri*. Photographs courtesy of Edward Netherlands (a-c) and Louis du Preez (d)

2.3 Field methods: Sample collection & handling

2.3.1 Biomarker response samples

For the purpose of biomarker response sample collection three separate surveys were conducted over a one-year period. April 2013 served as the first high flow survey, during which samples from all target species were collected for analysis. The second and third surveys ensued in November 2013 during the low flow period, and April 2014 as a follow up high flow survey, during which only *X. muelleri* samples were collected for analysis.

Frog collection was done through both active and passive collection. Active collection consisted of catching frogs by hand. As all of the target species are most active at night collection was thus also done at night using frog calls and a flashlight to locate frogs at the specific sites. This method of sampling is very effective, but is very dependent on weather conditions and the field experience level of the sampling team. The passive collection method consisted of placing traps at selected sites frequented by the target frog species. For aquatic species bucket traps or small fyke net traps were placed in water bodies at the sites and left overnight. The traps were baited with commercially bought chicken livers as *X. muelleri* is a predator/scavenger. This method proved fairly effective in collecting *X. muelleri*, but also in collecting catfish (*Clarias gariepinus*) and small terrapins (*Pelusios sinuatus*). This reduced the effectiveness of the method at some sites as both these organisms feed on *X. muelleri*. For terrestrial species pitfall traps set up with a drift fence made of industrial plastic sheeting were used. The drift fences were left in the field for the duration of the survey and checked on a daily basis. All organisms were then freed from the traps and target species were collected.

Upon collection frogs were placed in small plastic containers, with ventilation holes in the lid, containing some water to preference of the specific species. The animals were euthanized through double pithing as chemical euthanasia could possibly compromise the results of ecotoxicological analyses. Double pithing is done by cutting through the upper jaw of the frog behind the eyes with a strong pair of scissors and then destroying the spinal cord with a blunt needle (Amitrano & Tortora, 2012). The carcass' mass was recorded and dissection followed. The liver was removed, the gallbladder dissected out, the liver mass was recorded and the sample transferred into a labelled Eppendorf tube containing Hendrikson's buffer (40 mM tris-HCl, 10 nM 2-Mercaptoethanol, 1 mM 0.04% bovine serum albumin [BSA], 1 nM ethylene-diamine-tetraacetic acid [EDTA]) and then frozen in liquid N₂. A small hole (made using a needle) in the Eppendorf tube lid ensured that the tube did not burst or crack during the flash freezing process.

Muscle tissue from the right thigh was dissected out and the mass recorded, after which it was handled in the same manner as the liver samples. The samples were later transferred from liquid N₂ to a -80 °C freezer in the laboratory until analysis.

2.3.2 Organochlorine pesticide bioaccumulation samples

The remaining carcasses after the biomarker samples were removed were used for OCP analysis. Thus sample collection and handling was the same for both analysis types and biomarker response results can be compared to OCP analysis results per individual frog. After removal of the biomarker samples the remaining carcass was wrapped in aluminium foil and frozen at -20 °C until analysis.

2.3.3 Stable isotope samples

Samples for food web relationships using stable isotope analysis were collected during a separate survey in February 2014. During this survey samples representing 11 different food web components (Table 2.1) relating to *X. muelleri* were collected at sites inside and outside the reserve. Scoop sampling (Table 2.1) consisted of scooping sediment from various points along the edge of the water body with a 15 ml polypropylene tube, or picking up leaf litter by hand. Toothbrush collection (Table 2.1) consisted of using a clean toothbrush to brush biofilm from rocks and aquatic plants in undisturbed water. The biofilm was collected in marked 15 ml polypropylene tubes. Sweep net sampling (Table 2.1) consisted of using a 30 cm x 30 cm x 30 cm net with a 1 mm mesh size and sweeping repeatedly in different micro-habitats within a water body and sorting through the collected samples in a plastic tray filled with water. Individual organisms were then identified and collected using tweezers and transferred to marked 15 ml polypropylene tubes. Frog samples were collected in the same manner as described under heading 2.3.1. Muscle tissue was collected from frog carcasses and collected in 15 ml polypropylene tubes. After collection all tubes were frozen at -20 °C until analysis.

Table 2.1: Eleven Food web components collected for stable isotope analysis and the corresponding sampling methods used

Food web component	Sampling method
Sediment	Scooping
Leaf litter	Scooping
Biofilm	Toothbrush
Oligochaeta (aquatic worm)	Sweep net
Baetidae (aquatic worm)	Sweep net
Mollusca (aquatic snail)	Sweep net
Atyidae (aquatic shrimp)	Sweep net
Ghomphidae (aquatic insect)	Sweep net
Small fish	Sweep net
Tadpoles (<i>X. muelleri</i>)	Sweep net
Frogs (<i>X. muelleri</i> , <i>C. xerampelina</i> , <i>A. garmani</i> , <i>P. anchietae</i>)	Baited traps & night frogging

2.4 Laboratory methods

2.4.1 Biomarker analyses

All sample preparation was done on ice to keep the sample temperature below 4 °C. Three sample batches (A, B, & C) were prepared. Batch A was prepared by adding 0.05 g of liver tissue and 250 µl of Tris-Sucrose buffer (0.05 M Tris-HCl [pH 7.4], 0.2 M sucrose) to an Eppendorf tube. The sample was homogenised and centrifuged at 9,500 G for 10 minutes. The supernatant from this batch was used for AChE and MDA analysis. Batch B consisted of 0.05 g of liver tissue and 1,000 µl potassium phosphate buffer (PPB) (0.09 M, K₂HPO₄ + KH₂PO₄ [pH7.4]) once again added in to an Eppendorf tube. The sample was homogenised and centrifuged at 10,000 g for 30 minutes. This batch was used for CAT, SOD, CYP450 and PC analyses on. The third batch (C) was prepared by adding 0.2 g of muscle tissue and 400 µl of electron transport system (ETS) homogenising buffer (0.1 M Tris-HCl [pH 8.5], 0.2 % v/v Triton-X, 15 % w/v polyvinyl pyrrolidone, 153 µM MgSO₄) to an Eppendorf tube and homogenising the sample. This batch was used for all of the CEA analyses.

Due to the small size of frog livers some specimen samples were grouped together according to species and site providing a total of 52 samples with usable tissue sizes for laboratory use. In these groupings equal parts in mass from each frog liver in the grouping was used in order to assure that the responses measured would portray the group average. These groupings are accounted for in the combined statistical analysis of biomarker analysis results and OCP analysis results.

Acetylcholine esterase

Ellman *et al.* (1961) was used as basis for the AChE activity analysis method. Potassium phosphate buffer (210 µl), 10 µl *s*-acetylthiocholine iodide (30 mM), and 10 µl Ellman's reagent (10 mM 2,2-Dinitro-5,5-dithio-dibenzoic acid) was added to 24 of the wells (only the blank and seven samples were analysed per plate) in a 96 well microtitre plate. As Ellman's reagent is photosensitive the plate was covered in aluminium foil. The plate was incubated at 37 °C for five minutes after which 5 µl of the sample supernatant (PPB was used for blanks) was added to the wells and the absorbance at 405 nm was measured immediately using an automated microplate reader (BioTek ELx800). A kinetic read of six measurements with two minute intervals between reads was taken. Using the method of Bradford (1976) (method is explained under **Available energy reserves** later on in this chapter) protein content was determined separately.

Cytochrome P450

Cytochrome P450 activity analysis was done using the DetectX Demethylating P450 fluorescent activity kit (Arbor Assays). The sample, blank (Assay Buffer), or standard (95 µl) was added to a black flat bottom 96 well microtitre plate (Corning Costar 3694 plate), which was sealed and incubated at 37 °C for 15 minutes. Reconstituted nicotinamide adenine dinucleotide phosphate (NADPH; 5 µl) was then added where after the plate was resealed and again incubated at 37 °C for 30 minutes. Stop solution (5 µl) was then added to the wells to stop the P450 reaction. DetectX Formaldehyde Detection Reagent (25 µl) was then added and the contents of the wells mixed by lightly tapping the plate sides. The plate was then incubated for 30 minutes at 37 °C after which the fluorescent signal was read at 510 nm with excitation at 450 nm using an automated plate reader (BioTek FLx800).

Superoxide dismutase

The method used to determine SOD content was adapted from Greenwald (1989). The sample (4 µl), 242 µl Tris-HCl buffer (50 mM [pH 8.2]; 0.1 M Tris-HCl, 0.1 M diethylene-triamine-pentaacetic acid) and 4 µl of pyrogallol (24 mM in 10 mM HCl) was added to the wells of a 96 well microtitre plate to initiate the reaction. A kinetic absorbance read of seven readings over six minutes was done using an automated microplate reader. Using the method of Bradford (1976) the protein content was determined separately.

Catalase

The method from Cohen *et al.* (1970) was adapted for the analysis of CAT activity. Only the first 24 wells were used (Blank + seven samples in triplicate) in each plate due to the speed of the reaction. H₂O₂ (93 µl; 34%) was added to 10 µl of sample in each well and left to incubate for three minutes at room temperature. H₂SO₄ (19 µl; 96 %) was then added in order to stop the reaction, immediately followed by the addition of 130 µl KMnO₄ solution (2 mM) and the measurement of the absorbance at 490 nm using an automated microplate reader. Protein content was determined using the method of Bradford (1976).

Malondialdehyde

The methodology for determining MDA content as an indication of lipid peroxidation was adapted from Ohkawa *et al.* (1979) as it has been modified by Üner *et al.* (2005). Sample supernatant (25 µl) (the same volume of Tris-Sucrose buffer was used for the blanks), sodium dodecyl sulphate (50 µl; 8.1 % w/v), acetic acid (375 µl; 20 %), thiobarbituric acid (375 µl; 0.8 % w/v), and deionised water (175 µl) was added into an Eppendorf tube (3 ml tube required to fit volume) and incubated at 95 °C in a water bath for 30 minutes after which it was allowed to cool to room temperature. A further 250 µl deionised water was added along with 1,250 µl *n*-butanol:pyridine solution (15:1). Samples were vortexed and then centrifuged at 4,000 rpm for 10 minutes. The supernatant (245 µl) was added to the wells of a microtitre plate and the absorbance at 540 nm was measured using an automated microplate reader. The protein content was determined separately through the method of Bradford (1976).

Protein carbonyls

The methodology for PC content determination was adapted from Parves & Riasuddin (2005) as originally assayed by Levine *et al.* (1990) and modified by Floor & Wetzel (1998). Equal parts (500 µl) of the sample supernatant and 2,4-dinitrophenylhydrazine solution in 2 M HCl; 10 mM) was added into an Eppendorf tube and allowed to incubate for one hour at room temperature while being vortexed every 10-15 minutes. Trichloroacetic acid (500 µl; 6 % w/v) was added and the sample was centrifuged at 10,000 G for three minutes. The supernatant was discarded and the pellet washed thrice with ethanol:ethyl ether (1:1 v/v) standing for 10 minutes before centrifugation and discarding of the supernatant each time. Next 400 µl of guanidine hydrochloride (6 M in 50 % formic acid) was added and the sample left for 15 minutes at room temperature before being centrifuged at 16,000 G for five minutes. Supernatant (100 µl) was added to the wells of a microtitre plate and absorbance at 366 nm was measured using an automated microplate reader. Protein content was determined separately through the method of Bradford (1976).

Cellular energy allocation

The CEA analysis method used was adapted from De Coen & Janssen (1997) and De Coen & Janssen (2003). This method consists of the determination of protein content, lipid content, glucose content, and electron transport system activity. Batch C samples (100 µl) were diluted with a further 400 µl of ETS homogenising buffer and used for the available energy reserves assays, while another 100 µl of sample was diluted with 400 µl of deionised water and used for the energy consumption assay.

Available energy reserves

The Ea analysis consists of the combination three separate assays. For protein content determination the method of Bradford (1976) was used. For this method 5 µl of sample (deionised water was used for blank) along with 245 µl of Bradford reagent was added to the wells of a 96 well microtitre plate and left to incubate for five minutes at room temperature after which the absorbance was measured at 595 nm with an automated microplate reader. Glucose content analysis (representing the carbohydrate content) was done using the GOD-PAP 1 448 668 Roche glucose content test kit. For the standard CFAS 759 350 was used. The sample homogenate (2.5 µl) and 247.5 µl of the assay reagent was added to the microtitre plate wells and incubated at room temperature for 30 minutes. The absorbance was then measured at 540 nm using an automated microplate reader. The lipid content analysis method was adapted from Bligh & Dyer (1959). Chloroform (500 µl) was added to 250 µl of sample homogenate in an Eppendorf tube and vortexed. Next 500 µl of methanol and 250 µl of deionised water was then added and the sample once again vortexed, after which it was centrifuged for five minutes at 3,000 G at 4 °C. Afterwards 100 µl of the organic phase (chloroform was used for blank) and 500 µl of H₂SO₄ (96%) were added to a glass tube that was then covered with aluminium foil and incubated at 200 °C for 15 minutes. Deionised water (1 ml) was added and the samples were allowed to cool down. The sample (2.5 µl) was then added to the wells of a microtitre plate and the absorbance measured at 360 nm using an automated microplate reader.

Energy consumption

The Ec analysis consisted of an ETS activity assay. Samples were centrifuged at 3,000 G at 4 °C for 10 minutes. The supernatant (25 µl) was added to the wells of a microtitre plate along with 75 µl of buffered substrate solution (BSS) (0.3 % v/v Triton-X, 0.13 M Tris-HCl) and 25 µl of (NAD[P]H) solution (1.7 mM). The reaction was then initiated by adding 50 µl of *p*-iodonitro tetrazolium chloride (8 mM) and the absorbance was measured kinetically over five minutes (one minute intervals between reads) at 490 nm using an automated microplate reader.

Cellular energy allocation calculation

Using the combustion enthalpy of glycogen (17,500 mJ/mg), protein (24,000 mJ/mg), and lipids (36,500 mJ/mg) the Ea results were converted energy equivalents (De Coen & Jansen, 1997). The Ec results were converted by means of the stoichiometric relationship between formazan formation and oxygen consumption (1:2) inside the ETS. The oxygen consumption results were then converted into energy equivalents using the combustion enthalpy of O₂ (484 kJ/mol) (De Coen & Janssen, 1997). An energy budget was calculated using the equation: CEA = Ea – Ec with Ea being the sum of the protein, glucose and lipid energy equivalents and Ec being the ETS energy equivalents.

2.4.2 Organochlorine pesticide bioaccumulation

The chemical analyses for this study were performed at Hokkaido University, School for Veterinary Medicine in Japan. The method used for chemical residue analysis in this study was adapted from Yohannes *et al.* (2013).

For the last three surveys frog carcasses were cut into small pieces, 5 – 10 g of wet sample was weighed and mixed with excess anhydrous Na₂SO₄ for desiccation. Soxhlet extraction (160 ml acetone:hexane, 1:3 v/v; Extraction Temperature: 180 °C, Reduction interval: four minutes, Reduction pulse: three seconds, Extraction time two hours) was done on desiccated samples using a Gehardt Soxtherm. Polychlorinated biphenyl (PCB) #77 was used as surrogate marker. After extraction, lipid content was determined gravimetrically using 20 % of the sample volume. On the remaining 80 % Gel Permeation Chromatography (GPC) was done using hexane:dichloromethane (1:1 v/v) (WakoGel column) to separate excess lipids. Final clean-up was done using 6 g of 5 % deactivated (m/m; ddH₂O) Florosil tightly packed in a 40 cm column. Sample was eluted with 100 ml hexane:dichloromethane (3:7 v/v) and final extract was evaporated to dryness and reconstituted with 100 µl n-decane. Instrumental analysis was done using a Shimadzu GC-2014 Gas Chromatograph coupled with a micro Electron Capture Detector (µECD) according to the parameters described in Addendum Table A2 (GC-µECD; ENV-8MS, 0.25 mm ID, 0.25 µm, 30 m Cica column). Dr Ehrenstorfer pesticide mix 1037 containing 22 POPs pesticides (Addendum Table A3) was used as internal standard. Standard curve was calibrated from five concentrations between 10 µg/l and 500 µg/l (R² ranged between 0.997 and 0.999 for all analyses).

2.4.3 Stable Isotope analyses

Samples for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ SIA (approximately 1 g) were dried at 50 °C and ground to a fine powder using a sterile mortar and pestle. The powder was placed in 2 ml chloroform:methanol (2:1 v/v) solution for 12 hours at 4 °C (in darkness) for lipid removal. The sample was then centrifuged at 1,500 G for two minutes after which the supernatant of the organic solvent was carefully discarded. The sample was once again dried completely at 50 °C.

Using an IsoPrime100-vario MICRO cube (Jasco) initial samples from each food web component were weighed (using a Sartorius Cubis microbalance), wrapped in tin containers (Elemental Microanalysis, D1008, 8 x 5 mm), and analysed in triplicate at six different masses ranging from 0.8 – 2.0 mg in order to determine the optimal mass for analysis for each component. The mass with the lowest standard deviation per component group was then chosen and all samples were then analysed to determine the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ stable isotope ratios. Stable isotope analysis data is reported as $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, which refer to parts per thousand (‰) ratios relative to the standard (Pee Dee Belemnite in the case of C, and atmospheric N in the case of N) and is calculated through the equation: $\delta^{15}\text{N} = (R_{\text{sample}} / R_{\text{standard}}) - 1 \times 10^3$, (same equation for $\delta^{13}\text{C}$) with R referring to the ratio between the lesser abundant heavier isotope and more abundant lighter isotope (i.e. $^{15}\text{N} / ^{14}\text{N}$ & $^{13}\text{C} / ^{12}\text{C}$). Every seventh sample an L-alanine standard was run to ensure reading accuracy (total variation less than 0.02 ‰).

2.4.4 Statistical analysis

An analysis of variance (ANOVA) was performed for both the bioaccumulation data and biomarker response data in terms of seasonal, species and site (inside vs. outside) differences using GraphPad Prism 5. Firstly the D'Agostino & Pearson omnibus normality test was used to test whether the data sets conform to a Gaussian distribution, which in turn determined the type analysis performed. A one-way ANOVA along with Tukey's Multiple Comparison Post Hoc Test was performed if the distribution was normal, whilst The Kruskal-Wallis Test coupled with Dunn's Multiple-Comparison Post Hoc Test was performed for non-Gaussian distribution. Due to the pooling of liver samples that was required for the biomarker response analyses accurate statistical analysis of those results is not necessarily possible even though the amount of individual frogs collected and analysed were enough to be statistically valid. A Redundancy Analysis (RDA) was performed on *d* Log transformed data sets (as described specifically for multivariate analysis by Howel (2007)) combining OCP bioaccumulation and biomarker response analyses using Canoco 5. Nitrogen SIA data was used to calculate Tropic Positions (TP) though means of the following equation: $TP = ([\delta^{15}N_{comp} - \delta^{15}N_{ref}] / 2.8) + 1$, with $\delta^{15}N_{comp}$ referring to the $\delta^{15}N$ of the specific food web component and $\delta^{15}N_{ref}$ referring to the basal source (sediment in this case). The constant value (2.8) is the mean nitrogen enrichment difference between trophic levels (Jepsen & Winemiller, 2002).

3. Results

Data analysis reported on in this chapter deals with the assessment of OCPs and biomarker responses in four selected frog species collected in the Ndumo Game Reserve (high flow – April 2013), spatial assessment of OCPs and biomarker responses in *X. muelleri* collected from inside and outside the Ndumo Game Reserve (high flow – April 2013) and temporal assessment of OCPs and biomarker responses in *X. muelleri* collected from inside the Ndumo Game Reserve over consecutive flow periods (i.e. high flow – April 2013, low flow - November 2013, high flow – April 2014). All samples for stable isotope analyses were collected from inside and outside Ndumo Game Reserve during April 2014. To promote data comparison when considering the figures the specific sample sets were coded. These code sets consist of the species and survey date (flow period) sampled (Table 3.1).

Table 3.1: A list of the codes used to describe full data set names in Figures 3.1 - 3.6

Full name	Label code
<i>Amietophrynus garmani</i> 2013 high flow (April) inside Ndumo Game Reserve	<i>A. g. 13</i>
<i>Chiromantis xerampelina</i> 2013 high flow (April) inside Ndumo Game Reserve	<i>C. x. 13</i>
<i>Ptychadaena anchietae</i> 2013 high flow (April) inside Ndumo Game Reserve	<i>P. a. 13</i>
<i>Xenopus muelleri</i> 2013 high flow (April) inside Ndumo Game Reserve	<i>X. m. 13</i>
<i>Xenopus muelleri</i> 2013 high flow (April) outside Ndumo Game Reserve	<i>X. m. out 13</i>
<i>Xenopus muelleri</i> 2013 low flow (November) inside Ndumo Game Reserve	<i>X. m. 13 L</i>
<i>Xenopus muelleri</i> 2014 high flow (April) inside Ndumo Game Reserve	<i>X. m. 14</i>

3.1 Organochlorine pesticide bioaccumulation

Of the 22 OCPs (Addendum Table A2) analysed for in this study 12 were detected in amphibian carcasses from the lower Phongolo River floodplain. Bioaccumulation results (Table 3.2) are indicated as the mean concentrations as well as the standard error (SEM) and the full range for all detected OCPs. The body mass and lipid concentration of the carcasses are also provided. No correlation was found between body mass and total OCP concentrations. The 2014 high flow survey for *X. muelleri* had the highest total OCPs mean concentration as well as the highest maximum value, recorded as $8,689.5 \pm 2,037.1$ ng/g lipid and 21,399 ng/g lipid respectively. For the same sample set γ -HCH had the highest mean for any single compound detected at $6,349.8 \pm 1,803.1$ ng/g lipid. Low levels of δ -HCH (compared to γ -HCH) were detected only during the 2013 low flow survey in *X. muelleri*. Aldrin was also only detected during one survey (2014 high flow) in *X. muelleri*.

There were significant ($p < 0.05$) differences in species-specific bioaccumulation of OCPs $P. anchietae \leq A. garmani < X. muelleri \leq C. xerampelina$ (Figure 3.1a). For *X. muelleri* there was a significant increase in OCPs for 2013 high flow from inside the reserve to outside. A similar increase was seen between 2013 high flow and 2013 low flow with an even greater increase towards the 2014 high flow survey. The DDTs were the main contributing OCP group in the following sample sets: *A. garmani* 2013 high flow, *C. xerampelina* 2013 high flow, *X. muelleri* outside 2013 high flow, and *X. muelleri* 2013 low flow making up more than 58 % of the total OCPs. The HCHs were the main contributing OCP group for the remaining sample sets: *P. anchietae* 2013 high flow, *X. muelleri* 2013 high flow, and *X. muelleri* 2014 high flow making up more than 47 % of the total OCPs.

The composition of DDTs (Figure 3.1b) indicated slight differences in composition between *P. anchietae* and all the other species from the 2013 high flow survey. With *p,p*-DDT making up 81.37 % of the total DDTs detected in *P. anchietae* whereas *p,p*-DDT made up between 33 % and 39 % of the total DDTs detected in the other three species. This difference in composition for *P. anchietae* was largely due to a lower *p,p*-DDE: *p,p*-DDT ratio, however *P. anchietae* also showed slightly higher *o,p*-DDT percentage than other species of the same survey even though *o,p*-DDT still made up less than 10 % of the total DDTs for all species. For *X. muelleri* the ratios of DDT to its metabolites (DDD + DDE) between inside and outside the reserve were fairly similar, but the outside samples contained a higher percentage of *o,p*-DDT (29.20 % of total DDTs) while the inside samples contained mostly the *p,p*-DDT isomer with *o,p*-DDT making up only 2.89 % of the total DDTs.

The DDT metabolites for the inside samples also contained a higher percentage of *p,p*-DDD (11.45 % of total DDTs) whereas the outside samples consisted mainly of *p,p*-DDE (*p,p*-DDD for this sample set was only 2.56 % of total DDTs). *Xenopus muelleri* inside the reserve over consecutive surveys displayed a decrease in *p,p*-DDT over time from 2013 high flow through 2013 low flow towards 2014 high flow. The ratio of DDT metabolites were similar between 2013 low flow and 2014 high flow, but the DDT isomer composition changed from almost completely *p,p*-DDT (*o,p*-DDT was 0.62 % of total DDTs) towards consisting only of *o,p*-DDT for the 2014 high flow survey. The metabolite composition for this same time span showed an increase in *p,p*-DDD from 3.97 % in 2013 low flow to 39.84 % in 2014 high flow.

The total HCHs (Figure 3.1c) indicated γ -HCH as the main contributing HCH isomer making up more than 99 % of all HCHs found across species and surveys. The exception was for *P. anchietae* during the 2013 low flow survey where 91.50 % of the total HCHs were made up by γ -HCH with the rest being α -HCH. The residual <1 % in the other samples was made up by combinations of δ -HCH and α -HCH.

Table 3.2: Chemical analysis results for all sample sets (species + survey) showing the mean (ng/g lipid mass), standard error of the mean, range for body mass (g), lipid content (%), and all organochlorine pesticides detected. (for concentration in terms of wet mass refer to Addendum table A4) *ND = Not Detected (value below machine detection limit)

Survey	Inside 04/2013				Outside 04/2013	Inside 11/2013	Inside 04/2014
Species	<i>A. garmani</i>	<i>C. xerampelina</i>	<i>P. anchietae</i>	<i>X. muelleri</i>	<i>X. muelleri</i>	<i>X. muelleri</i>	<i>X. muelleri</i>
Sample size	n=11	n=10	n=9	n=8	n=3	n=13	n=13
Body mass	19.05	16.02	12.63	12.61	6.51	10.80	5.05
SEM	2.13	2.08	1.59	0.87	3.58	0.85	1.07
Range	(8.69 – 28.27)	(6.97 – 24.89)	(3.75 – 16.34)	(7.36 – 15.17)	(2.08 – 13.60)	(6.35 – 18.14)	(2.30 – 16.90)
Lipid content (mass % of body mass)	3.88	3.85	5.11	2.65	1.97	0.80	0.90
SEM	0.47	0.52	0.83	0.57	1.58	0.095	0.32
Range	(1.72 – 6.57)	(1.91 – 6.14)	(1.28 – 9.53)	(0.52 – 5.42)	(0.28 – 5.12)	(0.252 – 1.55)	(0.03 – 4.31)
α-HCH	0.09	ND	4.14	ND	2.6 x 10⁻³	ND	ND
SEM	0.09		1.58		2.6 x 10 ⁻³		
Range	(0 – 0.99)		(0 – 11.93)		(0 – 0.01)		
δ-HCH	ND	ND	ND	ND	ND	1.20	ND
SEM						1.08	
Range						(0 – 14.07)	
γ-HCH	12.74	6.89	44.45	86.62	977.2	198.7	6,349.7
SEM	3.66	1.56	18.95	31.37	781.4	27.15	1,803.1
Range	(0 – 33.84)	(1.37 – 16.17)	(8.71 – 180.74)	(16.06 – 271.3)	(85.59 – 2,534.6)	(38.37 – 345.5)	(236.8 – 19,364.1)
Aldrin	ND	ND	ND	ND	ND	ND	16.68
SEM							8.88
Range							(0 – 84.63)
trans-Heptachlor- epoxide	ND	ND	ND	13.48	57.99	ND	362.6
SEM				10.66	30.53		237.8
Range				(0 – 85.61)	(0 – 103.54)		(0 – 2,434.4)
cis-Chlordane	ND	ND	ND	ND	ND	20.15	280.8
SEM						12.45	238.4
Range						(0 – 153.1)	(0 – 3,095.1)
p,p-DDE	41.07	133.9	3.53	38.57	930.9	1,733.9	780.7
SEM	12.84	30.69	2.96	9.58	222.9	551.7	184.6
Range	(0.82 – 158.8)	(51.03 – 317.6)	(0 – 27.05)	(0 – 80.98)	(606.7 – 1,358.1)	(343.6 – 7,056.8)	(0 – 1,761.7)
o,p-DDT	3.38	5.01	0.57	2.26	430.5	12.85	229.8
SEM	1.56	1.28	0.32	1.97	338.8	4.53	178.3
Range	(0 – 17.3)	(0 – 14.31)	(0 – 2.22)	(0 – 15.91)	(13.2 – 1,101.6)	(0 – 43.24)	(0 – 2,274.5)
p,p-DDD	1.39	5.39	ND	10.05	37.88	81.91	669.2
SEM	1.17	1.76		10.05	6.92	28.37	555.9
Range	(0 – 12.89)	(0 – 20.07)		(0 – 80.40)	(24.76 – 48.24)	(0 – 254.9)	(0 – 7,219.0)

Table 3.2 (continued): Chemical analysis results for all sample sets (species + survey) showing the mean (ng/g lipid mass), standard error of the mean, range for body mass (g), lipid content (%), and all organochlorine pesticides detected. (for concentration in terms of wet mass refer to Addendum table A4) *ND = Not Detected (value below machine detection limit)

Survey	Inside 04/2013				Outside 04/2013	Inside 11/2013	Inside 04/2014
Species	<i>A. garmani</i>	<i>C. xerampelina</i>	<i>P. anchietae</i>	<i>X. muelleri</i>	<i>X. muelleri</i>	<i>X. muelleri</i>	<i>X. muelleri</i>
Sample size	n=11	n=10	n=9	n=8	n=3	n=13	n=13
p,p-DDT	22.82	92.89	4.31	32.51	74.72	233.5	ND
SEM	11.24	20.83	3.62	16.75	43.90	83.92	
Range	(0 – 123.2)	(48.04 – 271.0)	(0 – 32.83)	(0 – 138.4)	(0 – 152.0)	(0 – 878.2)	
ΣDDTs	68.66	237.2	8.40	83.40	1473.9	2,062.1	1,679.7
SEM	22.72	51.34	5.17	22.47	525.2	623.1	799.5
Range	(18.44 – 286.8)	(101.8 – 607.1)	(0 – 36.46)	(42.23 – 234.7)	(796.7 – 2,507.9)	(370.1 – 7,656.1)	(0 – 10,642.7)
ΣOCPs	81.49	244.1	56.99	183.5	2,509.2	2,282.1	8,689.5
SEM	22.17	50.80	17.49	55.67	1,330.4	623.9	2,037.1
Range	(35.99 – 293.7)	(109.9 – 609.9)	(8.71 – 180.7)	(72.84 – 528.2)	(882.3 – 5,146.0)	(408.4 – 7,859.7)	(465.9 – 21,399.0)

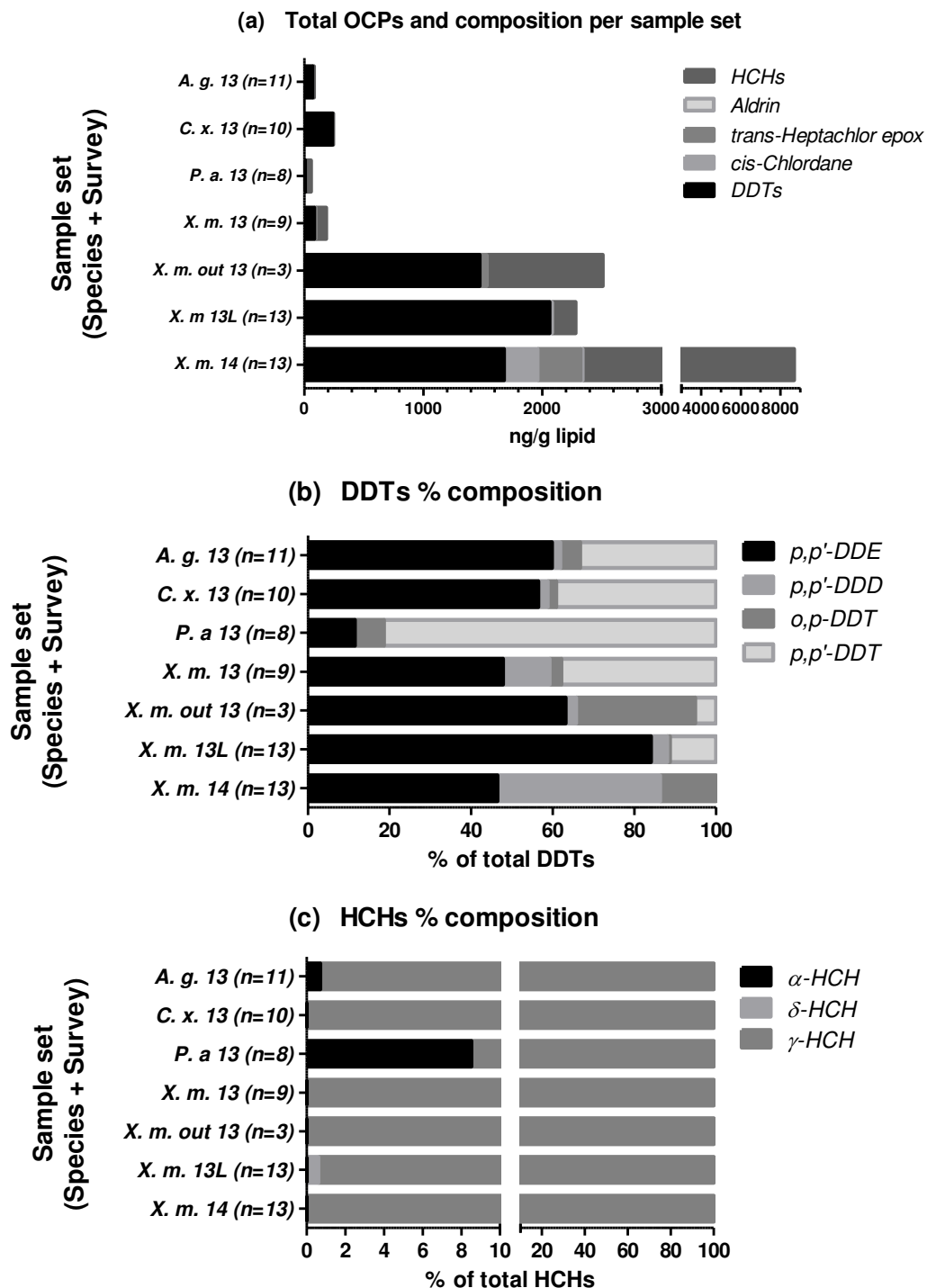


Figure 3.1: Bioaccumulation of the mean organochlorine pesticides (refer to Table 3.1 for description of label codes). Total organochlorine pesticides (a), percentage composition of dichlorodiphenyltrichloroethanes (b) and percentage composition of hexachlorocyclohexanes (c)

The temporal change observed in total OCP bioaccumulation for *X. muelleri* (Figure 3.2) indicated a statistically significant increase ($p < 0.05$) over the period of one year from April 2013 to April 2014. The variation in the OCP bioaccumulation also increased over time. When comparing the ratio between DDT and its metabolites, there were six cases in which the DDT concentration exceeded the sum of its metabolites for the 2013 high flow survey (Figure 3.3a). These samples were all found at mid-level exposure concentrations, however normality tests (D'Agostino & Pearson, & Shapiro-Wilk) revealed that the data do not conform to a Gaussian distribution. This is indicative of individuals exposed to recently applied DDT. No data points exceed the 1:1 ratio between DDT and its metabolites for the 2014 season (Figure 3.3b), indicating no recent exposure to DDT.

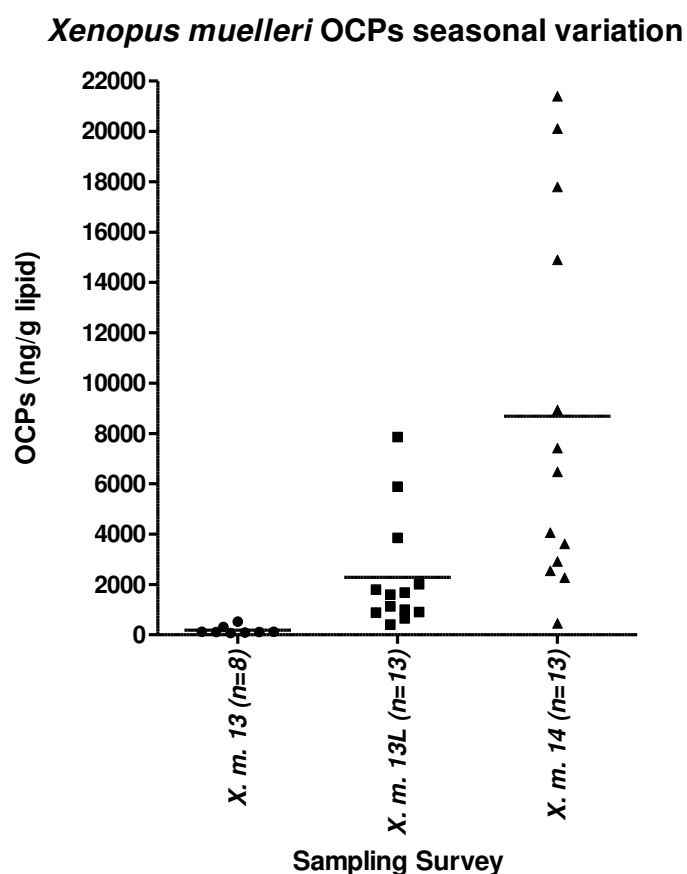


Figure 3.4: Total organochlorine pesticide bioaccumulation in *X. muelleri* from inside Ndumo Game Reserve from three surveys from April 2013 high flow to April 2014 high flow (refer to Table 3.1 for description of label codes)

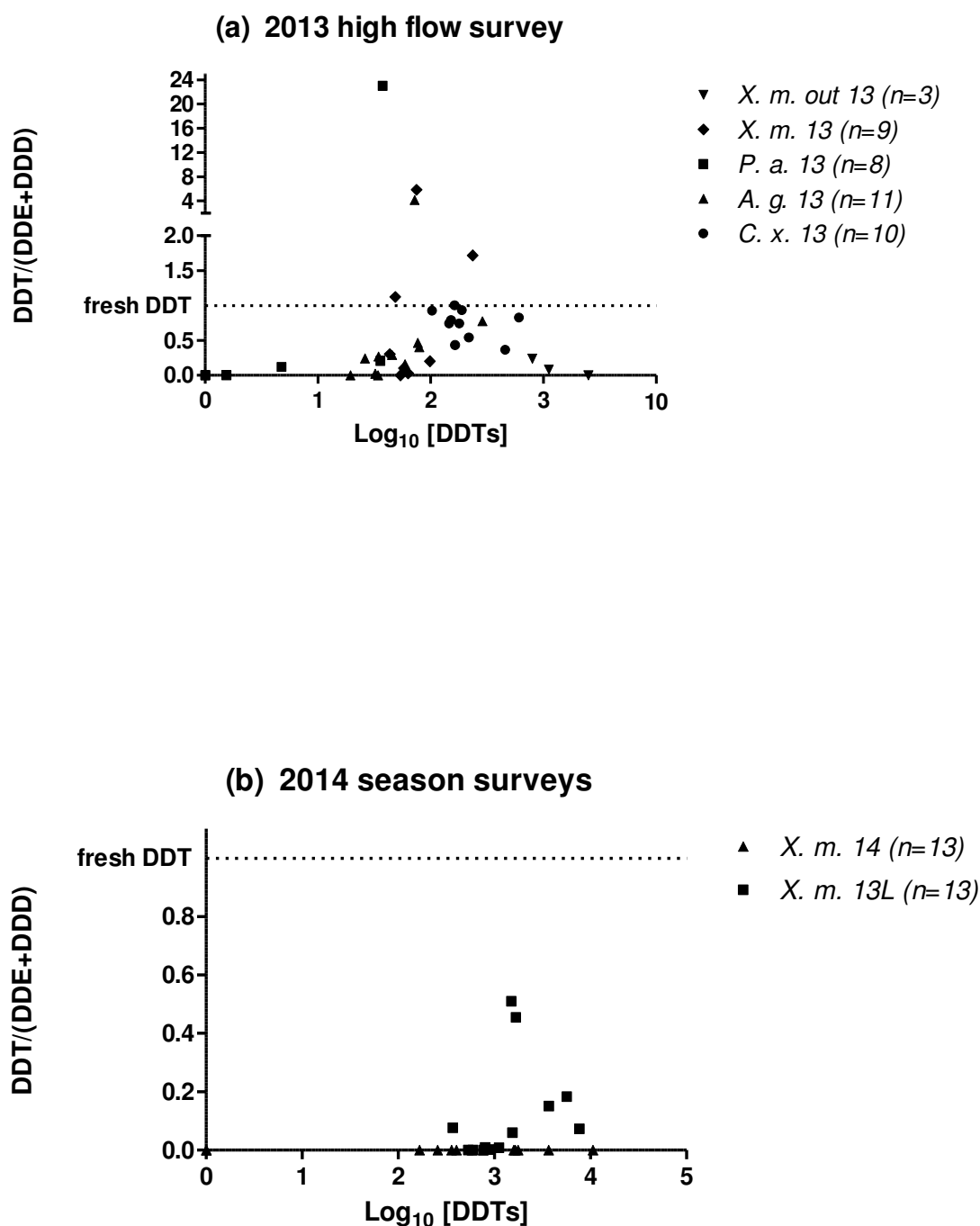


Figure 3.3: Recent dichlorodiphenyltrichloroethane use plots with the ratio between dichlorodiphenyltrichloroethane and its metabolites plotted against the Log_{10} dichlorodiphenyltrichloroethane concentrations for each sample set (refer to Table 3.1 for description of label codes). Data are split over different dichlorodiphenyltrichloroethane spraying seasons with (a) indicating the 2013 high flow survey data and (b) showing the 2014 season consisting of the 2013 low flow and the 2014 high flow surveys. The dotted lines at $y=1$ indicate that all samples above the line represent exposure to dichlorodiphenyltrichloroethane that was recently introduced into the environment (Strandberg & Hites, 2001). * Log_{10} of concentration was used for better distribution of data points

The ratio between hepato-somatic index (HSI) and γ -HCH exposure (Figure 3.4) showed an inhibition response through means of non-linear regression on a variable slope ($R^2 = 0.27$). The IC_{50} (\pm standard error) for this inhibition correlates to 539.2 ± 9.5 ng/g lipid of γ -HCH. Definite species differences in the HSI were observed only between *A. garmani* and *C. xerampelina* with all *C. xerampelina* samples having higher HSI values than those of *A. garmani* within a similar exposure range.

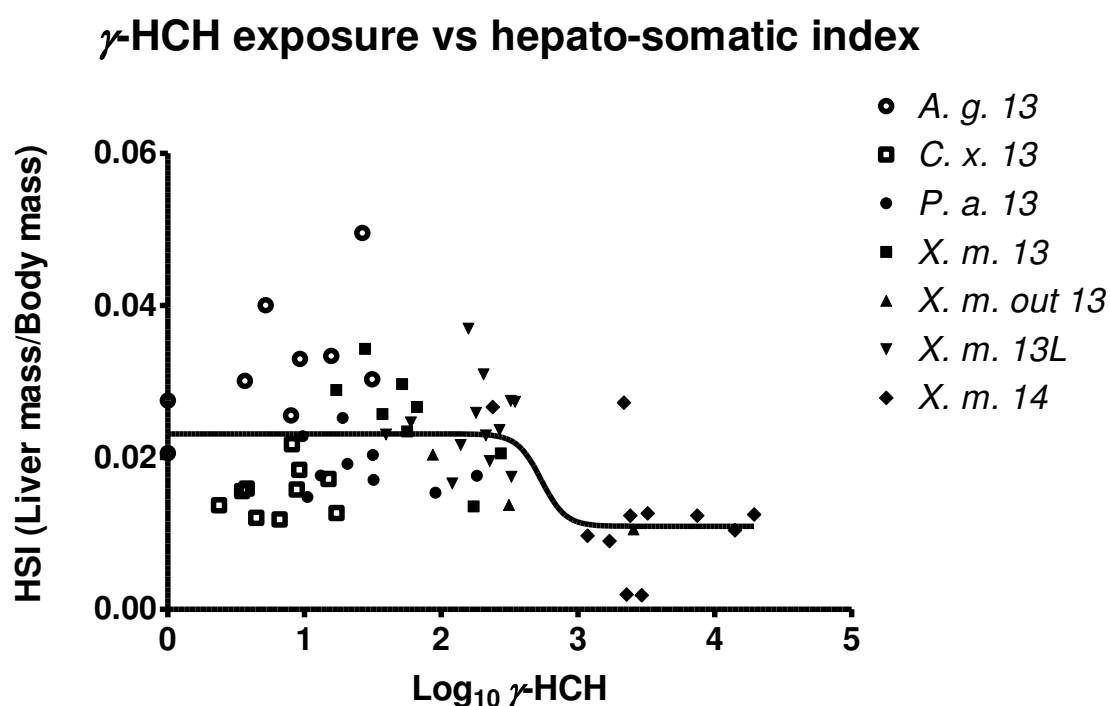


Figure 3.4: The Log₁₀ γ -hexachlorocyclohexane exposure concentrations of all sample sets plotted against the corresponding hepato-somatic index (HSI) (shown as fractions). A non-linear inhibitor vs. response regression line based on the total data is also plotted over the data points. (refer to Table 3.1 for description of label codes)

3.2 Biomarker responses

The results for AChE activity (Figure 3.5a) indicated no significant statistical difference between the species, or between inside and outside sites. A decrease in AChE activity was however noted for *X. muelleri* between the 2013 high flow (April) survey and 2013 low flow (November) survey. The following high flow survey (April 2014) activity showed closer resemblance to 2013 low flow than to the 2013 high flow survey. There were no significant statistical differences in CYP450 activity between comparable sample sets in terms of species differences, season, or inside and outside sites (Figure 3.5b), although an increase in CYP450 activity for *X. muelleri* was seen from the 2013 high flow survey to both the following surveys (2013 low & 2014 high). The mean CYP450 activity value for this species was also higher outside than inside Ndumo Game Reserve for the 2013 high flow survey.

Superoxide dismutase activity (Figure 3.5c) increased significantly from 2013 high flow to 2013 low flow as well as 2014 high flow for *X. muelleri*. Variation within the last two mentioned sample sets was very high, however the minimum activity level of both surveys were more than 10 fold the maximum of the 2013 high flow survey for *X. muelleri*. No significant species difference was noted. Catalase activity (Figure 3.5d) showed no significant statistical difference between species. A slight increase in CAT activity for *X. muelleri* was seen in both surveys following the 2013 high flow survey as well as outside the Reserve for the same survey similar to that of the CYP450 activity (Figure 3.5b).

Figure 3.5e displaying the PC content indicated a statistically significant decrease for *X. muelleri* between the 2013 high flow survey and 2013 low flow as well as the 2014 high flow surveys. There was no significant statistical difference between other data sets in terms of site (inside vs. outside) or species. Malondialdehyde content (Figure 3.5f) indicated the same statistically significant decrease for *X. muelleri* as the PC content (Figure 3.5e) for the surveys following the 2013 high flow survey. No other statistically significant differences were displayed between species or inside and outside sites.

The cellular energy allocation results for the available energy in the form of carbohydrates (Figure 3.6a) illustrated that there was statistical significant differences between the different species for the same survey (2013 high flow). *Amietophrynus garmani* had higher carbohydrate energy reserves than all other species, whilst *P. anchietae* had significantly lower reserves than all other species. There was however no significant difference between the carbohydrate energy reserves of *X. muelleri* and *C. xerampelina*. Furthermore *X. muelleri* showed a statistically significant decrease in carbohydrate energy reserves between 2013 high flow and 2013 low flow. Inside and outside samples showed no statistical difference.

Protein energy reserves (Figure 3.6b) indicated a significant decrease from 2013 high flow towards both 2013 low flow and 2014 high flow surveys for *X. muelleri*. No statistically significant differences were noted for species or inside and outside sites. Results for lipid energy reserves (Figure 3.6c) showed a significant increase for *X. muelleri* from 2013 high flow to 2013 low flow. Other comparable results showed no statistically significant differences in terms of either species or inside and outside samples, but 2014 high flow did display a notable increase from 2013 high flow levels.

When available energy reserves from the three sources described above were combined (Figure 3.6d) the only statistically significant difference between comparable sample sets was an increase for *X. muelleri* from 2013 high flow to 2013 low flow. The mean available energy for this species decreased again towards the 2014 high flow survey, but large variation in values voids this decrease of statistical significance. It was notable that even though the total energy reserves did not vary much between comparable sample sets, there was a definite change in the composition of these reserves (Figure 3.6a-c) for *X. muelleri* taking place between 2013 high flow and 2013 low flow surveys. No notable differences were observed between inside and outside, or between species.

The energy consumption indicated that *A. garmani* had significantly higher energy consumption levels than two other species for the 2013 high flow survey, namely *C. xerampelina* and *P. anchietae* (Figure 3.6e). No other statistically significant differences between comparable sample sets were shown for either seasonal change or inside and outside sites. The total CEA results (Figure 3.6f) showed no significant differences between species, sites (inside vs. outside), or season. A slight increase in the mean value for *X. muelleri* between 2013 high flow and 2013 low flow surveys was however noted.

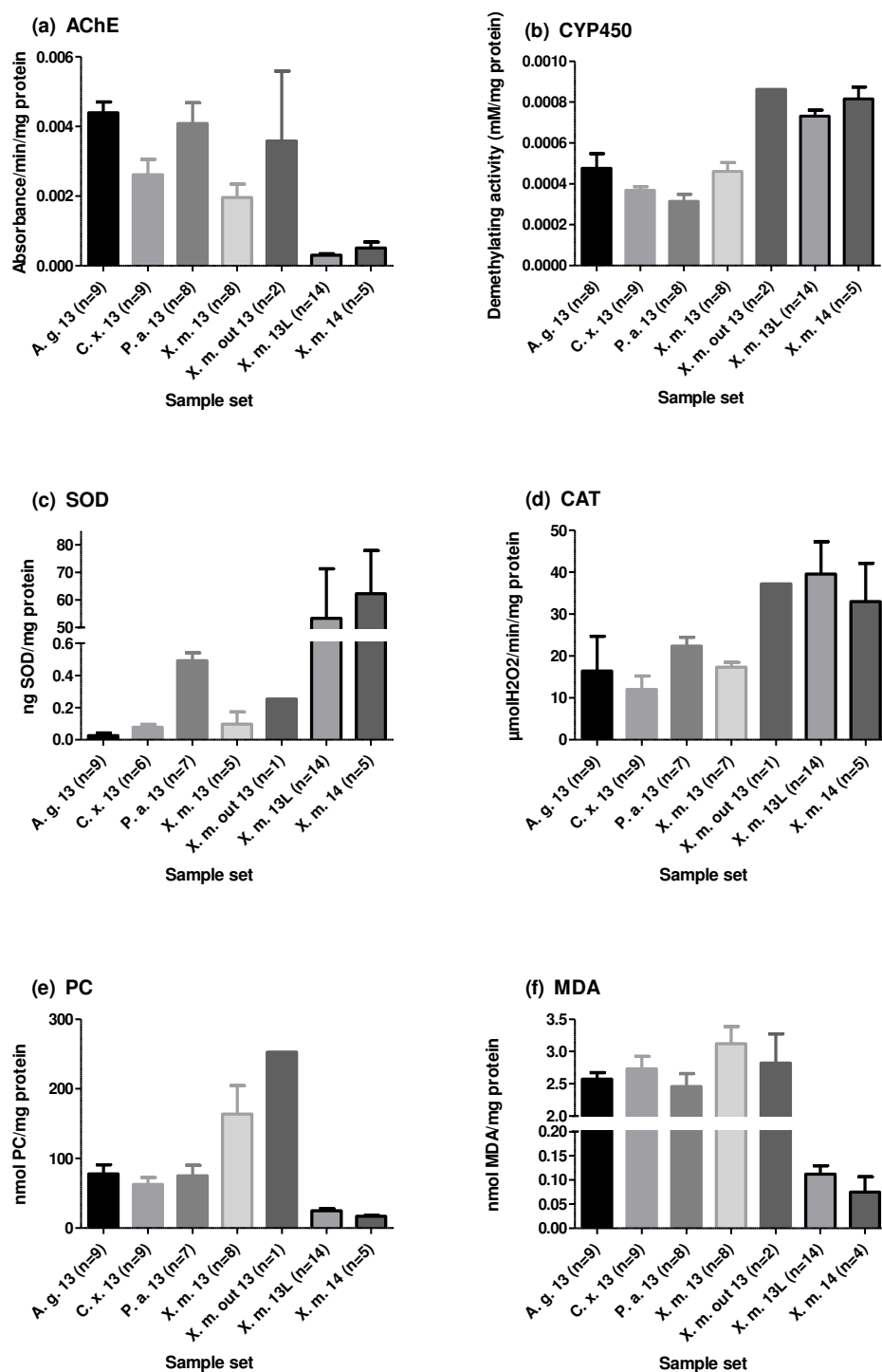


Figure 3.5: Biomarkers of exposure and oxidative stress for sample sets consisting of the species and survey (refer to Table 3.1 for description of label codes). (a) Acetylcholine esterase activity and (b) cytochrome p450 demethylating activity as biomarkers of exposure. (c) Superoxide dismutase activity and (d) catalase activity as oxidative stress response indicators, while (e) protein carbonyls, and (f) malondialdehyde content as oxidative damage indicators

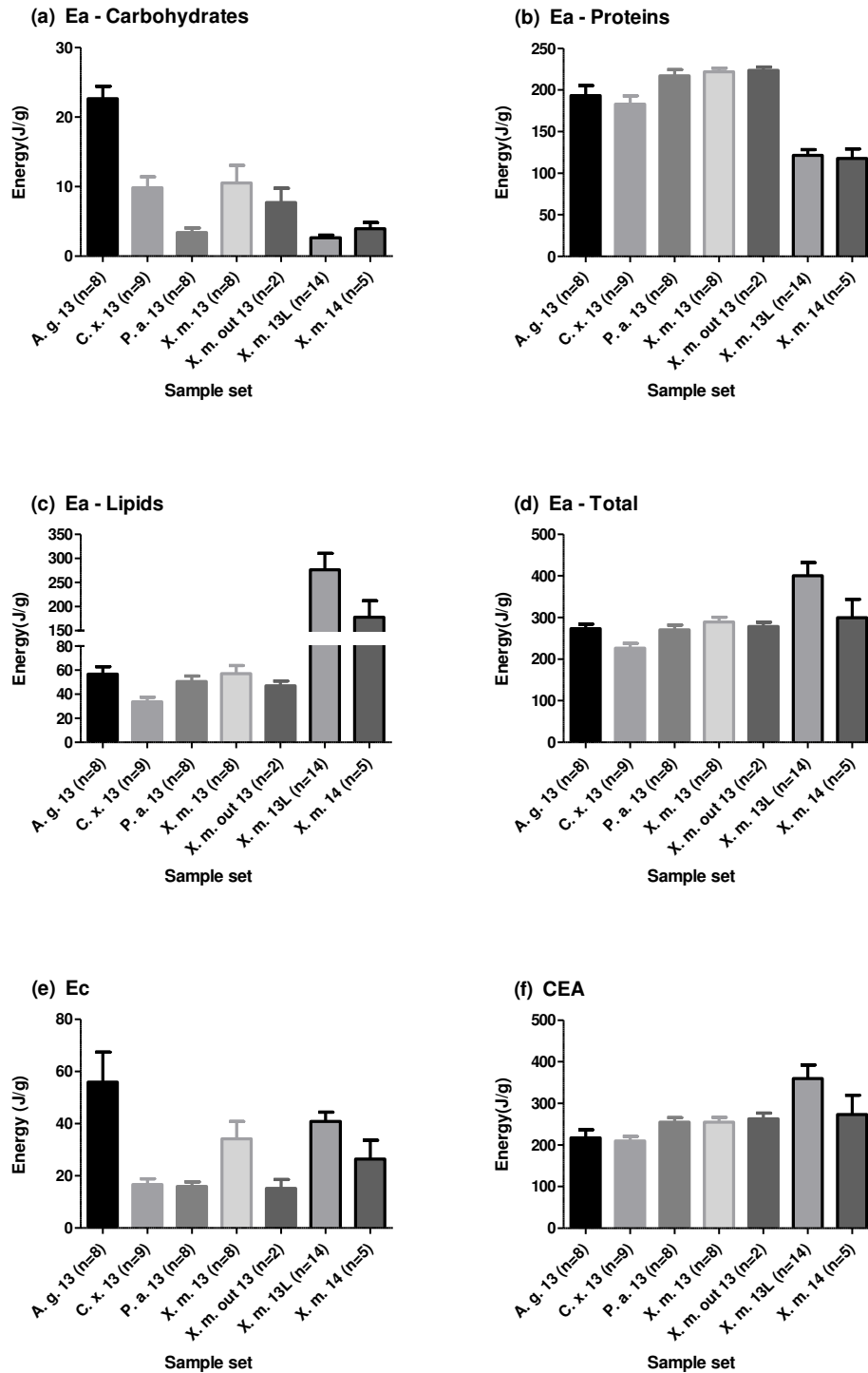


Figure 3.6: Cellular energy allocation per sample set consisting of species and survey (refer to Table 3.1 for description of label codes). Energy reserves of carbohydrates (a) proteins (b) and lipids (c). The total available energy (d) is the sum of values from (a)-(c) and (e) indicates the energy consumption in terms of the electron transport system activity. The total cellular energy allocation (f), which is determined by subtracting the values of (e) from those of (d)

3.3 Relationship between OCP bioaccumulation and biomarker responses

The following section reports on the correlations between OCP bioaccumulation and biomarker responses measured for all sample sets through means of redundancy analysis (RDA). Table 3.3 provides labels used in Figure 3.7.

Table 3.3: Functional names and corresponding labels used to describe data in the redundancy analysis results shown in Figure 3.7

Full name	Label
Sample sets	
<i>Amietophrynus garmani</i> 2013 high flow (April) inside Ndumo Game Reserve	<i>A13H</i>
<i>Chiromantis xerampelina</i> 2013 high flow (April) inside Ndumo Game Reserve	<i>C13H</i>
<i>Ptychadaena anchietae</i> 2013 high flow (April) inside Ndumo Game Reserve	<i>P13H</i>
<i>Xenopus muelleri</i> 2013 high flow (April) inside Ndumo Game Reserve	<i>X13H</i>
<i>Xenopus muelleri</i> 2013 high flow (April) outside Ndumo Game Reserve	<i>Xo13H</i>
<i>Xenopus muelleri</i> 2013 low flow (November) inside Ndumo Game Reserve	<i>X13L</i>
<i>Xenopus muelleri</i> 2014 high flow (April) inside Ndumo Game Reserve	<i>X14H</i>
OCPs	
Aldrin	Aldrin
α -HCH	α -HCH
δ -HCH	δ -HCH
γ -HCH	γ -HCH
<i>cis</i> -Chlordane	<i>cis</i> -Chl _r
<i>trans</i> -Heptachlor-epoxide	<i>trans</i> -Hept-epox
<i>o,p</i> -DDT	<i>o,p</i> -DDT
<i>p,p'</i> -DDT	<i>p,p'</i> -DDT
<i>p,p'</i> -DDD	<i>p,p'</i> -DDD
<i>p,p'</i> -DDE	<i>p,p'</i> -DDE
Biomarker Responses	
AChE activity	<i>AChE</i>
CYP450 demethylating activity	<i>CYP450</i>
SOD activity	<i>SOD</i>
CAT activity	<i>CAT</i>
PC content	<i>PC</i>
MDA content	<i>MDA</i>
CEA	<i>CEA</i>
Ec	<i>Ec</i>
Ea	<i>Ea</i>
Ea-Proteins	<i>Prot</i>
Ea-Carbohydrates	<i>Carb</i>
Ea-Lipids	<i>Lipids</i>

The RDA performed on the complete data set indicated strong correlations between OCP levels and biomarker responses. Analysis was performed with normalised values as described in Section 2.4.4. In Figure 3.7 axis one (eigen value: 0.18) and two (eigen value: 0.04) were plotted accounting for 22.14 % of the total variation.

Superoxide dismutase and CYP450 activities (Figure 3.7) showed weak positive correlations of similar strength to γ -HCH levels while both had almost zero correlation to one another. *trans*-Heptachlor-epoxide indicated relatively strong negative correlation to CYP450. Available energy in terms of lipids and SOD showed strong positive correlation to one another. Catalase and SOD displayed very similar tendencies, but SOD showed almost double the increase probability. Weak positive correlations were indicated between Ec and both *p,p*-DDD and *trans*-heptachlor-epoxide, whilst PC showed a strong correlation towards *p,p*-DDT. There was strong positive correlation shown for AChE, MDA, Ea- carbohydrates, and Ea-proteins with one another as well as with *p,p*-DDT, Aldrin, δ -HCH and *cis*-chlordane. Strong negative correlation was visible between this group and SOD, CAT and Ea-lipids, as well as *p,p*-DDD and γ -HCH although these compounds did not have such direct negative relations to the AChE containing group as SOD, CAT and Ea-lipids. There were no clear groupings visible within samples pertaining to specific species, or between *X. muelleri* samples pertaining to locality (inside vs outside).

3.4 Stable isotope analysis

To identify the food web structure components described in Figures 3.8 and 3.9 the labels given in Table 3.4 are used.

Table 3.4: The different food web components and their corresponding labels used in Figures 3.7 and 3.8 for identification of components in the food web structure biplots

Food web component	Label
<i>Xenopus muelleri</i>	<i>X. m.</i>
<i>Ptychadaena anchietae</i>	<i>P. a.</i>
<i>Amietophrynus garmani</i>	<i>A. g.</i>
<i>Chiromantis xerampelina</i>	<i>C. x.</i>
<i>Xenopus muelleri</i> tadpoles	<i>X. m. T</i>
Small Fish	F
Atyidae	At
Gomphidae	G
Oligochaeta	O
Baetidae	Ba
Molluscs	Mol
Leaf litter	L
Biofilm	Bio
Sediment	Sed

In Figure 3.8 the SIA results from inside the reserve showed clear distinctions between the primary sources and invertebrates with *X. muelleri* clearly depicted as the apex predator of the represented food web. Other frog species did not have as high nitrogen enrichment as *X. muelleri*, but carbon sources were similar. Biofilm and leaf litter were displayed as the primary food sources for all invertebrates, except molluscs for which the carbon source correlated to sediment. *Xenopus muelleri* tadpoles had similar nitrogen enrichment to Atyidae, but the tadpoles' carbon sources seemed to be more closely correlated to leaf litter whilst Atyidae correlated more closely to biofilm as primary carbon source.

When the food web structure outside the reserve (Figure 3.9) was compared to inside clear differences could be seen. The sediment carbon source shifted even further away from the rest of the food web and all primary sources had higher nitrogen enrichment than for the food web inside the reserve. There was no longer clear distinction between trophic groups and carbon levels of higher trophic position organisms did not correlate to the same primary sources, especially in the case of molluscs.

The tadpoles of *X. muelleri* still correlated to leaf litter as primary source, but nitrogen enrichment was higher than inside the reserve. Small fish collected showed fairly similar N and C levels to invertebrates in both food webs. *Xenopus muelleri* was still depicted as the apex predator, but the distinction between it and other food web components was smaller outside the reserve.

Trophic positions (TP) revealed higher TP values for primary sources, specifically biofilm (1.0 TP value increase), outside the reserve (Table 3.5). The variance in invertebrate TP values was higher outside the reserve due to lower values for molluscs and Baetidae while all other invertebrates showed higher values outside. The fish collected outside also had a higher TP value as well as *X. muelleri* tadpoles. *Xenopus muelleri* adults however had a lower TP value outside the reserve. For both inside and outside, *X. muelleri* was close to four trophic positions above the basal source (sediment in both cases).

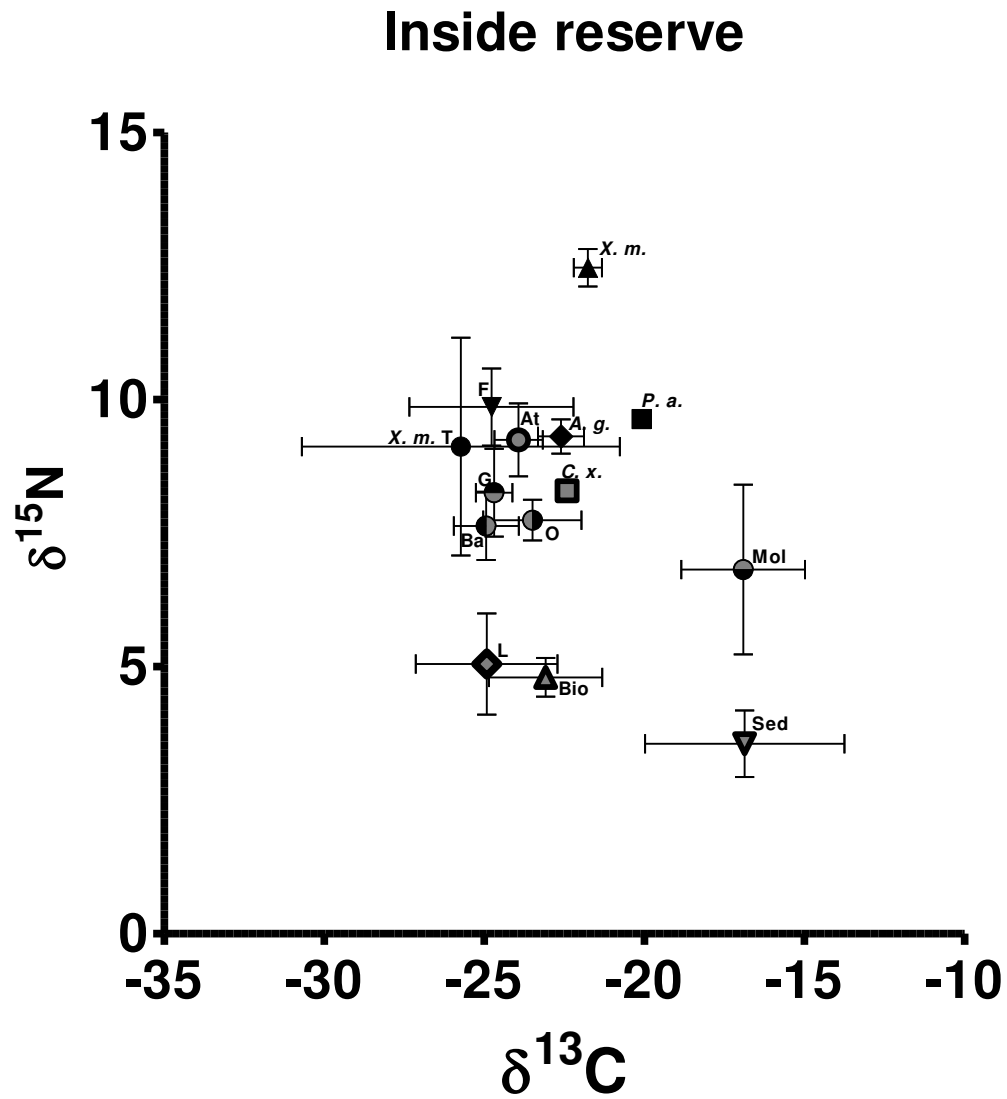


Figure 3.8: Stable isotope analysis biplot of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ isotope ratios for the food web components corresponding to *X. muelleri* from inside Ndumo Game Reserve. Data plots (mean \pm standard error of the mean) are a composition of all sites sampled within the reserve. (refer to Table 3.4 for label descriptions)

Outside reserve

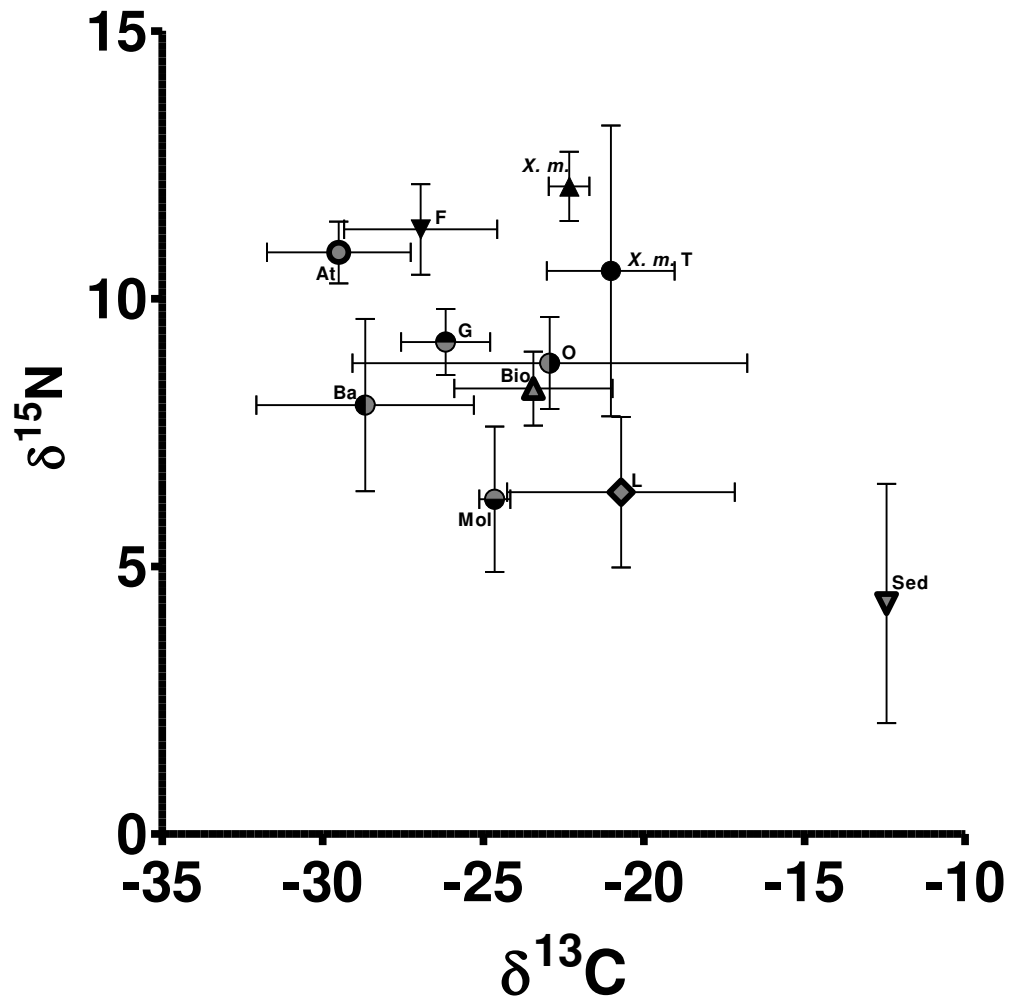


Figure 3.9: Stable isotope analysis biplot of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ isotope ratios for the food web components corresponding to *X. muelleri* from outside Ndumo Game Reserve. Data plots (mean \pm standard error of the mean) are a composition of all sites sampled outside the reserve. (refer to Table 3.4 for label descriptions)

Table 3.5: The mean Trophic Positions of all food web components analysed, organised according to trophic groups

Food web component	mean Trophic Position	
	Inside	Outside
<i>Amphibians</i>		
<i>Xenopus muelleri</i>	4.2	3.8
<i>Ptychadaena anchietae</i>	3.2	n/a
<i>Amietophrynus garmani</i>	3.1	n/a
<i>Chiromantis xerampelina</i>	2.7	n/a
<i>Xenopus muelleri</i> tadpoles	3.0	3.2
<i>Fish</i>		
small Fish (Barbs inside, <i>Tilapia sp.</i> outside)	3.3	3.5
<i>Invertebrates</i>		
Atyidae	3.0	3.3
Gomphidae	2.7	2.7
Oligochaeta	2.5	2.6
Baetidae	2.5	2.3
Molluscs (aquatic)	2.2	1.7
<i>Primary sources</i>		
Leaf litter (in contact with water body)	1.5	1.7
Biofilm (from rocks or reeds in water body)	1.4	2.4
Sediment (composite sample from water body)	1	1

4. Discussion

The results discussed in this chapter are related to the Ndumo Game Reserve and surrounding lower Phongolo River floodplain. Given the fact that only environmental samples were analysed in this study, chemical analysis and biomarker response results cannot be fully interpreted individually and should be considered as a whole in order to fully understand the state of the study area. Stable isotope analysis provides supporting data to be compared to literature in combination with chemical accumulation results. There are currently no international guidelines available for OCP tissue levels in amphibians, with literature mostly only reporting toxicity exposure studies and not bioaccumulation levels (Addendum Table A6), creating a challenge in terms of interpretation of the data. It is acknowledged that external exposure (i.e. LC_{50}) is not equivalent to internal bioaccumulation but for comparative purposes in this dissertation the internal levels (i.e. bioaccumulated OCPs) are related to the LC_{50} toxicity concentrations of the respective OCPs. Literature on biomarker responses in frogs is also scarce resulting in unanswered questions with regards to some of the possible accumulation-response reactions observed in this study.

4.1 Organochlorine pesticide bioaccumulation

When interpreting the chemical analysis results it is important to note that contaminants can interact with one another when an organism is exposed to a mixture (Newman, 2010; Haschek *et al.*, 2013). These interactions are mostly unknown and can result in either increased or decreased toxicity. Many other factors such as the metabolic rate or pathway followed within the target organism can affect the storage of persistent chemicals in the body. The ten contaminants detected in the frog tissue make up four contaminant groups namely DDTs, HCHs, chlordane, heptachlor and aldrin (Table 3.2). Although heptachlor itself is a natural derivative of chlordane (IPCS, 2006) it was considered as its own compound group for the purpose of this discussion.

Aldrin

Aldrin was only detected in one survey (2014 high flow) in *X. muelleri*, and at very low levels in comparison to all other compounds detected in that particular survey (Table 3.2). The maximum recorded level of aldrin was 0.55 ng/g wet mass (Addendum Table A4). The lowest toxicity concentrations measured in literature on amphibians (Addendum Table A6) yielded 50% mortality at 150 ng/g exposure after 96 hours of exposure (Sanders, 1970). With the exposure medium being almost 300 fold the maximum accumulated concentration in this study it is highly unlikely that the aldrin concentration in the environment causes any immediate threat to amphibians from this region. With regards to the dynamics of aldrin in this environment it is possible that flooding could have re-introduced traces of aldrin from upstream sediment. This is the most likely scenario as the accumulation levels are too low to indicate fresh introduction of high concentrations as would be expected from agricultural introduction of aldrin.

Mean aldrin concentrations of 0.09 ng/g lipid have been reported in *C. xerampelina* from the Kruger National Park, South Africa (Farquharson, unpublished data), compared to 16.68 ng/g lipid in *X. muelleri* from the current study. Edwards *et al.* (2015) sampled various fish species from the Phongolo River floodplain between November 2012 and September of 2013, and aldrin was not detected in any samples. This supports the fact that low levels of aldrin were somehow made bio-available between September and November of 2013, and that aldrin concentrations in this system are not cause for concern at this time.

Chlordane

Only the *cis*- isomer of chlordane was detected (Table 3.2) and this only in *X. muelleri* samples for the 2013 low flow and 2014 high flow surveys. Accumulation over time seems to increase and initial detection in low flow survey samples make re-introduction of dormant chlordane from sediment highly unlikely. In toxicity studies by Khan *et al.* (1979) the bioaccumulation factor of *cis*-chlordane in *X. laevis* was 108. A biological half-life of 3.3 weeks was seen with maximum accumulation of 0.207 ng/g after 96h exposure to 5 ppb (Addendum Table A6). Levels detected in the current study have maximum values close to 1 ppb (wet mass) (Addendum Table A4). Edwards *et al.* (2015) detected only the *trans*- isomer of chlordane in fish samples from the Phongolo River between November 2012 and September 2013. This indicates that chlordane might undergo some form of bio-transformation changing the isomer form when being transferred up to higher trophic levels in the food web, although Edwards *et al.* (2015) detected *trans*-chlordane in all surveys whilst, *cis*-chlordane was only detected during November 2013 and April 2014 in the current study.

Trans-chlordane accumulation in Tiger fish (*Hydrocynus vittatus*), an apex predator in the Phongolo River, was highest in September of 2013 at 6.95 ng/g lipid (Edwards *et al.*, 2015) compared to the mean accumulation of *cis*-chlordane in *X. muelleri* for 2013 low flow and 2014 high flow at 20.15 and 280.8 ng/g lipid respectively. The levels of *cis*-chlordane in this environment do not appear to be a cause for concern to wellbeing of the amphibian population, and although fresh introduction seems likely, the biological half-life determined by Khan *et al.* (1979) makes it difficult to determine exactly when and at what initial concentration chlordane was introduced.

Heptachlor

With regards to heptachlor only one metabolite, *trans*-heptachlor-epoxide was detected in only three samples indicating that exposure to heptachlor most likely did not occur recently (Table 3.2). Sources on the soil half-life of heptachlor vary extensively from less than one week up to three and a half years depending on the conditions (IPCS, 2006; ATSDR, 2007), but 120 days is generally accepted (Addendum Table A5). Higher order organisms can readily metabolise heptachlor into heptachlor-epoxide through oxidation (IPCS, 2006). From the results obtained in this study (Table 3.2) it seems likely that the target organisms were not exposed to freshly introduced heptachlor prior to the 2013 high flow sampling. How recent exposure was is difficult to determine considering the area's climate, as high temperature speeds up, but high humidity slows down heptachlor breakdown in the environment (ATSDR, 2007).

Heptachlor has been reported to completely break down to its epoxide form after three months in soil (ATSDR, 2007). The fact that *trans*-heptachlor-epoxide was not detected during the 2013 low flow survey, but again detected in the 2014 high flow survey at higher concentrations than the previous year might indicate dormant heptachlor in upstream sediment being re-introduced when washed downstream by floodwaters. Another possibility is that new heptachlor was introduced to the system in the few months between the last two surveys.

No guideline accumulation levels for *trans*-heptachlor epoxide exist for amphibians, but the maximum accumulated concentration is more than 10 fold lower than reported LD₅₀ levels in rats (IPCS, 2006). Toads exposed to heptachlor (Sanders, 1970) showed LC₅₀ levels of 0.44 ng/g after 96 hours of exposure (Addendum Table A6). The maximum accumulation level in the current study in terms of wet mass was 2.07 ng/g (Addendum Table A4). Farquharson (unpublished data) reports 1.9 ng/g lipid *trans*-heptachlor-epoxide bioaccumulation in *C. xerampelina* in a composite data set of 2010 – 2012 from the Kruger National Park, compared to 362.6 ng/g lipid in *X. muelleri* in April 2014 from the current study.

Heptachlor or any of its metabolites were not detected in any fish samples from the study by Edwards *et al.* (2015) in the same area over a similar sampling period. This might indicate the ability of higher level organisms to metabolize and excrete heptachlor and its derivatives completely. These results once again do not indicate *trans*-heptachlor-epoxide as holding any immediate threat to the amphibian population from the lower Phongolo River floodplain, although levels indicate that amphibians from the lower Phongolo River floodplain are subjected to higher levels of *trans*-heptachlor-epoxide than those from the Kruger National Park.

Hexachlorocyclohexanes

Hexachlorocyclohexane tissue residues were made up almost completely by γ -HCH (Figure 3.1c), which is also the most toxic isomer of HCH (ATSDR, 2005). The significant increase in accumulation towards 2014 high flow suggests fresh input of the compound into the aquatic system. The trace amounts of other isomers can be explained through the grade of HCH being introduced into the system (Itawa *et al.*, 1995; Strandberg & Hites, 2001). Technical HCH used for crop spraying can contain more than 8 % α -HCH and other isomers (ATSDR, 2005), while technical lindane contains almost 100 % γ -HCH (Itawa *et al.*, 1995). The increased exposure outside the reserve could possibly be due to active use by small subsistence farmers in the area. The site outside the Game Reserve was surrounded by village houses and small crop fields. As the use of lindane (γ -HCH) for agricultural purposes is banned internationally after the Stockholm Convention on POPs in 2001 (Ritter *et al.*, 1995), the increase in levels observed in this study is cause for concern.

The relationship between γ -HCH bioaccumulation and decreased HSI values as shown in Figure 3.4 indicates that accumulation of a high (but still sub-lethal) concentration of γ -HCH might cause a lower HSI value, which can most probably be attributed to liver atrophy. Liver atrophy due to γ -HCH exposure was reported in the Banded Gourami *Colisa fasciatus* (now *Trichogaster fasciatus*) by Verma *et al.* (1975). Fagotti *et al.* (2005) measured 2.38×10^{-3} ng/g wet mass of γ -HCH in *Rana esculenta* (now *Pelophylax esculentus*) from central Italy compared to 6.59 ng/g wet mass mean concentration in *X. muelleri* for the 2014 high flow survey of this study (Addendum Table A4). The HCH bioaccumulation in several frog species from the Kruger National Park between 2010 and 2012 found no γ -HCH in any frog species, although low levels (below 2 ng/g lipid) of other isomers were indeed detected (Farquharson, unpublished data).

Fish samples from the Phongolo River system sampled between November 2012 and September 2013 by Edwards *et al.* (2015) did not show γ -HCH as the dominant isomer, with dominance varying between species and surveys. *Clarias gariepinus* (Sharptooth catfish, a known predator of *X. muelleri*) showed the highest γ -HCH bioaccumulation and also most similar HCH composition to *X. muelleri* with a mean γ -HCH concentration of 150 ng/g lipid in April 2013 (Edwards *et al.*, 2015) vs. 86.62 ng/g lipid in *X. muelleri* from the current study for the same season. No γ -HCH was however reported in *C. gariepinus* for September 2013, which is interesting considering the increase in γ -HCH seen in *X. muelleri* in the current study between April 2013 and November 2013. Toxicity test results on amphibians vary greatly between sources, most likely due to species differences. The lowest reported LC₅₀ was for *Rana limnocharis* (now *Fejervarya limnocharis*), with the LC₅₀ being 0.94 ng/g after 48 hours exposure (Addendum Table A6; Pan & liang, 1993). Marchal-Segault & Ramade (1981) indicated reduced time to metamorphosis in *X. laevis* tadpoles exposed to 2 mg/l of γ -HCH. The 2014 high flow survey samples had a mean concentration of 6.59 ppb in terms of wet mass in *X. muelleri* (Addendum Table A4). If *X. muelleri* and *X. laevis* are considered to have similar reactions then the HCH bioaccumulation levels in adult frogs from this study are 300 times below the lowest exposure in tadpoles reported to have observable effects. Thus based on observable effects the HCH concentrations in this system are also considered to not pose any immediate threat to the amphibian population, although seasonal variation should be investigated further.

Dichlorodiphenyltrichloroethanes

Dichlorodiphenyltrichloroethanes (DDTs) were detected in all sample sets and contributed the largest or second largest percentage of total OCP bioaccumulation in all samples (Figure 3.1a). Only the metabolites of *p,p*-DDT were detected in tissue (Figure 3.1b). Based on its chemical structure the *o,p*-DDT isomer is a less stable molecule and should degrade more quickly in the environment (McMurry, 2008) and its metabolites are also more readily excreted (IPCS, 1979). Technical DDT used for indoor residual spraying consists of about 75 % *p,p*-DDT with the rest made up by *o,p*-DDT with traces of metabolites from both isomers (Yang *et al.*, 2005). *o,p*-DDT has lower direct toxicity, but has slight estrogenic effects while *p,p*-DDE has been reported to act as an anti-androgen (Haschek *et al.*, 2013).

Environmental OCP accumulation results for *X. muelleri* from outside the reserve indicate a much higher percentage of *o,p*-DDT than any other sample sets (Figure 3.1b). This can possibly be explained by this site being closer to the original contamination area (not necessarily a single site) as there would be less transfer time meaning less time for environmental breakdown of *o,p*-DDT which then leads to higher *o,p*-DDT uptake by organisms at the sampling site. This could also indicate Dicofol contamination, which contains higher percentages of *o,p*-DDT (Qui *et al.*, 2005). It is important to note that this does not necessarily indicate that the outside *Xenopus* community was exposed to DDT more recently, as shown by Figure 3.3a. How recently DDT was introduced into the environment is related more closely to the ratio between DDT and its metabolites as described by Strandberg & Hites (2001). Aguilar (2011) also indicated degradation of DDT in marine mammals over time using this ratio. The recent DDT plots in Figure 3.3 indicate that some frogs from the 2013 high flow season were recently exposed to fresh DDT input whilst the 2013 low flow and 2014 high flow seasons showed no recent exposure. This result is confusing when compared to bioaccumulation levels for the different surveys as one would not expect such a great increase in DDT concentration in organism tissue if there was no recent exposure. However the increase in bioaccumulation is coupled with a shift in DDT composition with the percentage of *p,p*-DDE increasing significantly towards the 2013 low flow survey (Figure 3.1a & b). The *p,p*-DDE is the most persistent form of DDT (Haschek *et al.*, 2013) leading to the possibility that the increase was due to DDTs already in the environment becoming bioavailable due to a change in environmental condition.

All detected total DDTs are well below the reported no observed effects concentration (NOEC) for *p,p*-DDT in *Rana temporaria* tadpoles of 110,000.0 ng/g lipid (Cooke, 1979). The highest concentration of *p,p*-DDE in this study in terms of wet mass was 73.35 ng/g in a *X. muelleri* sample from the 2013 low flow survey (Addendum Table A4) which is around 100 times lower than the LC₅₀ in *Rana temporaria* adults (Addendum Table A6; Harri *et al.*, 1979). Sanders (1970) however determined a 96h LC₅₀ in *Bufo woodhousii fowleri* (now *Anaxyrus woodhousii*) tadpoles exposed to 0.03 mg/l of DDT (Addendum Table A6). An analysis of HSI vs DDT accumulation similar to that of γ -HCH in Figure 3.4 did not yield the same negative relationship. Lincer *et al.* (1981) measured environmental accumulation levels to the equivalent of 70 ng/g in a dry weight sample of *P. mascariensis* (a relative of *P. anchietae*, also present in the research area of this study) in lakes from the Rift Valley in Kenya, where DDT was actively used for agricultural purposes at the time. The *p,p*-DDE levels measured in the 2013 low flow and 2014 high flow surveys in *X. muelleri* are similar to that found in Pacific chorus frogs (*Psudacris regilla*) from the Nevada Mountains in the United States of America (Smalling *et al.*, 2013). Smalling and co-authors (2013) refer to the similarity between their results and those measured in 1990 in the same region by Fellers *et al.* (2004). Although different frogs were used for these two studies (Fellers *et al.*, 2004 used the Yellow-legged frog *Rana muscosa*) the lack of degradation of *p,p*-DDE over time provided evidence to the persistence of this specific DDT metabolite in amphibians. This is of concern considering the increase in DDE accumulation seen in *X. muelleri* this study over the period of one year.

Other literature results however show much lower accumulation levels. Fagotti *et al.* (2005) detected 0.02 ng/g wet mass of *p,p*-DDT in the Italian *Rana esculanta* (now *Pelophylax ridibundus*). Total DDTs in the eggs of *Rana limnocharis* (now *Fejervarya limnocharis*) from the South of China had a maximum concentration of 1,930 ng/g lipid (Wu *et al.*, 2011). De Solla *et al.* (2002) detected *p,p*-DDT at 83.3 ng/g lipid and *p,p*-DDE at 333.3 ng/g lipid in the eggs of *Rana aurora* from the Sumas Prairie in British Columbia, Canada. Farquharson (unpublished data) reported total DDTs in *A. garmani* and *C. xerampelina* of 0.9 and 1.48 ng/g lipid respectively from composite data for 2010 to 2012 in the Kruger National Park. In the current study *A. garmani* and *C. xerampelina* had total DDT concentrations of 68.66 and 237.2 ng/g lipid respectively for the 2013 high flow survey (Table 3.2).

Xenopus muelleri from the 2013 low flow survey of this study had mean and maximum total DDTs accumulation of 16.06 and 79.59 ng/g wet mass respectively (Addendum Table A6). Through means of trophic accumulation it is expected that fish such as *C. gariepinus* or *H. vittatus* from the Phongolo River floodplain would show similar, if not higher bioaccumulation levels than those measured in *X. muelleri* in this study. McHugh *et al.* (2011) measured total DDTs of 5,537.41 ng/g lipid (mean value) in *H. vittatus* from the Pongolapoort Dam upstream of the floodplain in July of 2009, while Edwards *et al.* (2015) reported total DDT accumulation of 319 and 420 ng/g lipid in *C. gariepinus* and *H. vittatus* respectively for April 2013, with 626 and 966.7 ng/g lipid respectively for September 2013 from the lower Phongolo River floodplain. Yohannes *et al.* (2013) measured DDT levels in fish from the Ethiopian Rift valley where DDT is actively used for both malaria vector control and agriculture. Their results show mean and maximum total DDTs measurements of 9.35 and 30.84 ng/g wet mass respectively in *C. gariepinus*. Compared to mean concentrations of 83.40 and 2,062.13 ng/g lipid total DDTs in *X. muelleri* for the 2013 high flow (April) and 2013 low flow (November) surveys of this study respectively, it is clear that DDT levels in the environment increased over time, but also that the accumulation of DDT in the aquatic food web does not increase to the same extent over the same time period. The comparison between the results of Yohannes *et al.* (2013) and this study it is also clear that aquatic organisms from the lower Phonoglo River floodplain are exposed to higher levels of DDT than other areas with active DDT use. The DDT levels from this system are also markedly higher than those measured in the same species in Kruger National Park in 2010 – 2012 (Farquharson, unpublished data). The dynamics of DDTs in this system are of some concern considering the fluctuation of accumulation seems to be greater in lower level organisms such as amphibians.

Total organochlorine pesticides

Some extent of total OCP bioaccumulation was expected for all target organisms due to active malaria vector control and the type of agriculture practiced in the catchment. The variation in bioaccumulation between species was expected to follow a trend from aquatic through to terrestrial, however, this was not the case. Although significant differences were found between species (Figure 3.1), these differences do not show any tendencies correlating to the aquatic preference of the species. The octanol/water partition coefficient (Log K_{ow}) values for all OCPs (Addendum Table A5) indicate a greater affinity to bind to lipids or sediments than the water column. If this logic is followed one would then expect frog habitat preference relating to contact with sediments to determine the accumulation difference between species.

The results from this study do however not fully support this theory as the expected result would be *X. muelleri* ≥ *A. garmani* ≥ *P. anchietae* ≥ *C. xerampelina* based on habitat preference and behavioural traits taken from Du Preez & Carruthers (2009). The results from this study show a total OCPs accumulation gradient of *C. xerampelina* ≥ *X. muelleri* ≥ *A. garmani* ≥ *P. anchietae* (Table 3.2). In breeding season OCP bioaccumulation in *C. xerampelina*'s would be expected to increase due to its behavioural change as they sit in water leading to increased water contact. Although *C. xerampelina* still live in trees they do move to the water intermittently and during the breeding season they spend a lot more time in water than usual (Taylor, 1971). If this increase is significant enough the expected bioaccumulation order might change to resemble the gradient seen in the results from this study. It is however uncertain to what extent *C. xerampelina*'s bioaccumulation might be affected during the breeding season.

The samples from the 2013 high flow survey were taken at the end of *C. xerampelina*'s breeding season as well as being after the DDT spraying season. Dichlorodiphenyltrichloroethanes were the major contaminant group in all *C. xerampelina* samples. This could well indicate sediment contact as the main reason for accumulation differences measured between species, although bioavailability is an important factor that could greatly influence these results. Chemical analysis comparisons between frog tissue and sediment samples would be necessary to research this theory in full.

Gilliland *et al.* (2001) analysed OCPs in adult Green frogs (*Rana clamitans*; now *Lithobates clamitans*) from several populations in south-western Michigan. Chemical residues of total DDTs, *cis*-chlordane and, total HCHs measured 1.24; 0.01; and 0.12 ng/g wet mass respectively. The same compound groups measured 1.79; 0.0; and 1.34 ng/g wet mass for *X. muelleri* in the 2013 high flow survey (lowest total OCPs for *X. muelleri*) from the current study. Dichlorodiphenyltrichloroethane use has been banned in the U.S.A. since 1972 (Smalling, 2013) which speaks even further to the persistence of these compounds making the increase in total DDTs observed in *X. muelleri* in this study over the period of one year a cause for concern in terms of possible chronic effects.

Total OCP accumulation differed significantly between inside and outside Ndumo Game Reserve (Figure 3.1a). The site sampled outside the reserve has close proximity to a rural village with subsistence farming being practised all around the site. The site hosts a much larger water body than any of the inside sites, but has similar connection to the Phongolo River during low and high flow seasons. The results from this study indicate that proximity to anthropogenic activity does affect the OCP bioaccumulation in *X. muelleri* in this region.

Definite seasonal variation can be observed for both individual and total OCP levels (Figure 3.2). Over a one year period OCP concentrations in *X. muelleri* increased significantly. Even though the mean accumulation concentration of the 2013 low flow survey is not significantly different from either high flow surveys there is a clear tendency of increase in accumulation mean and range over time indicating that *X. muelleri* in this system are constantly exposed to OCPs from the analysed groups. If this increase tendency continues at the same rate shown in the results from this study, exposure levels of individual OCPs can increase above individual compound threshold levels in the near future. Bringing into account the mixed exposure effects of these pesticides, observable health effects might manifest well before those levels are reached. The application of OCPs for agricultural purposes is however not an annual activity, and there is the possibility of a rest period between exposure causing enough relief to the environment that tissue accumulation levels decrease again. It is however important to keep in mind the persistence of these contaminants in an aquatic environment and in aquatic organisms such as that of *p,p*-DDE in amphibians shown by Smalling *et al.* (2013) discussed in the previous section. DDT application for malaria vector control is an annual event in this region. The regulations under which DDT is applied are strict (DAFF, 2010), but as indicated by the results of this study there is still DDT input into the aquatic environment of the Phongolo River. This means that there is no rest period available for recovery increasing the chances of the annual increase tendency discussed above continuing at the same rate.

A comparison in terms of exposure mixtures and observed effects is not entirely possible as the specific sets of OCPs measured differ between the literature available, however, Edwards *et al.* (2015) measured the same set of OCPs in the same system, in higher trophic level organisms (fish). Although the results from this study and those reported by Edwards *et al.* (2015) show differences in the composition of OCPs with some compounds accumulating in fish, but not frogs, and *vice versa*, the general seasonal tendencies of total OCPs are very similar indicating an increase over time. This is cause for concern as the use of OCPs in this region are supposed to be limited to DDT only applied during the low flow season. The results from this study, supported by those reported by Edwards *et al.* (2015), however indicate an increase in total OCPs approaching the low flow season, with the increase continuing towards the following high flow season.

4.2 Biomarker responses

Biomarkers of exposure

The mode of action of OCPs results in an increase in ACh at nerve endings (Haschek *et al.*, 2013), but OCPs are not known to specifically activate or inhibit AChE activity. However if low-level exposure causes changes in ACh levels, a ripple effect may become noticeable in the AChE activity. The results from this study however, do not show any significant effects. A decrease in activity towards the 2013 low flow and 2014 high flow for *X. muelleri* is noted (Figure 3.5a). Although this decrease is not statistically significant this result compared to OCP accumulation results (Table 3.2) as well as other biomarker responses (Figures 3.5 & 3.6) still to be discussed indicates some level of inhibition that might be more prominent at higher accumulated concentrations. There is no decrease noted for the outside site, thus leading to the possibility that the decrease in the two previously mentioned surveys can either be the result of mixed exposure effects or be completely unrelated. As only OCP levels were measured the strong possibility exists that other known AChE inhibitors such as organophosphates (Haschek *et al.*, 2013) might have been present during the 2013 low flow and 2014 high flow surveys.

The CYP450 enzymes are expected to be activated by the presence of contaminants as it is part of the biotransformation detoxification mechanism (Garret & Grisham, 2010; Newman, 2010). Hexachlorocyclohexanes have been reported to increase CYP450 activity (ATSDR, 2005). The results from this study indicate a slight yet not statistically significant increase in CYP450 demethylating activity correlating to increased OCP accumulation in *X. muelleri* for the following surveys: 2013 high flow outside, 2013 low flow, and 2014 high flow (Figures 3.1 & 3.5b). Zaidi & Banerjee (1987) reported CYP450 reductase activity increase in rats after exposure to DDT at a single dose of 50 µg/g body weight, but with lower stimulation response observed at higher doses.

Biomarkers of effect

Oxidative stress is expected whenever an excess concentration of contaminant is present in the body of an organism due to increased metabolic rate combined with an increase in free cations in the body would increase the production of superoxides (Garret & Grisham, 2010). Following the physiological pathways the expected reaction would be indicated firstly by SOD activity, followed by CAT activity. If for some reason these enzymes cannot cope with the amount of superoxides produced the increase in superoxides would lead to oxidative damage in the formation of PC or MDA. The results from this study indicate a significant increase in oxidative stress in *X. muelleri* over the 2013 low flow, and 2014 high flow surveys (Figure 3.5c-f). This is shown through a significant increase in SOD activity, and a slight increase in CAT activity. The oxidative damage over these surveys is however significantly lower. A possible explanation for this observation is that the SOD specifically is somehow over-activated during these surveys. Banerjee *et al.* (1999) reported increased SOD and CAT activity in humans in response to lindane exposure. This could also be the result of mixed exposure effects. The chemical accumulation of the 2013 high flow outside sample set shows similar total levels of accumulation to the 2013 low flow survey, but with different compositions, and yet does not show the same SOD and oxidative damage tendencies. There is however slightly elevated oxidative damage compared to 2013 high flow inside samples and CAT levels are similar to the 2013 low flow and 2014 high flow surveys indicating the possibility that SOD activity might somehow have been inhibited in outside samples resulting in greater oxidative damage (specifically PC levels).

Cellular energy allocation indicates changes in energy storage and consumption. Changes in storage can be indicative of stress to the body resulting in dietary shifts or physiological changes increasing or decreasing energy storage of a specific type due to stressors within the body (De Coen & Janssen, 1997, Verslycke *et al.*, 2004). Organochlorine pesticides have not yet been shown to act as such stressors, however the results from this study indicate tendencies within the CEA that correlate with OCP accumulation levels and other previously described biomarker responses (Figure 3.6). *Xenopus muelleri* shows a shift in energy storage from proteins as major energy source to lipids as main energy source with a corresponding decrease in carbohydrate energy availability over the 2013 to 2014 season. There is a concomitant decrease in lipid mass for *X. muelleri* over the same period (Table 3.2).

These results indicate that the frogs were under dietary stress for the previously mentioned surveys, with the body increasing lipid storage in muscle for use as main energy source, while depleting overall lipid reserves in the process. Energy consumption however shows no significant changes over this period. As the changes observed correlate to increased OCP accumulation a possible reason for this shift could be due to exposure stress, however because the ETS activity (representing metabolic rate in this case) shows no significant change over the discussed period the reason behind the shift is most likely due to a change in the major food source. Such a change would also explain the increase in OCP accumulation if the new food source had higher accumulated levels than those previously consumed. As SIA analysis was only performed on a single survey sample set confirmation of a change in food source cannot be done in this manner. The reason behind such a change can include population shifts in lower level organisms that serve as food sources for amphibians, with either an influx of a previously less abundant species, or population decrease of a previously major food source (Begon *et al.*, 2006), but can unfortunately also not be confirmed with the information available from this study.

Combination of the biomarker results from this study for *X. muelleri* specifically shows a definite response to changes in environmental conditions with tendencies that relate to OCP accumulation levels. Mixed exposure effects seem to result in slight differences in the observed response, however these differences cannot with certainty be attributed to specific mixtures of OCPs and the possible influence of other unmeasured contaminants must be considered.

4.3 Relationship between OCP bioaccumulation and biomarker responses

The redundancy analysis performed (Figure 3.7) indicates that only a small portion of the variation in the results from this study can be explained through the combined analysis of biomarker responses and chemical analysis. The tendencies and correlations in the data set do however become more clearly visible when this analysis is performed. Definite oxidative stress responses can be seen correlating to almost all detected OCPs except *trans*-heptachlor-epoxide. Slight CYP450 activation by γ -HCH is shown. As this compound had the highest measured levels of all detected OCPs the results suggest that a more prominent CYP450 reaction might be expected at γ -HCH levels higher than those measured in this study, as well as the possibility of the other contaminants inducing a response at higher concentrations. Results also suggest that the shift in primary energy source correlates to some extent to the *p,p*-DDD levels measured. The strong correlations (both negative and positive) found between CAT, SOD, Ea-lipids, AChE, MDA, Ea-carbohydrates, Ea-proteins, *p,p*-DDT, *p,p*-DDD, aldrin, δ -HCH, and γ -HCH provide evidence that some extent of chemical bioaccumulation response is indicated by the biomarker responses of amphibians from this region. There is however a complexity towards the interactions of the OCP compounds these organisms are exposed to that makes individual accumulation-response reactions almost impossible to interpret accurately without performing laboratory based controlled exposures.

4.4 Stable Isotope analysis

Based on the SIA results for this study (Figures 3.8 & 3.9) there are significant differences in the food web structure between inside and outside Ndumo Game Reserve. Large variation is expected, as results are compositional from three sites inside and three sites outside the reserve. The inside sites show clearer distributions between the different levels of consumers and producers. The non-aquatic frogs do not show as high nitrogen enrichment as *X. muelleri*, but this is expected as they would most likely feed on other food sources that are not aquatic. *Xenopus muelleri*'s dietary change from tadpole to adults can be seen through a shift in carbon source between the two stages. The nitrogen enrichment between these two overlaps for the outside sites is most likely due to the fact that tadpoles from one site were at a later Gosner stage (Gosner, 1960) of development when sampled.

The main reason for a shift in food web structure as observed with the outside sites is most likely due to eutrophication of the water body causing an increase in the nitrogen enrichment of biofilm as primary food source and causing a chain reaction at higher trophic levels (Graening & Brown, 2003). This is supported by the TP value difference observed for biofilm between inside and outside the reserve (Table 3.5). Eutrophication could also affect the bioavailability of contaminants in the water (Newman, 2010). This introduces the possibility that exposure between inside and outside the reserve might not differ much, but that the bioavailability and thus bioaccumulation factors are different leading to the differences in accumulation observed. However even with the shift in food web structure the TP of *X. muelleri* is fairly similar between inside and outside indicating that no major dietary change has occurred or that the shift occurred recently before sampling and has not yet affected the apex predator to the same extent as other food web components.

5 Conclusion & Recommendations

5.1 Conclusion

Chemical bioaccumulation analysis of OCPs in several frog species from the lower Phongolo River floodplain was done in compliance with the first objective of this study: To determine OCP levels in frog tissue on a spatial and temporal scale to determine the influence of seasonal variation (i.e. different flow periods) and human activity on bioaccumulation levels. This analysis revealed the presence of aldrin, *cis*-chlordane, *trans*-heptachlor-epoxide, α -, δ - & γ -HCH, *o,p*- & *p,p*-DDT, *p,p*-DDD and *p,p*-DDE. Statistical analysis of chemical data for *X. muelleri* over three surveys covering a period of one year revealed a significant increase in both the mean concentration and variance between samples, along with significant changes in the OCP composition. Species differences were expected to follow a trend correlating to aquatic preference, but instead revealed a higher probability of sediment contact being the main contributing factor. *Xenopus muelleri* samples from outside Ndumo Game Reserve had significantly higher OCP levels with a different composition to those inside the reserve during the 2013 high flow survey. This indicates the possibility of different exposure sources relating to the proximity towards, and severity of, anthropogenic activity. The results also revealed a high probability of active γ -HCH introduction into the environment, which is cause for concern as it is an internationally banned substance with the only exception being for medical use. Bioaccumulation levels were markedly higher than those measured in similar frog species from the Kruger National Park between 2010 and 2012.

Biomarker response assays were performed for the objective of measuring biochemical responses of frogs to OCP bioaccumulation on a spatial and temporal scale to determine the influence of seasonal variation (i.e. different flow periods) and human activity. These assays revealed definite oxidative stress responses with changes in CYP450 and AChE following the same tendencies. A significant shift in CEA in terms of energy storage also conformed to the tendencies seen in the other assays. These responses were not correlated to the flow level of the river, and only slight differences between inside and outside sites were shown. No significant differences between species were observed.

To achieve the objective of determining if any relationships exist between chemical bioaccumulation and biomarker responses on a spatial and temporal scale, multivariate analysis was performed on the full data set which confirmed a complex set of correlations between bioaccumulation of the different OCPs and biomarker responses, most of which related to the oxidative stress biomarkers. The strength of correlations between OCPs and biomarker responses were not dependant on temporal or spatial factors. The objective to determine if there was a relationship between the frog species' water dependence and chemical bioaccumulation and concomitant biomarker responses was achieved by the use of both OCP bioaccumulation analysis and biomarker responses and the relationship between the two in terms of species differences. The water dependence of the different species were not indicated as the main contributing factor for observed species differences in either the OCP bioaccumulation- or biomarker response analyses.

The final objective—to collect stable isotope data for different food web components involved in the diet of *X. muelleri* to determine the trophic interactions of those components, stability of the food web structure. To investigate the possible connection between food web structure and OCP bioaccumulation—was determined using SIA to determine the food web structure and trophic positioning of the different components. The food web structure of inside and outside sites revealed nitrogen enrichment of the primary sources (biofilm in particular) outside Ndumo Game Reserve. This was attributed to the possibility of external nitrogen input from small scale agriculture around the outside sites. The nitrogen enrichment outside caused less distinct separations in trophic groups, but not to such an extent that the trophic position of the apex predator (*X. muelleri*) was affected leading to the conclusion that the food web structure and external effects thereon would not be likely to affect the OCP and biomarker response results in *X. muelleri* from outside the reserve.

None of the frogs analysed showed any physical deformities, and other than the biomarker responses seen only one effect was observed where high γ -HCH accumulation levels indicated slight liver atrophy. Based on these results it is clear that amphibians from the lower Phongolo River floodplain area are to some extent affected by environmental exposure to various OCPs, but these effects do not appear to be life threatening as of yet.

The severe increase in OCP levels observed in just one year combined with the known persistence of these compounds and the effects indicated at the current accumulated levels is however cause for concern in terms of future amphibian health in this area. Thus the hypothesis for this study stating that frogs from the lower Phongolo River floodplain area are exposed to potentially dangerous levels of DDT, its derivatives and other OCPs, is not rejected. The hypothesis statement that amphibians from this region exhibit biochemical (biomarker) responses towards changes in environmental condition is therefore not rejected. The hypothesis statement that these biomarker responses indicate biochemical stress to amphibians due to exposure to environmental contaminants is also not rejected.

5.2 Recommendations

The following factors are suggested to be included in future studies in order to confirm the tendencies observed in this study and better understand the dynamics of OCPs in amphibians of this region.

- Sampling two high flow and two low flow surveys for all species in order to determine if individual species have similar seasonal tendencies.
- Chemical analysis of OCP levels in *X. muelleri* from annual surveys over a 5 year period in order to track accumulation tendencies over a longer time period.
- A wider spectrum organic contaminant chemical analysis that includes organochlorine pesticides (OCPs), organophosphates (OPs), polychlorinated biphenyls (PCBs) polychlorinated dibenzo-dioxins and -furans (PCDD/Fs), pyrethroids, perfluorooctanesulfonic acid (PFOS) and polycyclic aromatic hydrocarbons (PAHs) of at least one survey combined with biomarker responses in order to confirm the accumulation-response correlations observed.
- Chemical analysis of at least one survey should include all component levels of the food web structure involving *X. muelleri* in order to determine chemical uptake from the basal source (sediment) and bio-magnification rates through the food web.
- Inclusion of adenosine triphosphatase (ATPase), *gamma*-aminobutyric acid (GABA), and glutathione-S-transferase (GST) assays in the biomarker response array as they could be more directly influenced by most OCPs' mode of toxicity.
- Laboratory based controlled exposure experimentation where OCPs are introduced individually and in different environmentally relevant mixtures in order to confirm biomarker response reactions observed.
- Such experiments should include micro-array ribonucleic acid (RNA) analysis in order to confirm gene expression caused by OCP exposures.
- Chemical analysis of soils and sediments from various sites in the Phongolo River floodplain coupled with bio-availability assays in order to pinpoint pollution sources that are of concern to the environment.

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Addendum

Table A1: A List of the chemicals and suppliers used in this study

Chemical	Supplier
2,2'-Dinitro-5,5'-dithio-dibenzoic acid	Sigma-Aldich
2,4-Dinitrophenylhydrazine	Sigma-Aldich
2-Mercaptoethanol	Sigma-Aldich
Acetic acid	Rochelle Chemicals
Acetone	Cica
Bovine serum albumin	VWR international
Bradford reagent	Sigma-Aldich
Chloroform	Merck
Diatomaceous earth	ThermoFisher Scientific
Dichloromethane	Cica
Diethyl ether	Rochelle Chemicals
Diethylene-triamine-pentaacetic acid	Sigma-Aldich
Dr. Ehrenstorfer pesticide mix 1037	LGC-Standards
Envirogel GPC column	Waters
Ethanol Absolute	Merck
Ethylene-diamine-tetra-acetic acid	Rochelle Chemicals
Florosil	Cica
Formic Acid	Rochelle Chemicals
Guanidine hydrochloride	Sigma-Aldich
H ₂ O ₂	Rochelle Chemicals
H ₂ SO ₄	Rochelle Chemicals
Hydrochloric acid	Rochelle Chemicals
K ₂ HPO ₄	Rochelle Chemicals
KH ₂ PO ₄	Rochelle Chemicals
KMnO ₄	Rochelle Chemicals
Liquid N ₂	Afrox
Methanol	Rochelle Chemicals
MgSO ₄	Rochelle Chemicals
Na ₂ SO ₄	Cica
<i>n</i> -Butanol	Sigma-Aldich
<i>n</i> -Decane	Cica
<i>n</i> -Hexane	Cica
Nicotinamide adenine dinucleotide phosphate	Sigma-Aldich
PCB #77	LGC-Standards
p-Iodonitro tetrazolium chloride	Sigma-Aldrich
Polyvinyl-pyrrolidone	Sigma-Aldich
Pyridine	Sigma-Aldich
Pyrogallol	Sigma-Aldich
s-Acetylthiocholine iodide	Sigma-Aldich
Sodium dodecyl sulphate	Merck
Sucrose	Merck
Thiobarbituric acid	Sigma-Aldich
Trichloroacetic acid	Merck
Tris-HCl	Sigma-Aldich
Triton-X	Sigma-Aldich
WakoGel	Wako

Table A2: A List of persistent organic pollutant pesticides, metabolites, and isomers present in the certified reference material used for chemical analysis (Dr Ehrenstorfer pesticide mix 1037) as specified by supplier

Chemical compound
Aldrin
<i>cis</i> -Chlordane (<i>alpha</i>)
<i>trans</i> -Chlordane (<i>gamma</i>)
oxy-Chlordane
2,4'-DDD
4,4'-DDD
2,4'-DDE
4,4'-DDE
2,4'-DDT
4,4'-DDT
Dieldrin
Endrin
<i>alpha</i> -HCH
<i>beta</i> -HCH
<i>gamma</i> -HCH
<i>delta</i> -HCH
Heptachlor
Heptachlor- <i>exo</i> -epoxide (<i>cis</i> -, isomer B)
Heptachlor- <i>endo</i> -epoxide (<i>trans</i> -, isomer A)
Hexachlorobenzene
<i>cis</i> -Nonachlor
<i>trans</i> -Nonachlor

Table A3: Shimadzu 2014 gas chromatograph with micro electron capture detector machine parameters used for organochlorine pesticide bioaccumulation analysis

Parameters	
Carrier gas	He (99.99 %)
Makeup gas	N ₂ (99.99 %)
Flow	2 mL/min
Needle wash	Acetone, <i>n</i> -Hexane, sample
Injection	1 µL, Splitless at 260 °C
Oven program	100 °C, hold 1 min; ramp at 20 °C/min to 200 °C, hold 0 min; ramp at 3 °C/min to 260 °C, hold for 4 min
ECD temperature	310 °C

Table A4: An alternate version of Table 3.2 presenting chemical bioaccumulation data of whole frog samples in ng/g wet mass

Survey	Inside 04/2013				Outside 04/2013	Inside 11/2013	Inside 04/2014
Species	<i>A. garmani</i>	<i>C. xerampelina</i>	<i>P. anchietae</i>	<i>X. muelleri</i>	<i>X. muelleri</i>	<i>X. muelleri</i>	<i>X. muelleri</i>
Sample size	n=11	n=10	n=9	n=8	n=3	n=13	n=13
Body mass (g)	19.05	16.02	12.63	12.61	6.51	10.80	5.05
SEM	2.13	2.09	1.59	0.88	3.58	0.85	1.07
Range	(8.69 – 28.27)	(6.97 – 24.89)	(3.75 – 16.35)	(7.36 – 15.18)	(2.09 – 13.60)	(6.35 – 18.14)	(2.30 – 16.90)
Lipid content (mass % of body mass)	3.89	3.86	5.11	2.65	1.97	0.80	0.90
SEM	0.47	0.52	0.83	0.57	1.58	0.10	0.32
Range	(1.72 – 6.57)	(1.91 – 6.14)	(1.28 – 9.54)	(0.52 – 5.42)	(0.28 – 5.12)	(0.25 – 1.55)	(0.03 – 4.31)
α-HCH	4.57 x 10⁻³	ND	0.25	ND	0.14 x 10⁻³	ND	ND
SEM	4.57 x 10 ⁻³		0.09		0.14 x 10 ⁻³		
Range	(0 – 0.05)		(0 – 0.64)		(0 – 0.41 x 10 ⁻³)		
δ-HCH	ND	ND	ND	ND	ND	0.02	ND
SEM						0.02	
Range						(0 – 0.22)	
γ-HCH	0.53	0.23	1.38	1.34	4.33	1.44	6.59
SEM	0.17	0.05	0.33	0.18	1.58	0.20	0.90
Range	(0 – 1.65)	(0.07 – 0.50)	(0.38 – 3.79)	(0.85 – 2.30)	(1.56 – 7.04)	(0.40 – 2.66)	(2.04 – 13.50)
Aldrin	ND	ND	ND	ND	ND	ND	0.05
SEM							0.04
Range							(0 – 0.55)
trans-Heptachlor-epoxide	ND	ND	ND	0.16	0.21	ND	0.29
SEM				0.14	0.11		0.19
Range				(0 – 1.15)	(0 – 0.35)		(0 – 2.07)
cis-Chlordane	ND	ND	ND	ND	ND	0.17	0.15
SEM						0.10	0.10
Range						(0 – 0.95)	(0 – 1.04)

Table A4 (continued): An alternate version of Table 3.2 presenting chemical analysis data of whole frog samples in ng/g wet mass

Survey	Inside 04/2013				Outside 04/2013	Inside 11/2013	Inside 04/2014
Species	<i>A. garmani</i>	<i>C. xerampelina</i>	<i>P. anchietae</i>	<i>X. muelleri</i>	<i>X. muelleri</i>	<i>X. muelleri</i>	<i>X. muelleri</i>
Sample size	n=11	n=10	n=9	n=8	n=3	n=13	n=13
<i>p,p</i>-DDE	1.35	5.08	0.32	1.22	13.00	13.53	1.36
SEM	0.31	1.46	0.28	0.43	9.03	5.36	0.36
Range	(0.02 – 3.93)	(1.65 – 16.85)	(0 – 2.58)	(0 – 3.39)	(3.77 – 31.06)	(3.61 – 73.36)	(0 – 4.42)
<i>o,p</i>-DDT	0.13	0.20	0.04	0.01	1.54	0.11	0.15
SEM	0.08	0.05	0.03	0.01	0.76	0.03	0.10
Range	(0 – 0.88)	(0 – 0.45)	(0 – 0.21)	(0 – 0.08)	(0.68 – 3.06)	(0 – 0.29)	(0 – 1.16)
<i>p,p</i>-DDD	0.04	0.22	ND	0.05	0.54	0.65	0.39
SEM	0.03	0.10		0.05	0.37	0.27	0.24
Range	(0 – 3.11)	(0 – 1.10)		(0 – 0.42)	(0.13 – 1.27)	(0 – 3.13)	(0 – 2.41)
<i>p,p</i>-DDT	0.58	3.83	0.26	0.51	2.71	1.79	ND
SEM	0.27	1.27	0.20	0.17	2.54	0.76	
Range	(0 – 3.05)	(1.38 – 14.81)	(0 – 1.77)	(0 – 1.49)	(0 – 7.78)	(0 – 8.04)	
ΣDDTs	2.11	9.33	0.62	1.80	17.79	16.07	1.90
SEM	0.51	2.82	0.40	0.41	11.51	5.95	0.53
Range	(0.90 – 7.10)	(3.22 – 33.19)	(0 – 3.33)	(0.71 – 4.07)	(5.60 – 40.79)	(3.68 – 79.59)	(0 – 6.18)
ΣOCPs	2.64	9.56	2.25	3.29	22.33	17.70	8.98
SEM	0.49	2.80	0.46	0.38	11.59	6.04	1.09
Range	(1.31 – 7.27)	(3.39 – 33.34)	(0.38 – 4.42)	(5.19 – 2.00)	(7.52 – 45.17)	(4.68 – 81.70)	(3.70 – 17.17)

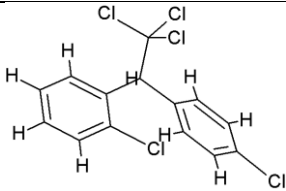
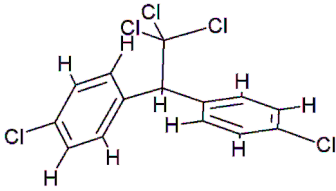
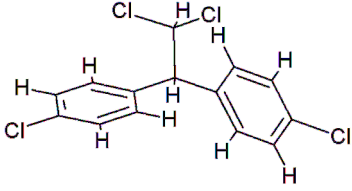
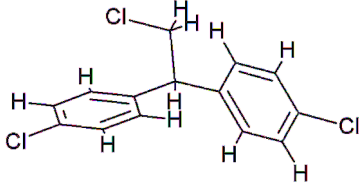
Table A5: Physical properties of the organochlorine pesticides detected in this study. a: Log of octanol/water partition coefficient (log K_{ow}). b: Half-life data available varies greatly based on the conditions of exposure. c: Value reported from soil surface (value in brackets reported from saturated water solution) for γ -hexachlorocyclohexane only, similar properties are assumed for the *alpha*- and *delta*-hexachlorocyclohexane isomers. d: Value is reported as the mean half-life in soil for total dichlorodiphenyltrichloroethanes, individual reports range between 22,5 days and 30 years

IUPAC name	Common name	Chemical structure	Log K_{ow} ^a	Water solubility mg/L (25 °C)	Half-life in soil (days) ^b
(α)-1,2,3,4,5,6-Hexachlorocyclohexane	α -HCH		3.8	10	—
(γ)-1,2,3,4,5,6-Hexachlorocyclohexane	γ -HCH (Lindane)		3.72	17	24.8 ^c (191) ^c
(δ)-1,2,3,4,5,6-Hexachlorocyclohexane	δ -HCH		4.14	10	—
1,2,3,4,10,10-Hexachloro-1,4,4a,5,8,8a-hexahydro-exo-1,4-endo-5,8-dimethanonaphthalene	Aldrin		6.5	0.01	53
(1 α ,2 α ,3 α ,4 β ,7 β ,7 α)-1,2,4,5,6,7,8,8-Octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methano-1 <i>H</i> -indene	<i>cis</i> -Chlordane		5.54	0.06	154
(1 α ,1 β ,2 α ,5 α ,5 β ,6 β ,6 α)-2,3,4,5,6,7,7eptachloro-1a,1b,5,5a,6,6a-hexahydro-2,5-methano-2 <i>H</i> -indeno(1,2-b)-oxirene	<i>trans</i> -Heptachlor-epoxide		5.40	0.28	120

Chemical structures rendered with ChemSketch (ACDlabs)

Sources: (^{a,b}ATSDR, 1994; ^{a,b}ATSDR, 2002; ^{a,b,d}ATSDR, 2002b; ^{a,b,c}ATSDR, 2005; ^{a,b}ATSDR, 2007)

Table A5 (continued): Physical properties of the organochlorine pesticides detected in this study. a: Log of octanol/water partition coefficient. b: Half-life data available varies greatly based on the conditions of exposure. c: Value reported from soil surface (value in brackets is reported from saturated water solution) for γ -hexachlorocyclohexane only, similar properties are assumed for the *alpha*- and *delta*-hexachlorocyclohexane isomers. d: Value is reported as the mean half-life in soil for total dichlorodiphenyltrichloroethanes, individual reports range between 22,5 days and 30 years

IUPAC name	Common name	Chemical structure	Log K_{ow} ^a	Water solubility mg/L (25 °C)	Half-life in soil (days) ^b
1,1,1-Trichloro-2,4- <i>bis</i> (4-chlorophenyl)ethane	<i>o,p</i> -DDT		6.79	0.09	—
1,1,1-Trichloro-2,2- <i>bis</i> (4-chlorophenyl)ethane	<i>p,p</i> -DDT		6.91	5.5×10^{-3}	$\pm 2000^d$
1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene	<i>p,p</i> -DDD		6.02	0.09	—
1,1- <i>bis</i> -(4-chlorophenyl)-2,2-dichloroethene	<i>p,p</i> -DDE		6.51	0.04	—

Chemical structures rendered with ChemSketch (ACDlabs)

Sources: (^{a,b} ATSDR, 1994; ^{a,b} ATSDR, 2002; ^{a,b,d} ATSDR, 2002b; ^{a,b,c} ATSDR, 2005; ^{a,b} ATSDR, 2007)

Table A6: Toxicity data available for organochlorine pesticides (adapted from Pauli *et al.*, 2000 and other individual sources)

Acute Toxicity Tests					
Chemical compound	Endpoint (Experiment type)	Exposure Concentration <small>*units not given, mg/l assumed</small>	Target organism	Life stage	Source
β-HCH (also given as Lindane)	LC ₅₀ (immersion, 96h)	4.4*	<i>Bufo woodhousii fowleri</i>	Tadpoles (4-5 weeks)	Sanders (1970)
	LC ₅₀ (immersion, 96h)	2.7*	<i>Pseudacris triseriata</i>	Tadpoles (1 weeks)	Sanders (1970)
γ-HCH (Lindane)	LC ₅₀ (immersion, 48h)	24 mg/l	<i>Bufo bufo japonica</i>	Tadpoles	Hashimoto & Nishiuchi (1981)
	LC ₅₀ (immersion, 48h)	0.94*	<i>Rana limnocharis</i>	Adult	Pan & Liang (1993)
	LC ₅₀ (immersion, 48h)	5.88*	<i>Rana temporaria</i>	Tadpoles	Thybaud (1990)
	LC ₅₀ (immersion, 96h)	23.37*	<i>Microhyla ornata</i>	Embryo	Pawar & Katdare (1984)
	LC ₅₀ (immersion, 96h)	7.27*	<i>Microhyla ornata</i>	Tadpoles	Pawar & Katdare (1984)
Aldrin	LD ₅₀ (immersion, 48h)	2.4*	<i>Rana hexadactyla</i>	Adult	Vijay & Jayantha Rao (1990a)
	LC ₅₀ (oral administration, 24h)	2.2 mg/kg	<i>Rana hexadactyla</i>	Adult	Vijay & Jayantha Rao (1990b)
	LC ₅₀ (immersion, 96h)	0.15*	<i>Bufo woodhousii</i>	Tadpoles (4-5 weeks)	Sanders (1970)

Table A6 (continued): Toxicity data available for organochlorine pesticides (adapted from Pauli *et al.*, 2000 and other individual sources)

Chemical compound	Endpoint (Experiment type)	Exposure Concentration <small>*units not given, mg/l assumed</small>	Target organism	Life stage	Source
Heptachlor	LC ₅₀ (Immersion, 96h)	0.44*	<i>Bufo woodhousii fowleri</i>	Tadpoles (4-5 weeks)	Sanders (1970)
DDT	LC ₅₀ (Immersion, 48h)	0.38*	<i>Rana limnocharis</i>	Adult	Pan & Liang (1993)
	LC ₅₀ (Immersion, 48h)	31 mg/l	<i>Bufo bufo japonica</i>	Tadpoles	Hashimoto & Nishiuchi (1981)
	LC ₅₀ (Immersion, 36h)	0.62 mg/l	<i>Acris crepitans</i>	Young Adult	Ferguson & Gilbert (1967)
	LC ₅₀ (Immersion, 72h)	0.4 mg/l	<i>Bufo bufo</i>	Tadpoles (26-37days)	Marchal-Segault (1976)
	LC ₅₀ (Immersion, 36h)	0.4 mg/l	<i>Bufo woodhousii</i>	Young Adult	Ferguson & Gilbert (1967)
	LC ₅₀ (Immersion, 96h)	0.03*	<i>Bufo woodhousii fowleri</i>	Tadpoles (1-7 weeks)	Sanders (1970)
	LC ₅₀ (Immersion, 96h)	0.8*	<i>Pseudacris triseriata</i>	Tadpoles (1 week)	Sanders (1970)
	LC ₅₀ (Oral dosing, 20d)	7.5 mg/l	<i>Rana temporaria</i>	Adult	Harri <i>et al.</i> (1979)
DDD	LC ₅₀ (Immersion, 96h)	0.14*	<i>Bufo woodhousii fowleri</i>	Tadpoles (4-5 weeks)	Sanders (1970)
	LC ₅₀ (Immersion, 96h)	0.4*	<i>Pseudacris triseriata</i>	Tadpoles (1 week)	Sanders (1970)

Table A6 (continued): Toxicity data available for organochlorine pesticides (adapted from Pauli *et al.*, 2000 and other individual sources)

Other Toxicity Tests					
Chemical compound	Endpoint / Exposure route (Effect or Result)	Exposure Concentration	Target organism	Life stage	Source
β-HCH (also given as Lindane)	Mortality / Immersion (>50 % mortality)	0.4 $\mu\text{g}/\ell$	<i>Bufo arenarum</i>	Tadpoles	Ferrari <i>et al.</i> (1997)
	Residue / Immersion (Decreased accumulation through skin over time)	10 $\mu\text{g}/\ell$	<i>Rana pipiens</i>	Adult	Kaiser & Dunham (1972)
γ-HCH (Lindane)	Development / Immersion (reduced time to metamorphosis)	2 mg/ ℓ	<i>Xenopus laevis</i>	Embryo	Marchal-Segault & Ramade (1981)
Aldrin	Residue / Immersion (Decreased skin absorption over time)	11 $\mu\text{g}/\ell$	<i>Rana pipiens</i>	Adult	Kaiser & Dunham (1972)
	Behaviour / Immersion (mortality at 30d)	0.30 mg/ ℓ	<i>Rana pipiens</i>	Adult	Kaplan & Overpeck (1964)
	Physiological / Immersion (protein depletion at 1wk, increased amino acids at 2 wks, increased protease activity and amino transferases at 4wks)	0.24 mg/ ℓ	<i>Rana hexadactyla</i>	Not specified	Joseph & Rao (1991)
Chlordane	Mortality / Immersion (100% mortality at 20d)	5 mg/ ℓ	<i>Bufo arenarum</i>	Embryo	Juarez & Guzman (1984)
	Behaviour / Immersion (48 % mortality, hyperactivity at lower concentrations)	0.5 mg/ ℓ	<i>Rana pipiens</i>	Adult	Kaplan & Overpeck (1964)

Table A6 (continued): Toxicity data available for organochlorine pesticides (adapted from Pauli *et al.*, 2000 and other individual sources)

Chemical compound	Endpoint / Exposure route (Effect or Result)	Exposure Concentration	Target organism	Life stage	Source
Chlordane (continued)	Residue / Immersion (10 g/L biomass exposure, bioaccumulation factor for <i>cis</i> -chlordane = 108; maximum accumulation = 0.207 ppm at 96h, half-life 3.3 wks)	5 µg/ℓ	<i>Xenopus laevis</i>	Adult	Khan <i>et al.</i> (1979)
Heptachlor	Development / Immersion (5 ppm = 100% mortality at 15d, 1ppm = reduced time to metamorphosis)	1 & 5 mg/ℓ	<i>Bufo arenarum</i>	Embryo	Juarez & Guzman (1984)
	Residue / Immersion (Decreased accumulation through skin over time)	6 µg/ℓ	<i>Rana pipiens</i>	Adult	Kaiser & Dunham (1972)
DDT	Mortality / Dermal exposure (Expression of resistant genotypes suggested).	0.01 g/mℓ	<i>Acris crepitans</i>	Adult	Boyd <i>et al.</i> (1963)
	Development / Immersion (5 ppm = 100% mortality at 16d, 1ppm = reduced metamorphosis time)	1 & 5 mg/ℓ	<i>Bufo arenarum</i>	Embryo	Juarez & Guzman (1984)
	Physiological / Immersion (<i>beta</i> -Glucuronidase activity in tail fin)	21 mg/ℓ	<i>Bufo arenarum</i>	Tadpole (31d)	Juarez & Guzman (1986)
	Residue / Immersion (0.09 ppm DDT, 0.22 ppm DDE at 24h)	0.005 mg/ℓ	<i>Bufo bufo</i>	Tadpoles	Cooke (1972)

Table A6 (continued): Toxicity data available for organochlorine pesticides (adapted from Pauli *et al.*, 2000 and other individual sources)

Chemical compound	Endpoint / Exposure route (Effect or Result)	Exposure Concentration	Target organism	Life stage	Source
DDT (continued)	Residue / Immersion (High accumulation in liver and fat, trace residues in head, lung, gut and rest of body)	0.001 mg/l	<i>Rana sylvatica</i>	Adult	Licht (1976a)
	Residue / Immersion (Liver levels reached maximum at 24h)	0.001 mg/l	<i>Rana sylvatica</i>	Tadpoles	Licht (1976b)
	Residue / Injection Liver 16.9 ppm; muscle 1.4 ppm.	12 mg/l	<i>Rana temporaria</i>	Adults	Cooke (1974)