Determination of organic pollution in Phongola and Vaal Rivers, and the possible effects to wildlife—a comparison

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List of acronyms and abbreviations

%TOC	Percentage total organic carbon
δ13C	carbon stable isotope ratio
δ15N	Nitrogen stable isotope ratio
2,3,7,8-TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin

A

AChE	Acetylcholinesterase
AhR	Aryl hydrocarbon receptor
ATSDR	Agency for Toxic Substances and Disease Registry

B

BDE	Brominated diphenylether
BFR	Brominated flame retardant
BSA	Bovine serum albumin

C

CAT	Catalase
CEA	Cellular energy allocation
Chlors	Chlordanes
CN	Cis-nonachlor
CNS	Central nervous system
CYP450	Cytochrome P450

D

DCM	Dichloromethane
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DLC	Dioxin-like compound
DNA	Deoxyribose nucleic acid

E

Ea	Energy allocation
Ec	Energy consumption
EC	Electrical conductivity
EDC	Endocrine disrupting compounds
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immune-sorbent assay

G

GC	Gas chromatography

H

HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane

I

IARC	International Agency for Research on Cancer
IBR	Integrated biomarker response

K

K_{ow}	Octanol-water coefficient
KZN	KwaZulu-Natal

L

LOD	Limit of detection
LOQ	Limit of quantification

M

MDA	Malondialdehyde

N

NADPH	Nicotinamide adenine dinucleotide phosphate
NIST	National Institute of Standards and Technology

O

OCP	Organochlorine pesticide
OD	Optical density
OxC	Oxychlordane

P

PAH	Polycyclic aromatic hydrocarbon
PBDE	Polybrominated diphenyl ether
PC	Protein carbonyls
PCB	Polychlorinated biphenyl
PCDD/F	Polychlorinated dibenzo-*p*-dioxins and furans

POPs	Persistent organic pollutants
PPB	Potassium phosphate buffer

S

SA	South Africa
SIA	Stable isotope analysis
SOD	Superoxide dismutase
SRM	Standard reference material

T

TOC	Total organic carbon
TN	Trans-nonachlor

W

WHO	World health organisation
wm	wet mass

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Abstract

The aquatic environment is impacted by a wide range of anthropogenic activities, these include application of agricultural products like herbicides, insecticides and pesticides, and fertilisers; sewage; and industrial waste. These activities introduce various pollutants into the environment including: Polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs), organochlorine pesticides (OCPs) including dichlorodiphenyltrichloroethane (DDT), and metals. These pollutants inevitably enter aquatic environments where aquatic organisms are exposed to these compounds and the various detrimental effects they induce.

The main aims of this study were to determine presence of persistent organic pollution (POP) and the potential effects these have on biological life from three sites in the industrially impacted Vaal River (Vischgat, Barrage, and Thabela Thabeng), and the agriculturally impacted Phongola Floodplain, and to compare these two systems to each other. The objectives were to: (i) Determine the concentration of POPs from biotic matrices in each river system; (ii) evaluate the health of the sampled organisms by determining the biomarker responses; (iii) investigate whether the analysed compounds bioaccumulated in the organisms from each system.

Concentrations of target POPs were determined in fish muscle tissues by instrumental analyses. The target compounds were extracted from the muscle tissue via accelerated solvent extraction, followed by solid phase extraction. The POPs were quantified with gas chromatography (GC) coupled to either a mass spectrometer (MS) or electron capture detector (ECD) depending on the compound class. The effects of environmental stressors on the organisms were assessed by using biomarker assays at the biochemical level. The biomarkers of exposure, oxidative stress, oxidative damage, and cellular energy allocation were performed. The trophic position of the organisms were determined by stable isotope analyses and possible bioaccumulation of the POPs were assessed using the trophic magnification factor.

The organisms from the Vaal River were burdened predominantly with industrially associated pollutants. The Barrage site was the most impacted site the Σxenobiotics ranged from 3.0–35 ng/g, and the fish from Vischgat had the lowest concentrations with concentrations that ranged from 0.61–9.6 ng/g. PCBs and DDTs contributed the highest proportions to the Σxenobiotics. The biomarker assessment indicated that fish from Barrage were in the poorest state and the likely contributing factors to this was the presence of ΣPCBs, polybrominated

diphenyl ethers (ΣPBDEs), and ΣDDTs. The biomarker responses at Thabela Thabeng responses were explained by the environmental factors, and the fish from Vischgat were in the best condition with the responses mostly attributed to the presence of hexachlorobenzene (HCB), chlordanes, and hexachlorocyclohexanes (ΣHCHs). The common organisms between the three sites often had very different trophic positions which is explained by the presence of allochthonous nitrogen inputs that differ between the sites. There was also an indication of biomagnification of most of the compounds, particularly at Barrage and Thabela Thabeng.

The organisms sampled from the Phongola Floodplain had higher ΣOCPs during the April (high flow) sampling event. The red claw crayfish (*Cherax quadricarinatus*) followed by brown squeaker (*Synodontis zambezensis)* had the greatest ΣOCP burdens of <LOD–33 ng/g and 25–170 ng/g respectively. The predominant OCP class was the DDTs, these were mostly from aged sources. The biomarkers indicated that the organisms were in a poorer state during the April sampling event. The responses were explained more by the environmental parameters than the presence of OCPs. The only OCP class to biomagnify in the Phongola Floodplain were the total of the DDTs and metabolites (DDx). The Phongola system is less impacted than the Vaal River system based on the results from this study.

Keywords: bioaccumulation, biomarker, DDT, PCBs, PBDEs, POPs, OCPs, stable isotope, toxicity

1. General introduction

1.1. Persistent organic pollutants (POPs)

These are a wide range of compounds that all share similar characteristics, such as hydrophobicity, volatility, and persistence: adhering to organic matter in abiotic matrices and partition to lipids in organisms (Jones and De Voogt, 1999). POPs are slow to metabolise, able to bioaccumulate and biomagnify, and are toxic (Vallack *et al.*, 1998; Jones and De Voogt, 1999). This allows them to cause deleterious effects through the entire food web. POPs can volatilise into the atmosphere and be redeposited far distances away, a phenomenon termed "long range transport" (Vallack *et al.*, 1998; Jones and De Voogt, 1999; Jaikanlaya *et al.*, 2009). This means that POPs are commonly found in places they were never used or produced (Vallack *et al.*, 1998; Jones and De Voogt, 1999).

Some of these are anthropogenic compounds that have been produced as accidental by-products during combustion or production of other compounds, but most have been specifically produced for industrial applications or agricultural uses (Jones and De Voogt, 1999). They enter the environment either by intentional application, in the case of agricultural compounds, or unintentionally by volatilisation, leakage, or leaching during storage or disposal, such as the industrial POPs (Vallack *et al.*, 1998). Once in the environment they cause various detrimental effects to organisms exposed to them, including cancer, endocrine disruption, and liver damage (Bouwman, 2003).

Some POPs can be partially metabolised, by becoming hydroxylated. These can have detrimental effects that differ from those of the parent compound, such as interference with the endocrine pathways (Vallack *et al.*, 1998). In the case of polychlorinated biphenyls (PCBs) they have been found to be able to cross the placental and blood brain barriers. The structure of the hydroxylated-PCB resembles thyroxine, and has competitive displacement of the natural thyroid hormone. They have also been shown to have anti- and oestrogenic, and tumour promotion potential (Vallack *et al.*, 1998).

The Stockholm Convention on POPs is a treaty designed to phase out the production and use of chemicals, manage the waste of these chemicals, and prevent the introduction of new compounds with POP-like characteristics (Stockholm Convention, 2019). The convention became international law in 2004 (Stockholm Convention, 2019). Initially, 12 compounds and compound classes were listed and are known as the "dirty dozen". These POPs are PCB, polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs) and organochlorine pesticides

(OPCs): Aldrin, chlordane, dichlorodiphenyltrichloroethane (DDT), dieldrin, endrin, heptachlor, hexachlorobenzene (HCB), mirex, and toxaphene (Stockholm Convention, 2017). Since then more have been added some of which include: α-, β-, and γ- hexachlorocyclohexane (HCH), some brominated diphenyl ethers (BDEs), perfluoronated compounds, endosulfan and its related isomers, hexabromobiphenyl (Stockholm Convention, 2019).

1.1.1 Pesticides

The use of OCPs started declining in developing countries in the 1990s, however, the developing countries, specifically those in the tropical regions continued to use these pesticides for agriculture, and in veterinary and medical practices for the control of arthropods (Vallack *et al.*, 1998; Fatoki and Awofolu, 2004). The common pesticides for this use were DDT, HCH, chlordane, and heptachlor (Vallack *et al.*, 1998). All POP pesticides have now been banned, deregistered, their registration withdrawn, or their import and/or export subjected to import permit requirements in South Africa (DEA, 2011). The manufacturing of all POP pesticides within South Africa had ceased, however DDT was still formulated in the country for malaria vector control programmes until mid-2010 (DEA, 2011).

Pesticides can be absorbed directly by plants, or indirectly from the soil. Animals that feed on these plants accumulate the pesticides, and in agriculture this is a route of exposure for humans (Vallack *et al.*, 1998). Alternatively, these compounds deposit into aquatic systems via runoff where they bioaccumulate and biomagnify (Vallack *et al.*, 1998). These compounds are capable of mimicking hormones, which causes an activation or inhibition of the hormone receptors (Soto *et al.*, 1994; Waldbillig, 1998; Vinggaard *et al.*, 1999; Tessier and Matsumura, 2001; Cocco, 2002; Ralph *et al.*, 2003; Bulayeva and Watson, 2004; Lemaire *et al.*, 2004). Male vertebrates feminise due to exogenous chemicals that block the androgen receptor rather than exposure to increased oestrogens (Walker, 2009)

1.1.1.1 Dichlorodiphenyltrichloroethane (DDT)

This pesticide was used in large quantities historically (between the 1950s and early 1980s) for agriculture and was aerially sprayed to control mosquitoes, flies, and lice that spread malaria, typhus, and typhoid (Vallack *et al.*, 1998). Its use was banned in 1974 in South Africa (Van Dyk *et al.*, 1982; Wells and Leonard, 2006; DEA, 2011), but it is believed that illegal use continued due to available stockpiles (Wells and Leonard, 2006). It has been used since 1946 for malaria control where it was used both as a larvicide and for indoor residual spraying (IRS). However, in 1956 its use as a larvicide was discontinued, and for 1996–2000 DDT use was discontinued altogether (DEA, 2011). During this time pyrethroid insecticides were used.

However, malaria cases and deaths increased because a resistant mosquito (*Anopheles phenestus*) emerged, and DDT was reintroduced (Wells and Leonard, 2006; DEA, 2011). In 1999 the number of people infected with malaria was over 50 000, whereas prior to the introduction of pyrethriods the highest number of people infected was never higher than 14 000 since 1971 (Bouwman, 2003). In South Africa its use is only allowed in provinces where malaria is endemic: KwaZulu-Natal (KZN), Limpopo, and Mpumalanga (Dalvie *et al.*, 2004). Other African countries and India also use DDT for malaria vector control (Bouwman, 2004). The mixture used for IRS has a composition of approximately 72–75% of the active ingredient *p,p'*-DDT and the remaining consisting of *o,p'*-DDT (Bouwman *et al.*, 2006). It is applied by spraying on walls and ceilings of houses and animal shelters to kill the malaria vector mosquito (DEA, 2011). The malaria control programme used 15 785 kg DDT in 2001, 14 508 kg in 2002, and 6 483 kg in 2003 in KZN (Bouwman *et al.*, 2006). In 2014 approximately 18 000 kg of the active ingredient was used in South Africa, and 12 000 kg was used in Mozambique, which neighbours South Africa on the border of KZN. These masses differ yearly: In 2006 as much as 75 000 kg were applied in South Africa, and 300 000 kg were applied in Mozambique in 2009 (van den Berg *et al.*, 2017).

DDT is metabolised via dehydrochlorination into the persistent *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE) in aerobic environments, and it is highly bioaccumulative in lipids (Fisher, 1999; Bornman *et al.*, 2010). In anaerobic conditions DDT readily undergoes reductive dechlorination to form dichlorodiphenyldichloroethane (DDD) (Fisher, 1999). DDT can also be metabolised to DDD and DDE by the cytochrome P450 enzymes CYP2B, CYP3A, and minimally by CYP1A (Kitamura *et al.*, 2002).

The active ingredient, DDT, and its metabolites can cause many detrimental effects: It is an endocrine disrupting compound (EDC) and has been classified as a probable carcinogen by the International Agency for Research on Cancer (IARC, 2017). The metabolite *p,p'*-DDE is a potent androgen antagonist, while the two DDT isomers are slightly less inhibitory (Danzo, 1997; De Jager *et al.*, 2006). The *o,p'*-isomers have all been shown to be weak oestrogenic activators in human *in vitro* oestrogen receptor assays (Vallack *et al.*, 1998). Because of these endocrine disrupting effects, exposure to DDT or metabolites have been shown to cause reproductive abnormalities in humans and wildlife like decreased fertility, still births, urogenital birth defects, and congenital defects (WFPHA, 2000; Wells and Leonard, 2006; Bornman *et al.*, 2010). It has been documented for decades that exposure to DDE affects the egg shell thickness of birds (Ratcliffe, 1967; Ratcliffe, 1970; Bowerman *et al.*, 1998; Vasseur and Cossu-Leguille, 2006). Also, *o,p'*-DDT has the potential to influence the common biomarkers of xenobiotic exposure: gonadosomal index (GSI) and hepatosomatic index (HIS) in exposed fish

(Leaños-Castañeda *et al.*, 2004). It has been documented that DDT can stimulate and depress the central nervous system, cause neuro-developmental effects, cause tremors and convulsions, and damage the liver (Wells and Leonard, 2006).

This pesticide and its metabolites have been detected in regions across South Africa where spraying of DDT is no longer permitted (Vetter *et al.*, 1999; Fatoki and Awofolu, 2004; Batterman *et al.*, 2008; Sibali *et al.*, 2008; Amdany *et al.*, 2014; Pheiffer *et al.*, 2018c; Vogt *et al.*, 2018). This may be as a result of illegal usage, or long-range transport, or historic use.

1.1.1.2 Chlordane

Chlordane has been used to control arthropods important to medical and veterinary causes, and as a broad-spectrum contact insecticide on crops such as grains, maize, potatoes, sugarcane, fruits, nuts, and cotton (Vallack *et al.*, 1998; Fisher, 1999; Batterman *et al.*, 2008). In South Africa the use of this pesticide was restricted for pest protection of stem-crops and buildings, and all uses were banned in 2000 (Batterman *et al.*, 2008). In its technical form, chlordane is comprised of two chlordane isomers α- and γ-chlordane (38–48%), heptachlor (3–13%), α- and γ-nonachlor (5–11%), and the remainder consists of other chlordane isomers (17–25%) (Batterman *et al.*, 2008). Chlordane, α-nonachlor, and γ-nonachlor metabolise to mainly oxychlordane and heptachlor (Bondy *et al.*, 2000; ATSDR, 2007; Batterman *et al.*, 2008). Exposure to chlordane causes changes in immune system responses, liver damage, destruction of muscle and nerve membranes; it is a possible carcinogen, and can eventually lead to death (Frear, 1955; Fisher, 1999).

1.1.1.3 Heptachlor

Heptachlor was used widely to control termites, ants, and soil insects on seed grains and crops throughout the 1960s and 1970s (Vallack *et al.*, 1998). In 1976 its registration was withdrawn in South Africa (Bouwman, 2003), and most other countries have now also banned the pesticide. In its technical form it consists of approximately 72% heptachlor and the remaining portion is comprised of γ-chlordane and γ-nonachlor (ATSD, 2007). Via abiotic or biotic transformation heptachlor epoxide is formed, and can occur within the space of a few hours (Fisher, 1999; ATSDR, 2007). It is the metabolite that is more likely to occur in the environment considering the parent compound had been banned (ATSDR, 2007). Exposure to heptachlor and/or heptachlor epoxide had been shown to cause: hyperexcitation of the central nervous system (CNS), liver damage, lethargy, tremors, convulsions, stomach cramps, coma, and reproductive effects (ATSDR, 2007; Smith, 1991; Fisher, 1999; 2007).

1.1.1.4 Hexachlorobenzene (HCB)

Hexachlorobenzene has been used as a fungicide on onion, sorghum, wheat and other grain seeds, in military pyrotechnics, as a fluxing agent in aluminium smelting, as a porosity controller for the manufacturing of graphite electrodes, as a peptisising agent in rubber production, and as an intermediate in dye manufacturing (Vallack *et al.*, 1998; Bailey, 2001; Roos *et al.*, 2011). It had been used since 1945, but its use as a fungicide was banned in 1983 in South Africa (Batterman *et al.*, 2008). It is also formed as by-product during combustion processes, in metal industries, and during the manufacture of chlorine containing chemicals and is therefore a common contaminant in pesticide formulations, (Vallack *et al.*, 1998; Bailey, 2001; Bouwman, 2003). It has been documented that HCB is a potent reproduction toxicant, especially in humans (Schade and Heinzow, 1998; Jarrel and Gocmen, 2000; Fenster *et al.*, 2006). Neurological issues have been reported in rodents with effects such as tremors, paralysis, muscle incoordination, weakness, and convulsions (Edwards *et al.*, 1991). Tumours of the lungs, thyroid, liver, and spleen have also been described in animals (Edwards *et al.*, 1991).

1.1.1.5 Hexachlorocyclohexane (HCH)

There are eight possible isomers of HCH, however, γ-HCH or lindane, is the only one with notable insecticidal activity (Willett *et al.*, 1998). It has been produced as a broad spectrum insecticide for seed and soil treatment, foliar application, tree and wood treatments, and against ectoparasites of animals and humans since the 1940s (Shatalov *et al.*, 2004; Stockholm Convention, 2017). The technical mixture of HCH contains 60–70% α-HCH, 5–12% β-HCH, 10–15% γ-HCH, and the remaining comprising of other isomers (Iwata *et al.*, 1995). The production of lindane is highly ineffective: for every ton of γ-HCH manufactured 6–10 tons of α- and β-HCH are produced as by-products (Vijgen *et al.*, 2006; Stockholm Convention, 2017). The production of HCHs has been declining due to regulations in several countries before it was added to the new list of Stockholm Convention POPs (Stockholm Convention, 2017). Because γ-HCH is one of the most volatile OCPs it is particularly prone to distribution by long-range transport (Shatalov *et al.*, 2004). The CNS is most affected by HCH because it is an irritant and leads to sensibilising, but it also causes mutagenic, embryotropic, reproductive, immunotoxic, and developmental effects (Shatalov *et al.*, 2004; Stockholm Convention, 2017). The “waste” isomers (α- and β-HCH) are capable of bioaccumulating and are potentially carcinogenic (Fisher, 1999). Five isomers of HCH have been quantified from samples across South Africa (Vetter *et al.*, 1999; Batterman *et al.*, 2008; Sibali *et al.*, 2008; Amdany *et al.*, 2014; Buah-Kwofie and Humphries, 2017; Pheiffer *et al.*, 2018c).

1.1.1.6 Cyclodiens (Drins)

1.1.1.6.1 Aldrin

It has been used widely on crops such as corn and potatoes for protection from soil insects, and has also been used to prevent termites on wooden structures (Fisher, 1999). All uses were withdrawn in 1992 (Bouwman, 2003). Aldrin degrades in the environment into dieldrin (Fisher, 1999; Batterman *et al.*, 2008). Exposure to aldrin can cause muscle twitching, myolonic jerks, and convulsions. It may also be associated with liver and biliary cancer, can affect the immune response, and cause possible endocrine disruption (Fisher, 1999).

1.1.1.6.2 Dieldrin

This pesticide was used in agriculture to control soil insects and as a control of several insect vectors for human health (Fisher, 1999). South Africa restricted the use of dieldrin in 1970, withdrew it as a stock remedy in 1974, and in 1979 it was restricted for use only as a moth-proofing agent, and tsetse fly and harvester termite control (Fisher, 1999). It was completely banned in the country in 1982 (Van Dyk *et al.*, 1982). The health implications for dieldrin is similar to aldrin's (Fisher, 1999).

1.1.1.6.3 Endrin

Endrin has been used as a foliar insecticide mainly on field crops like cotton and grains (Batterman *et al.*, 2008). It was also used as a rodenticide (Fisher, 1999). It was voluntarily withdrawn in 1980 (Bouwman, 2003). It is slightly less accumulating than aldrin and dieldrin (Fisher, 1999). Epileptic convulsion and a coma can result from exposure to endrin, and there is also evidence that it can suppress the immune response (Fisher, 1999).

1.1.2 Polychlorinated biphenyls (PCBs)

These compounds comprise of two benzene rings covalently linked between two carbons in the neighbouring rings. Chlorine atoms, up to 10, can take the place of hydrogens on the benzene rings. A total of 209 PCB congeners is possible, each different by the number and position of chlorines (Vallack *et al.*, 1998; Jones and De Voogt, 1999). Their water solubility, vapour pressure, and biodegradability tend to be indirectly proportional to the number of chlorine atoms (Vallack *et al.*, 1998).

They have been produced intentionally for various industrial applications like lubricants, paint stabilisers, dielectric fluids, pesticide extenders, flame retardants, and polymers and adhesives since the 1930s (Vallack *et al.*, 1998; Shatalov *et al.*, 2004; Cardellicchio *et al.*, 2007; Han, *et*

al. 2017; Dhakal *et al.,* 2018). It is estimated that the global production of PCBs was approximately 1–2 million tons between 1930 and 1993, nearly all having been produced in the northern hemisphere (de Voogt and Brinkman, 1989; Breivik *et al.*, 2002). However, the PCBs can also be formed unintentionally as by-products during incineration, combustion, and industrial processes including steel manufacturing (Buekens *et al.*, 2001; El-Shahawi *et al.*, 2010).

The production of PCBs was banned in the 1970s and 1980s in many countries due to the detrimental effects they have on environmental health (Khim *et al.*, 1999; Tuomisto, 2019), and have been listed on the Stockholm Convention since 2001 (UNEP, 2009). Some of these effects include carcinogenicity, mutagenicity, endocrine disruption, hepatoxicity, reproductive toxicity, and immunotoxicity (Murk *et al.*, 1996). The most toxic PCBs are the coplanar- or dioxin-like (DL) PCBs, which is mediated through the aryl hydrocarbon receptor (AhR) (Tuomisto, 2019). Because PCBs are resistant to heat, light, and acid degradation they are highly persistent (Van Ael *et al.*, 2012; Reddy *et al.,* 2019) and therefore are still found in environmental matrices despite their ban decades ago (Khim *et al.*, 1999; Van Ael *et al.*, 2012). There is an estimated 10% of the 1.5 million metric tons that was produced remaining in the environment Reddy *et al.,* 2019. These PCPs have leached from historical sources such as electrical transformers, hazardous waste sites, and end up in the environment due to improper disposal of industrial waste, and incineration of waste (Vallack *et al.*, 1998; Jaikanlaya *et al.*, 2009; Needham and Ghosh, 2019; Reddy *et al.,* 2019).

South Africa never manufactured PCBs but Eskom, the main electricity supplier in the country, still uses transformers and capacitors that contain PCBs. The large steel refinery, Arcelor Mittal that was previously known as Iscor, and petroleum producer, Sasol, have also used oils containing PCBs. These companies have replaced most, if not all of their PCB containing oils (Bouwman, 2003). These oils have largely been sent overseas for incineration. Any PCBs present in the environment are due to their use in industries, or their waste (De Kock and Randall, 1984).

1.1.3 Polybrominated diphenyl ethers (PBDEs)

This group of brominated diphenyl ethers (BDEs) has been used as additives in flame retardants because of their ability to suppress combustion in plastics, electronic equipment, textiles, and in furniture (Siddiqi *et al.*, 2003; La Guardia *et al.*, 2006; Lorber, 2008; Han, *et al.* 2017), and can contribute up to 30% of the mass of these product (Siddiqi *et al.*, 2003). These synthetic compounds have been in use since the 1960s (Siddiqi *et al.*, 2003). The common

congeners used for flame retardation are the tetra-, penta-, hepta-, octa-, and deca-BDEs, but penta-, octa, and deca-BDEs are the most common in commercial mixtures (Darnerud, 2003; Siddiqi *et al.*, 2003; La Guardia *et al.*, 2006; Stockholm Convention, 2017). In the early 2000s it was estimated that between 67 000 and 150 000 metric tons of brominated flame retardants (BFRs) are produced per year globally (De Wit, 2002; Siddiqi *et al.*, 2003), however, more recent production information is hard to come by.

The BDEs with fewer bromines tend to have higher bioaccumulation and biomagnification potential than those with many bromine atoms (Siddiqi *et al.*, 2003; La Guardia *et al.*, 2006). It is also possible that BDEs with many bromines can be transformed in the environment to BDEs with fewer bromines (La Guardia *et al.*, 2006). Octa- and deca-BDEs degrade photolytically under high temperature, like fires, to toxic brominated dibenzofurans and dioxins (Darnerud, 2003; Siddiqi *et al.*, 2003). The toxic effects of BDEs include endocrine disruption, hepatic tumours, and neurodevelopmental and thyroid dysfunctions (Siddiqi *et al.*, 2003). An example of its endocrine disruptive effect is the oestrogenic activity of especially PBDE-100, -75, and -51and some of the hydroxylated BDEs have been shown to be a more potent oestrogen activator than oestradiol at high concentrations (Meerts *et al.*, 2000). Congeners of BDEs also have been documented to interact with the AhR, causing both activation and inhibition (Behnisch *et al.*, 2003; Eljarrat and Barceló, 2003; Kuiper *et al.*, 2004).Some methoxy-PBDEs have been determined to occur from natural sources, however, this does not mean that they do not pose detrimental effects (Teuten & Reddy, 2005).

1.2 Fish as a model for environmental stressors

Fish have been utilised to assess the state of environmental impacts to freshwater systems for some time, and the use of endpoint studies on fish are now common for ecological risk assessments (Adams, 2001; Van der Oost *et al.*, 2003). They are an important tool to monitor environmental stressors because they are long-lived, accumulate xenobiotics, and have high positions in the trophic structure (McHugh *et al.*, 2013). These fish characteristics make it possible to investigate the presence, and possible effects of the xenobiotics that bioaccumulate and biomagnify.

1.3 Biomarkers

Organisms are exposed to a wide range of the xenobiotics including the ones already mentioned and are exposed to various other environmental stressors, like temperature fluctuations, extreme temperatures, limited food availability, and depleted dissolved oxygen

concentration (Benedetti *et al.*, 2015; Lee *et al.*, 2015). Effects that are caused by these stressors can be measured biochemically using various markers of stress and exposure—oxidative stress, oxidative damage, cellular energy allocation. Biomarkers are being used as a sensitive early warning tool to determine biological effects due to these stressors before these effects cause detrimental effects to higher levels of biological organisation (Cajaraville *et al.*, 2000; Van der Oost *et al.*, 2003; Newman, 2010; Lee *et al.*, 2015). This technique is capable of giving an indication that an organism has been exposed to xenobiotics, and the magnitude of the organism's response to the pollutant (Cajaraville *et al.*, 2000). Because environmental stressors are seldom singular in occurrence, organisms are exposed to multiple agents that can cause detrimental effects—it is often impossible to predict how this mixture of stressors could influence an organism, possibly synergistically, antagonistically, or additively (Hilscherova *et al.*, 2000; Hecker and Giesy, 2011). By utilising biomarkers, these effects can be determined by measuring the end-points of the exposure. Biomarkers have been implemented into various pollution monitoring schemes such as North Sea Task Force Monitoring Master plan, Joint Monitoring Programme of Convention for the Protection of the Marine Environment of the North-East Atlantic, and National Oceanographic Atmospheric Administration's National Status and Trends Programme in the United States (Cajaraville *et al.*, 2000). Locally, there have been several studies that have implemented biomarkers to assess the health of organisms in the environment (Wepener *et al.,* 2012; Gerber *et al.,* 2018; Jansen van Rensburg *et al.,* 2020)

1.4 Study area

1.4.1 Phongola River

The Phongola River flows through the Ndumo area in the north east of KwaZulu-Natal, one of the malaria endemic provinces of South Africa, close to the borders of both Mozambique and Swaziland (Coetzee *et al.*, 2015). The river's origin is near Utrecht in the north of KwaZulu-Natal; it flows east through the town of Phongola, and then flows north where it joins the Maputo River; the river has a catchment of 7 000 km^2 (Lankford *et al.*, 2011). The area receives summer rainfall, from October to March, of 670–1000 mm annually, and has a subtropical climate with temperatures between 13°C to 40°C (Bewsher, 2005; Morgenthal *et al.*, 2006). High evaporation occurs in summer with rates of 103–156 mm per month. The river has its highest flow from November through March, and the greatest discharge usually occurs in February. The lowest flow season is expected during June to September (Dube *et al.*, 2015).

The area surrounding the river is mostly rural with informal settlements which have limited infrastructure and facilities. Despite its remoteness, there are a number of industrial activities in the area, including saw and paper mills (Venter *et al.*, 2010), and the Klipwal Gold Mine (Tate, 2014). The area is highly agricultural, dominated by forestry and commercial agriculture, mainly sugarcane and subtropical fruit plantations (McHugh *et al.*, 2011). Many of the locals also practice subsistence farming of maize, cattle, goat, and poultry.

The Pongolapoort dam, situated between the Lebombo and Ubombo mountains, was constructed in 1972 to improved irrigation of the highly fertile soils (Wepener *et al.*, 2012). The dam is the fifth largest impoundment in South Africa with a water holding capacity of close to 2.5 million m^3 (Wepener *et al.*, 2012) and the area below the dam is referred to as the Phongola floodplain. The floodplain extends for roughly 50 km and has a width that varies between 0.8 and 4.8 km (Lankford *et al.*, 2011). When construction of the dam began, flooding regimes to maintain the downstream ecology and integrity, as well as supply locals with water, had been planned. However, this did not materialise, and the flooding of the floodplain now happens on a demand basis, which does not simulate the natural seasonal flooding that occurred before the dam was constructed (Heeg and Breen, 1982). Altering the flow of rivers causes the natural environment to deteriorate, habitats are destroyed, and ecological functioning is compromised (Lankford *et al.*, 2011). Downstream areas are affected by reduced flow rates and this can often lead to the floodplain streams being permanently or intermittently dry. This can impact water quality and ecological integrity (Stanford 1996), as well as changing the physiochemical parameters of the system, including the water temperature and sediment flux. And in turn this affects the ecological processes and services of the system (Hughes 1988, Stanford 1996). Water is also pumped from the river and floodplain pans for irrigation and this practice has increased in recent years, however because this is unmetered the extent of the abstraction is unknown (Lankford *et al.*, 2011).

Communities in the Umkhanyakude District have increased in size, with a population of approximately 325 052 people and 59 511 households. These communities rely heavily on the resources in the area. Approximately 89% of locals utilise the fish in the river as their third most consumed protein source, which they eat twice a week on average (Coetzee *et al.*, 2015). The most frequently consumed species in the system are *Coptodon rendalli, Oreocromis mossambicus, Synodontis zambezensis, Clarias gariepinus, Schilbe intermedius*, *Hydrocynus vittatus, and Cherax quadricarinatus* (Coetzee *et al.*, 2015; Smit *et al.*, 2016). Households also rely on water from the Phongola River for irrigation for small-scale, or subsistence agriculture (Heeg and Breen, 1982). They also make use of the nutrient rich sediment that replenishes the soil fertility during a flooding event (Lankford *et al.*, 2011). Locals use of the fruits, reeds,

thatch grass, and firewood for their daily needs (Dube *et al.*, 2015). All this has led to increased exploitation of resources, including land and water. Additionally, there is accounts of sewage discharge into the river (Venter *et al.*, 2010). All this has had ecological ramifications with declines in resources and negative influences on the integrity and quality of the water resource. Because many of the households rely on the water sources, which are unsafe, frequent cholera and other water-borne outbreaks occur. And considering a large proportion of the population is infected with HIV, and therefore are immunocompromised, the poor water quality is an added health problem for the infected (Lankford *et al.*, 2011).

The region was ranked as having the highest incidence of malaria in South Africa around the time the study was conducted (Lankford *et al.*, 2011), and is defined as an "endemic to low-risk" malaria area (McHugh *et al.*, 2011; Wepener *et al.*, 2012). Although banned by the Stockholm Convention, DDT is allowed to be sprayed around and in the residences of locals from January to the end of March annually (Bouwman *et al.*, 1990). This had been done almost uninterrupted since 1946 to control the malaria vector, the *Anopheles* mosquito (Bouwman *et al.*, 1990; Sereda and Meinhardt, 2005). For a short period between 1996 and 2000, it was replaced by pyrethroids, but the mosquitos became resistant to the pesticide, and DDT was reintroduced (Van Dyk *et al.*, 2010). As such, DDT, and its metabolites had been detected in organisms in the area for decades (Bouwman *et al.*, 1990; McHugh *et al.*, 2011; Wepener *et al.*, 2012).

The Ndumo Game Reserve is located on the border between KZN and Mozambique, and portion of the Phongola River flows through the reserve. The game reserve is listed as one of South Africa's 21 Ramsar sites because of its unique wetlands and was proclaimed a reserve in 1924 with the objective of protection of biodiversity (Dube *et al.*, 2015). The regions of the river within the reserve are therefore also protected. The reserve is of high ecological value because of a diverse array of habitats, including lagoons, oxbow lakes, levees, marshes, forests, and floodplain grasslands (Heeg and Breen, 1982; Van Vuuren, 2009). This results in a rich biodiversity of fish, birds, and other animals who utilise the area as a refuge (Heeg and Breen, 1982).

However, the area is impacted largely because of the upstream activities that include tourism (fishing, boating, game drives, and birding), fisheries, industries, agriculture, lack of managing flow regimes, and over utilising the resources (Lankford *et al.*, 2011). Many of these are of great economic value and it resulted in loss of habitat, diversity, and ecological health in the system (McHugh *et al.*, 2011). The construction of the dam, and the poor management of controlled floods negatively influenced the floodplain and associated wetlands both in- and

outside of the nature reserve, as well as the biodiversity (Heeg and Breen, 1982; Dube *et al.,* 2015).

For at least two decades there had been a decline in the populations of tigerfish in South Africa, and the species has therefore been placed on the threatened and protected species list. The decline had been attributed to the pollutant burdens these fish, and its prey are exposed to (Steyn *et al.*, 1996). Aside from the DDT contamination, previous studies demonstrated that fish in the system are exposed to other pesticides, PCBs and flame retardants (Bouwman *et al.*, 1990; McHugh *et al.*, 2011; Wepener *et al.*, 2012; McHugh *et al.*, 2013).

1.4.2 Vaal River

The Vaal River is the second largest river in South Africa. It has its source in Mpumalanga province, east of Gauteng, and spans 1 120 km. It borders the North West province and Gauteng on its northern bank and the Free State on its southern bank (Moja *et al.*, 2013). It flows westwards to its confluence with the Orange River in the Northern Cape, and is the largest tributary of the Orange River. The area receives approximately 683 mm rainfall annually with the peak in summer (October to April). Summers are warm with average maximum temperatures of 30–35°C (South African Weather Service, 2018).

The Vaal River flows through the highly industrialised region known as the Vaal Triangle which includes the cities of Vereeniging and Vanderbijlpark in Gauteng, and Sasolburg in the Free State (Nieuwoudt *et al.*, 2009; Pheiffer *et al.*, 2014). Industries were developed in the Vaal Triangle in 1882 after coal was discovered in the beds of the Vaal River (Leigh, 1968), resulting in industrial pollution through the river for over a century. Some of these industries include a coal mine, cement factories, a coal fired power station, an oil refinery, a paper and pulp plant, and a liquid petroleum company (Nieuwoudt *et al.*, 2009; Quinn *et al.*, 2009). Historically there were gold and other mineral mining processes in the region. Many tributaries of the Vaal River, including the Blesbok Spruit, Riet Spruit, Suikerbosrand, and Klip Rivers flow through similarly industrialised and urbanised areas. The Klip River flows through the “steel capital” of the country that has a wide array of iron processing and smelting, steel mills, and steel production companies (Quinn *et al.*, 2009; Rimayi *et al.*, 2016). The Vaal and its tributaries have over 90 major man-made impoundments, and have become one of the country’s most regulated rivers (DWAF 2009; Weyl and Martin, 2016). However, the 63 km stretch downstream of the Vaal Dam to the Vaal Barrage receives high loading of contaminants from the tributaries, the large economic footprint with as much as 37.6% of the gross national products contributed from this area. The infrastructure in the area is insufficient and approximately 13 600 wet industries’

water flow into the river (Tempelhoff, 2009). Added to these impacts there are an estimated 10 million people that reside within the region, which equates to approximately 18.1% of the South African population (Tempelhoff, 2009; Moja *et al.*, 2013). This area in the river receives effluent from the major water treatment plants: Johannesburg Water, East Rand Water, and Metsi-a-Lekoa (Wepener *et al.*, 2011). This is also the stretch of the river that supplies potable water to Gauteng, for irrigation, human use, and industrial activities (Moja *et al.*, 2013). Even though this part of the river accounts for less than 5% of the total catchment of the Vaal River, it is regarded as the hardest working river region in the country (Tempelhoff, 2009).

Due to the various industrial, agricultural, and urban activities around the river system numerous pollutants are present (McCarthy and Venter, 2006; Roychoudhury and Starke, 2006; Jooste *et al.*, 2008; Pheiffer *et al.*, 2014; Rimayi *et al.*, 2016). Dioxin-like compounds (DL-PCBs, PCDD/Fs), and other PCBs have been detected in the region (Vosloo and Bouwman, 2005; Bouwman *et al.*, 2008; Jooste *et al.*, 2008; Quinn *et al.*, 2009; Wepener *et al.*, 2011; Rimayi *et al.*, 2016). The area around Alberton (south of Johannesburg) had been determined a hotspot for these compounds. The mineral mining in the areas had been named the cause (Jooste *et al.*, 2008; Rimayi *et al.*, 2016). Polycyclic aromatic hydrocarbons (PAHs) had been detected in sediment (Quinn *et al.*, 2009), and water from this region (Moja *et al.*, 2013). The concentrations in the water of the latter study of two PAHs, benzo(*b*)fluoranthene, and indeno(*1,2,3-cd*)pyrene), were concerning because of their carcinogenicity (Moja *et al.*, 2013). A range of OCPs had been detected in various matrices (Bouwman *et al.*, 2008; Burger, 2008; Wepener *et al.*, 2011; Pheiffer *et al.*, 2018c) including DDT, which is banned in the area (Bouwman *et al.*, 2008; Quinn *et al.*, 2009). The herbicides atrazine, and terbuthylazine had been recorded in water in the Barrage region (Burger, 2008). Metals had been detected frequently in the region in sediment, fish, and aquatic birds (Wepener *et al.*, 2011; Pheiffer *et al.*, 2014; Van der Schyff *et al.*, 2016). The concentrations of metals that were detected in fish are predicted to be capable of causing a health threat to humans who consume these fish (Pheiffer *et al.*, 2014). The Vaal River that flows through the Vaal Triangle has been facing a wastewater crisis for the last two years. This is because raw sewage is flowing directly into the Vaal River and its tributaries due to dysfunctional water treatment plants, and from formal and informal settlements in the area (Wepener *et al.*, 2011; Hosken, 2018; Bega, 2019; Monteiro, 2019). This problem was blamed on the rapid growth of populations and cities, and the inability of the wastewater treatment plants to deal with these loads. Studies reported faecal pollution in the water prior to the “crisis” (Jordaan and Bezuidenhout, 2013; Teklehaimanot *et al.*, 2014). The poor water quality resulted in disease outbreaks in the people who use these water sources (Teklehaimanot *et al.*, 2014). Detectable concentrations of perfluorinated compounds

(PFASs) have also been discovered in various matrices around the Vaal River (Lesch *et al.*, 2017; Groffen *et al.*, 2018). Groffen *et al.* (2018) sampled these matrices concurrently with this study, utilising the same fish. Aquatic birds and fish from the Vaal River were documented to contain brominated flame retardants (Polder *et al.*, 2008; Wepener *et al.*, 2011; Vogt *et al.*, 2015). The wetlands along the Vaal catchment deteriorated, which in turn reduces the system's ability to filter the water, damaging the ecological resilience of the system (McCarthy and Venter, 2006). Mass fish kills are also common in the region (Wepener *et al.*, 2011).

Although pollution, and in turn poor water quality are the main threats to biota in the region, several other factors affect the fish communities in the area as well. These include habitat alterations, flow regime modifications, barriers preventing migration, disturbance to wildlife, and non-endemic alien and or introduced fishes into the system (Wepener *et al.*, 2011).

1.5 Hypotheses, aims, and objectives

The two river systems, Vaal and Phongola Rivers, were chosen because they are impacted by different anthropogenic sources. The Vaal River and its tributaries flow through the central South Africa subjecting it to industrial, urban, and agricultural pollutants, while the Phongola Floodplain is located in a predominantly agricultural area exposing it to mainly agricultural related pollutants.

Hypotheses:

1. There will be high concentrations of POPs in the Vaal River, while Phongola Floodplain will be impacted by agriculturally relevant OCPs.
2. It is expected that organisms from the Vaal River will experience high levels of stress due to excessive burdens of a wider range of xenobiotics.

To test the hypotheses the aims of this study are to:

1. Determine the concentrations of various organic contaminants from the Vaal and Phongola systems in various matrices;
2. Determine the degree of stress organisms in these systems are experiencing via biomarker assays
3. Compare the two systems considering the different impacts and activities in the areas.

Chapter 1: General introduction

Provides the background of the study is discussed and the hypothesis, aims, and objectives are provided.

Chapter 2: Persistent organic pollutants in fish tissue from three sites in the Vaal River

Concentrations of various POPs were determined from fish species collected from three sites in the Vaal River, namely Vischgat, Barrage, and Thabela Thabeng. The hypothesis in this chapter is that the POP concentrations would be high, especially the industrially relevant PCBs and PBDEs. It is also expected that the Barrage site would be the most impacted by the POPs because of its location downstream of heavily polluted tributaries.

Chapter 3: Organochlorine pesticides in aquatic biota tissue from the Phongola Floodplain

Concentrations of OCPs from aquatic biota collected from the Phongola Floodplain during a low- and high low season are investigated. The hypothesis is that concentrations of agriculturally relevant OCPs would be present in high concentrations in biota collected from the Phongola Floodplain, especially DDT which is actively applied in the northern section of KwaZulu-Natal, and the neighbouring Mozambique.

Chapter 4: Biomarker responses from organisms in the Vaal River and Phongola Floodplain

In this chapter biomarker responses from the aquatic biota from the Vaal River and the Phongola Floodplain are assessed. Spatial trends will be evaluated between the sites in the Vaal River, and temporal differences will be determined between the high- and low flow seasons for the Phongola Floodplain. It is hypothesised that the fish from the Barrage site will have the greatest stress responses due to the high POP concentrations present in fish from this site and the fish from Vischgat would experience the least stress. Within the Phongola system it is expected that fish sampled during the high flow period would have greater stress responses due to the higher OCP concentrations in this flow period. These hypotheses were tested by determining the biomarkers of exposure, and effect. Chemical concentrations and physicochemical parameters were used to explain the biomarker responses, and the integrated biological response (IBR) was utilised to compare the overall stress levels in sampled organisms: between sites in the Vaal River, and spatially and between species in the Phongola Floodplain.

Chapter 5: Stable isotope analyses to determine bioaccumulation

Food web structures of the Phongola Floodplain, and the three sites from the Vaal River were assessed. Tropic positions of the species and trophic magnification factors were calculated to determine bioaccumulation of the xenobiotics through the food web. It is expected that compounds with a K_{ow} greater than five will tend to bioaccumulate.

Chapter 6: Conclusions and recommendations

A summary of the results obtained, conclusions, and recommendations future studies are discussed.

Chapter 7: References

List of all references used throughout the thesis.

2 Persistent organic pollutants in fish tissue from three sites in the Vaal River

2.1 Introduction

The Vaal River is the main river in central South Africa, with a total length of 1 120 km, and is the second largest in the country. It extends from Mpumalanga in the east, and flows westward to its confluence with the Orange River in the Northern Cape. The river flows through the Vaal Triangle, which is the most industrial region in southern Africa. It consists of iron and steel works, coal powered electricity generation plants, a petrochemical plant, and coal-based synthetic chemical manufacturing (Nieuwoudt *et al.*, 2009; Quinn *et al.*, 2009). The region also has numerous wet industries, gold mines, agricultural regions, and three large metropolitan cities (Sasolburg, Vanderbijilpark, and Vereeniging) where run-off, and rivers and streams flowing through these areas are tributaries to the Vaal River (Tempelhoff, 2009). In the upper catchment of the Vaal River there are large scale commercial farming activities (Quinn *et al.*, 2009). The river has been described as a work horse river due to all the activity (Braune and Roger, 1987).

The industries within the Vaal River catchment are known to release a wide range of pollutants into the environment. Pollutants of industrial origin and use like polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs), brominated flame retardants (BFR) like brominated diphenyl ethers (BDEs) had been detected in the Vaal Triangle, the Vaal River and its tributaries system (Polder *et al.*, 2008; Nieuwoudt *et al.*, 2009; Quinn *et al.*, 2009; Nieuwoudt *et al.*, 2011; Wepener *et al.*, 2011; Moja *et al.*, 2013; Vogt *et al.*, 2015; Pheiffer *et al.*, 2018b). Although South Africa ratified the Stockholm convention in 2001 and enforced it from 2004 (DEA, 2011) many banned POPs are still detected in this region, including aldrin, chlordane, dichlorodiphenyltrichloroethane (DDT), dieldrin, endrin, heptachlor, and hexachlorobenzene (HCB) (Quinn *et al.*, 2009; Wepener *et al.*, 2011; Pheiffer *et al.*, 2018c). These compounds are all persistent in the environment, hydrophobic, and adhere to organic matter like organic content in abiotic matrices and lipids in organisms (Jones and De Voogt, 1999). Once an organism takes up the contaminant it is slow to metabolise, and they tend to bioaccumulate (Vallack *et al.*, 1998; Jones and De Voogt, 1999). Often the metabolites, or breakdown products are more toxic than the parent compound (Eljarrat *et al.*, 2008; Yang and Chan, 2015; Reddy *et al.,* 2019).

These compounds can bioaccumulate and biomagnify, resulting in a food web being exposed to their highly toxic potential which includes cancer, endocrine disruption, and toxicities to the developmental, immune, and reproductive systems (Bouwman, 2003). Because of the detrimental effects these compounds cause, they have been listed in an international treaty to protect the environment and humans from these persistent organic pollutants (POPs) (Stockholm Convention, 2019). This treaty–The Stockholm Convention–initially listed 12 compounds/compound classes known as the “dirty dozen” and an additional 16 have been added since. There are over 180 parties that have ratified the convention with the intention of eliminating (Annex A), restricting (Annex B), or reduce unintentional production (Annex C) of POPs (Stockholm Convention, 2017; Stockholm Convention, 2019). DDT is the only restricted compound listed by the convention, and can only be used to combat the malaria vector mosquito in regions where malaria is an epidemic. South Africa ratified the convention in May 2001, and became a party in September 2002 (DEA, 2011).

Many studies in the Vaal River catchment had shown that because of the pollution the environment is negatively impacted in this area (Polder *et al.*, 2008; Tempelhoff, 2009; Wepener *et al.*, 2011; Rimayi *et al.*, 2016; Van der Schyff *et al.*, 2016). This is problematic because water from the catchment provides potable water for Gauteng, and fish, which are exposed, are an important food source for locals. If these fish and water are compromised with these compounds it is likely that it will cause detrimental effects to higher food web species (Gerber *et al.*, 2016) . There is also a risk to human health because they utilise the river for recreational activities and subsistence fishing (Pheiffer *et al.*, 2014).

The aim of this study was to determine the concentrations of various POPs in fish species from three regions in the Vaal River, namely Vischgat, Barrage, and Thabela Thabeng. It is expected that the fish would contain high concentrations POPs, particularly the industrial related BDEs and PCBs. It is expected that Barrage site would be the most contaminated due to the inflow of tributaries in that area that drain highly contaminated and industrial regions.

2.2 Materials and methods:

Fish were sampled in October 2014 from the Vaal River. The target fish were: *Clarias gariepinus, Labeobarbus aeneus, Cyprinus carpio*, and *Labeo capensis*, which are all omnivorous. The fish were caught by means of electro-shocking and fyke nets. The samples were collected from three sites in the Vaal River, Vischgat (28.062, -26.819) below the Vaal Dam, and Barrage (27.681, -26.767) and Thabela Thabeng (27.296, -26.864) further downstream (Figure 2.1). Muscle tissue samples were stored in precleaned aluminium foil, and

frozen at -80°C until analysis. The tissue samples were extracted and analysed in Belgium at the Toxicology Centre, University of Antwerp. The target compounds were: PCB congeners (PCB# 28, 49, 52, 74, 95, 99, 101, 105, 110, 118, 128, 138, 146, 149, 151, 153, 156, 170, 171, 174, 177, 180, 183, 187, 194, 195, 196/203, 199, 206, and 209), dichlorodiphenyltrichloroethane: *p,p'*-DDT and *o,p'*-DDT and metabolites: *p,p'*-DDD, *o,p'*-DDD, *p,p'*-DDE, and *o,p'*-DDE which will be referred to as DDx, β- and γ- HCH isomers, chlordane metabolites: oxychlordane (OxC), *cis*-nonachlor (CN) and *trans*-nonachlor (TN) which will be referred to as chlors, HCB, and polybrominated diphenyl ethers (PBDEs) (PBDE# 28, 47, 99, 100, 153, 154, and 183).

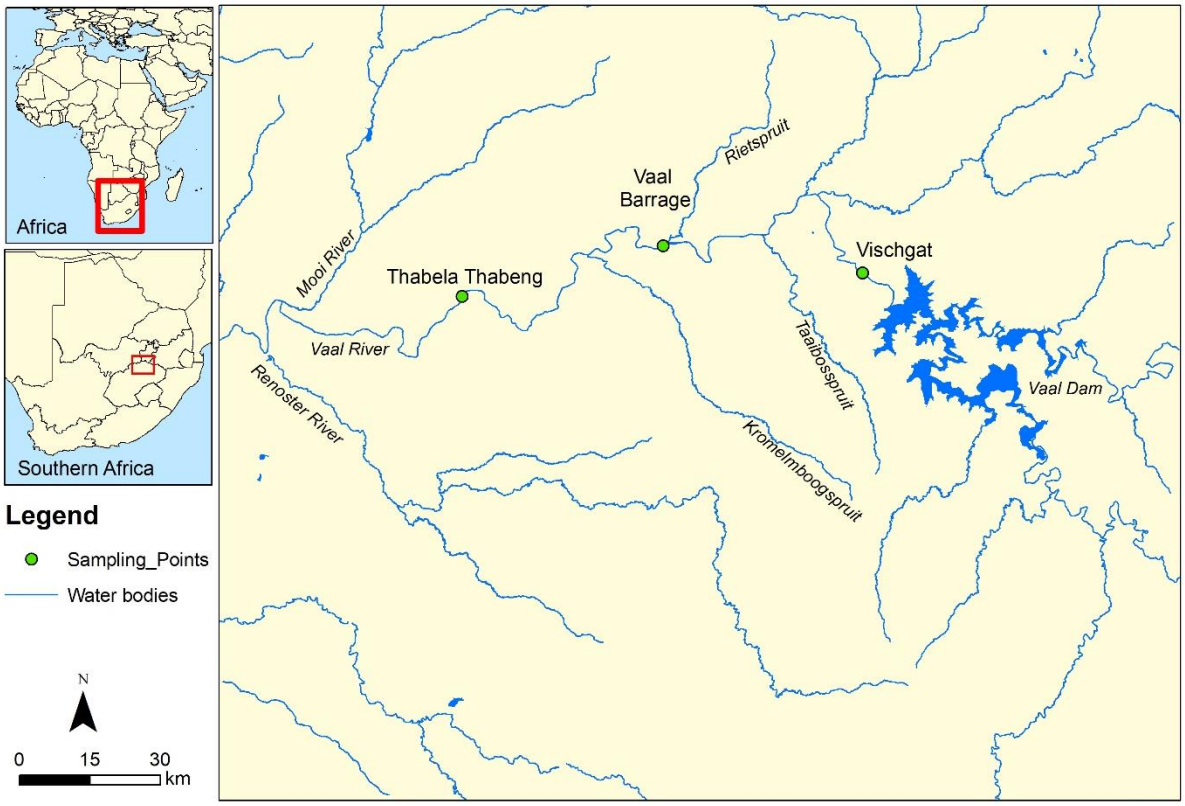


Figure 2.1 Vaal River sampling sites and area overview

Individual PCB standards were obtained from Dr. Ehrenstorfer Laboratories (Augsburg, Germany), OCP standards were obtained from Accustandard (New Haven, CT, USA). PBDE standard mixture was obtained from Wellington Laboratories (Guelph, Ontario, Canada). Analytical grade solvents, and silica gel (0.036–0.200 mm) were obtained from Merck, Darmstadt, Germany. Empty polypropylene columns for clean-up (25 mL) were purchased from Alltech (Lokeren, Belgium).

2.2.1 Quality assurance/control

The retention times, ion chromatograms, and relative abundance of the monitored ions were used as identification criteria. A deviation of ion abundance ratios within 15% of the mean values for calibration standards was considered acceptable. Quantification was based on five-point calibration curves. The peaks were positively identified as target compounds if: (1) the retention time matched that of the standard compound within ± 0.1 min and (2) the signal-to-noise ratio (S/N) was higher than 3:1. Procedural blanks were analysed simultaneously with every batch of seven samples to check for interferences or contamination from solvent and glassware. Procedural blanks were consistent (relative standard deviation (RSD) < 30%) and therefore the mean value was calculated for each compound and subtracted from the values in the samples. The limit of detection (LOD) was calculated as three times the standard deviation of the mean of the blank measurements and limits of quantification (LOQs) were calculated as 10 times the standard deviation of the mean of the blank (Verhaert *et al.*, 2013).

Mean ± standard deviations (SD) recoveries of the internal standards PCB 143, BDE 77 and ε-HCH were 86 ± 6%, 93 ± 10%, and 98 ± 8%, respectively. The analytical procedures were validated through the analysis of standard reference material in whale blubber (NIST, SRM 1945) for which deviations from certified values were less than 10%. The quality control scheme is assessed through successful participation to the Interlaboratory Comparison Exercise Program for Organic Contaminants in Marine Mammal Tissues organised by the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA).

2.2.2 Extraction and analyses

Methods used were adapted from those previously described by Verhaert *et al.* (2013). Approximately 5 g of wet fish muscle was homogenised with anhydrous sodium sulphate (Na_2SO_4), and enough Na_2SO_4 was added that the muscle was completely dry. The mixture was placed in extraction thimbles (25 x 100 mm, Whatman, England) and spiked with internal standards (PCB 143, PBDE 77, and ε-HCH). The samples were extracted in an automated hot Soxhlet (Buchi) for 2 hours with 100 mL 3:1 hexane:acetone (v:v). Lipid content was determined gravimetrically using an aliquot of the sample. The remaining sample was subjected to 8 g acidified silica (44% sulphuric acid, w:w), and analytes were eluted with 20 mL hexane, followed by 15 mL dichloromethane (DCM). The eluent was concentrated with a rotary evaporated and further concentrated under a gentle stream of nitrogen gas and reconstituted in 100 µL isooctane.

PBDEs, chlordanes, and higher mass PCBs were quantified with an Agilent 6890-5973 gas chromatograph coupled with a mass spectrometer system (GC-MS). The GC was equipped with a 30 m x 0.25 mm x 0.25 µm DB-5ms capillary column (J&W Scientific, Folsom, CA, USA) and the MS was operated in electron capture negative ionisation (ECNI) mode. Methane was used as reagent gas. The ion source, quadrupole, and interface temperatures were set at 170, 150 and 300°C, respectively. The MS was used in the selected ion-monitoring (SIM) mode with ions *m/z* = 79 and 81 for PBDEs acquired during the whole run, and with 2 most intense characteristic ions for every chlordane or PCB. Dwell times were set at 30 ms. One µl of the cleaned extract was injected in solvent vent mode (injector temperature: 90°C, held for 0.05 min, then with 700°C/min to 300°C and kept for 25 min; vent flow was set at 75 mL/min and the purge vent opened at 1.5 min). Helium was used as carrier gas at constant flow (1.0 mL/min). The temperature of the DB-5ms column was kept at 90°C for 1.50 min, then increased to 300°C at a rate of 10°C/min, kept for 20 min (Verhaert *et al.*, 2013).

For the measurements of lower mass PCBs, DDTs, and HCB, an Agilent 6890 GC–5973 MS system operated in electron ionisation (EI) mode equipped with a 25 m x 0.22 mm x 0.25 µm HT-8 capillary column was used (SGE, Zulte, Belgium). The ion source, quadrupole and interface temperatures were set at 230, 150 and 300°C, respectively. One µl of the cleaned extract was injected in cold pulsed splitless mode (injector temperature 90°C (0.03 min) rising to 300°C with 700°C/min), pressure pulse 25 psi (172 kPa) and pulse time 1.50 min. The splitless time was 1.50 min. Helium was used as the carrier gas at constant flow (1.0 mL/min). The temperature of the HT-8 column was kept at 90°C for 1.50 min, then increased to 180°C at a rate of 15°C/min (kept for 2.0 min), further increased to 280°C at a rate of 5°C/min and finally raised to 300°C at a rate of 40°C/min, and kept for 20 min. The MS was used in the selected ion-monitoring (SIM) mode with 2 ions monitored for each PCB homologue group or individual OCP. Dwell times were set to 30 ms.

For concentrations <LOQ a surrogate value of detection frequency x LOD was used (Verhaert *et al.*, 2013). All results are expressed as ng/g wet mass (wm). GraphPad Prism 6 was utilised for statistical analyses. Mann-Whitney U tests were used to determine significance between medians in data, with significance set at $p<0.05$.

2.3 Results

PCBs were the organic pollutant class with greatest concentrations at all sample areas, and had the greatest frequency of detection of all compounds in fish tissues, followed by DDx, and

BDEs (Table 2.1). HCB was the least frequently quantified compound, and was only found in *L. aeneus* and *L. capensis* from Barrage.

At Vischgat chlors and HCHs were detected at low concentrations and they were only quantifiable in *L. capensis* (Table 2.1). DDx and PCBs were quantified in all the fish species samples at Vischgat. *L. aeneus* was the only species at Vischgat that did not have any PBDEs.

PCBs were the dominant contaminant in all the fish species from Thabela Thabeng, with the highest concentrations in *C. gariepinus,* followed by *C. carpio, L. aeneus, and L. capensis* (Table 2.1). DDx and BDEs were also found in all fish sampled, at lower concentrations than the PCBs. HCHs were quantified at low concentrations in all species except *C. carpio*. Chlors were only detected at low concentrations in *C. gariepinus,* and HCB was not detected at all.

PCBs and DDx had the highest concentrations in fish from Barrage. *L. capensis* had the highest concentrations of the fish sampled with mean concentration of 16.4 ng/g wm (Table 2.1). All organic contaminant groups were detected at this site, with the exception of chlors and HCB in *C. carpio.* Barrage had the highest concentrations of the sampled sites.

Because there were few individuals per species sampled at the three sites the species have been pooled for the remainder of the chapter. This could be done because the fish belong to a similar trophic level, and there were no statistically significant differences between species from the respective sampling sites.

Table 2.1 Mean (±SD) and minimum–maximum concentrations (ng/g wm) of organic contaminants in various fish species from the three sample sites in the Vaal River: Vischgat, Barrage, and Thabela Thabeng

			ΣChlors	HCB	ΣDDx	ΣHCHs	ΣOCPs	ΣPCBs	ΣBDE	ΣXenobiotics
Vischgat	*C. gariepinus* (n=2)	Mean ± SD Range	ND	ND	0.77 ± 0.16 0.65–0.89	ND	0.85 ± 0.16 0.73–0.96	1.1 ± 0.94 0.41–1.8	0.04 ± 0.02 0.03–0.05	2.0 ± 1.2 1.2–2.9
	L. aeneus (n=3)	Mean ± SD Range	ND	ND	0.74 ± 0.21 0.52–0.93	ND	0.82 ± 0.21 0.6–1.0	1.0 ± 0.97 0.37–2.1	ND	1.9 ± 1.2 0.94–3.2
	L. capensis (n=9)	Mean ± SD Range	0.02 ± 0.04 0.01–0.13	ND	0.63 ± 0.88 0.2–2.9	0.09 ± 0.22 0.02–0.67	0.77 ± 1.1 0.26–3.7	1.0 ± 1.8 0.31–5.8	0.03 ± 0.05 0.01–0.16	1.8 ± 3.0 0.61–9.6
	Mean	Mean ± SD Range	0.02 ± 0.03 <LOD–0.13	ND	0.71 ± 0.69 0.24–2.9	0.06 ± 0.18 0.01–0.67	0.79 ± 0.88 0.26–3.7	1.1 ± 1.5 0.34–5.8	0.03 ± 0.04 0.01–0.16	1.9 ± 2.4 0.61–9.6
Barrage	*C. carpio* (n=3)	Mean ± SD Range	ND	ND	2.4 ± 0.98 1.9–3.6	0.16 ± 0.08 0.11–0.26	2.7 ± 0.95 2.1–18	8.2 ± 4.4 5.6–13	0.35 ± 0.23 0.2–0.61	12 ± 5.5 8.2–18
	L. aeneus (n=3)	Mean ± SD Range	0.09 ± 0.16 0–0.27	0.09 ± 0.1 0.03–0.2	2.6 ± 1.7 0.98–4.3	0.29 ± 0.29 0.08–0.62	5.2 ± 3.3 1.7–35	8.4 ± 4.1 4.4–13	0.39 ± 0.19 0.23–0.6	20 ± 14 5.8–35
	L. capensis (n=2)	Mean ± SD Range	0.39 ± 0.17 0.27–0.52	0.3 ± 0.36 0.05–0.56	7.0 ± 3.7 4.3–9.6	0.57 ± 0.07 0.52–0.62	5.9 ± 7.4 0.7–11	16 ± 5.3 13–20	0.94 ± 0.47 0.6–1.3	18 ± 21 3.0–33
	Mean	Mean ± SD Range	0.2 ± 0.18 0.08–0.52	0.11 ± 0.19 0.03–0.56	3.84 ± 3.21 0.49–9.58	0.28 ± 0.19 0.11–0.52	4.4 ± 3.6 0.7–11	11 ± 8.2 2.2–20	0.56 ± 0.47 0.13–1.3	16 ± 12 3.0–35
Thabela Thabeng	*C. gariepinus* (n=5)	Mean ± SD Range	0.15 ± 0.13 0.08–0.38	ND	1.5 ± 1.4 0.3–3.7	0.04 ± 0.05 0.02–0.13	1.6 ± 1.6 0.29–4.3	2.5 ± 2.0 0.84–5.5	0.12 ± 0.11 0.05–0.29	4.1 ± 3.8 0.93–10
	C. carpio (n=2)	Mean ± SD Range	ND	ND	0.54 ± 0.15 0.43–0.65	ND	0.62 ± 0.15 0.51–0.73	2.1 ± 0.43 0.71–1.3	0.06 ± 0.06 0.03–0.1	1.8 ± 0.7 1.3–2.3
	L. aeneus (n=5)	Mean ± SD Range	ND	ND	0.9 ± 0.64 0.3–1.9	0.09 ± 0.07 0.04–0.18	0.98 ± 0.72 0.29–2.1	1.5 ± 0.97 0.69–3.1	0.66 ± 1.23 0.07–2.86	3.1 ± 2.1 0.98–5.4
	L. capensis (n=5)	Mean ± SD Range	ND	ND	0.32 ± 0.31 0.12–0.84	0.05 ± 0.07 0.02–0.17	0.49 ± 0.34 0.29–1.1	0.65 ± 0.43 0.28–1.3	0.02 ± 0.02 0.01–0.06	1.5 ± 0.72 0.93–2.7
	Mean	Mean ± SD Range	0.05 ± 0.09 0.02–0.38	ND	0.88 ± 0.91 0.25–3.74	0.06 ± 0.06 0.02–0.19	1.0 ± 1.0 0.29–4.3	1.5 ± 1.3 0.59–5.6	0.25 ± 0.68 0.04–2.9	2.8 ± 2.5 0.93–10

ND = no detect

2.3.1 Organochlorine pesticides (OCPs)

2.3.1.1 Chlordanes

Concentrations of Σchlors were significantly higher ($p<0.05$) in fish at Barrage compared to the concentrations of chlors at the other two sites (Table 2.2). Fish at Thabela Thabeng had quantifiable concentrations of all chlor compounds analysed, whereas the very high concentrations in the fish from Barrage were only made up of two of the chlor compounds, TN and CN. In all the fish samples across the three sites the most dominant chlor compound was TN (Figure 2.2). In fish from Barrage, CN had a similar proportion to TN.

Table 2.2 Mean (±SD) and minimum–maximum concentrations (ng/g wm) of ∑chlordanes and the chlordane congeners in various fish species from the three sample sites in the Vaal River: Vischgat, Barrage, and Thabela Thabeng

		OxC	TN	CN	ΣChlors
Vischgat (n=14)	Mean ± SD Range	ND	0.02 ± 0.04 0.01–0.13	ND	0.02 ± 0.03 0–0.13
Barrage (n=8)	Mean ± SD Range	ND	0.11 ± 0.11 0.04–0.31	0.09 ± 0.07 0.04–0.21	0.2 ± 0.2 0.08–0.52
Thabela Thabeng (n=17)	Mean ± SD Range	0.01 ± 0.02 0.01–0.1	0.03 ± 0.04 0.01–0.2	0.01 ± 0.02 0.01–0.1	0.05 ± 0.1 0.02–0.38

ND = no detect

ΣChlors = Oxychlordane (OxC), *trans*-nonachlor (TN), and *cis*-nonachlor (CN).

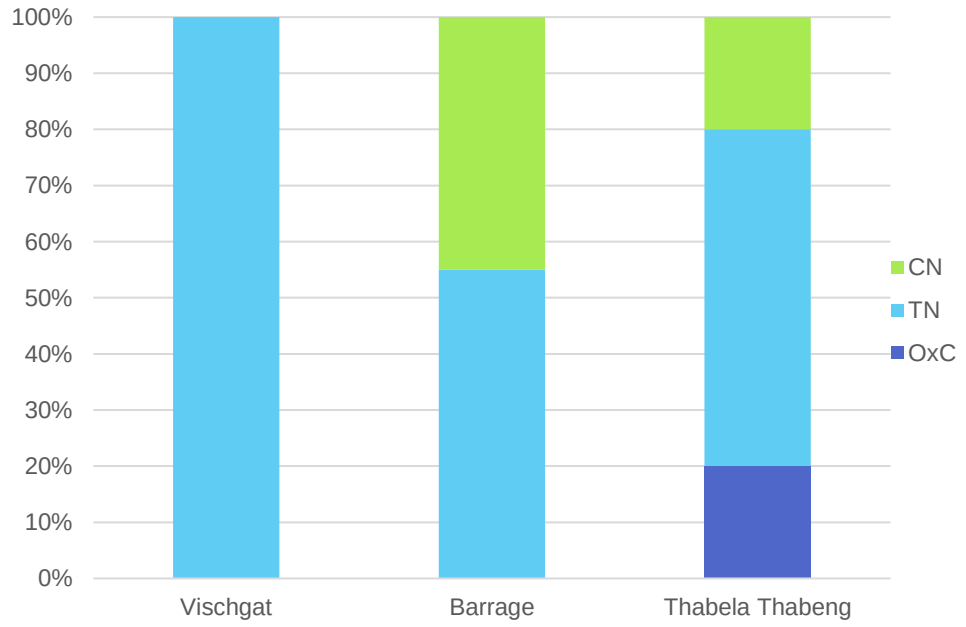


Figure 2.2 Contributions of the chlordanes in fish sampled from the three sites in the Vaal River

2.3.1.2 Hexachlorobenzene (HCB)

Only fish sampled at Barrage had quantifiable concentrations of HCB in the muscle tissue (Table 2.2).

2.3.1.3 Dichlorodiphenyltrichloroethane (DDx)

The concentrations of DDx in fish were the greatest at Barrage (Table 2.3), and was significantly greater ($p<0.05$) than concentrations in fish from Thabela Thabeng. The lowest concentrations were in fish sampled from Vischgat, however it also had the most DDx congeners detected (Table 2.3), and was the only site to have quantifiable concentrations of *p,p'*-DDT. This is also the form of DDT that contributes the greatest (72–75%) to technical DDT. The most dominant DDTs in fish from all sites was *p,p'*-DDE, and to a much lesser extent *p,p'*-DDD (Figure 2.3).

Table 2.3 Mean (±SD) and minimum–maximum concentrations (ng/g wm) of DDTs and metabolites in various fish species from the three sample sites in the Vaal River: Vischgat, Barrage, and Thabela Thabeng.

		***o,p'*-DDE**	***p,p'*-DDE**	***o,p'*-DDD**	***p,p'*-DDD**	***o,p'*-DDT**	***p,p'*-DDT**	**ΣDDx**
Vischgat (n=14)	Mean ± SD Range	ND	0.6 ± 0.55 0.17–2.2	ND	0.05 ± 0.1 0.02–0.4	ND	0.07 ± 0.08 0.04–0.28	0.71 ± 0.7 0.24–2.9
Barrage (n=8)	Mean ± SD Range	ND	3.2 ± 2.8 0.26–7.9	ND	0.6 ± 0.49 0.23–1.6	ND	ND	3.8 ± 3.2 0.49–9.6
Thabela Thabeng (n=17)	Mean ± SD Range	ND	0.8 ± 0.8 0.21–3.2	ND	0.08 ± 0.13 0.04–0.53	ND	ND	0.88 ± 0.91 0.25–3.7

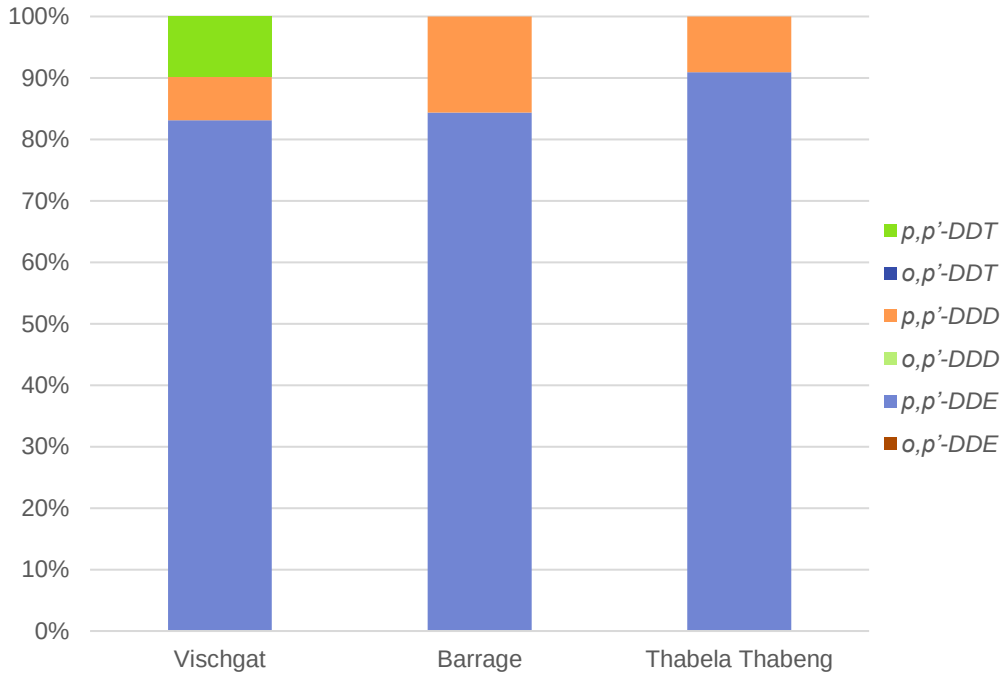


Figure 2.3 Contributions of DDTs and its metabolites in fish sampled from the three sites in the Vaal River

2.3.1.4 Hexachlorocyclohexane (HCH)

Concentrations of HCHs at Barrage were the highest reported in fish across the study sites (Table 2.4), it was also significantly greater ($p<0.05$) than the concentrations at Vischgat. The concentrations in fish sampled from Thabela Thabeng and Vischgat were approximately the same (0.06 ng/g wm). Of the two analysed HCH isomers, γ-HCH was dominant and contributed 66–79% of ΣHCH.

Table 2.4 Mean (±SD) and minimum–maximum concentrations (ng/g wm) of HCHs in various fish species from the three sample sites in the Vaal River: Vischgat, Barrage, and Thabela Thabeng.

		β-HCH	γ-HCH	ΣHCH
Vischgat (n=14)	Mean ± SD	0.02 ± 0.06	0.04 ± 0.12	0.06 ± 0.2
	Range	0.01–0.23	0.01–0.45	0.01–0.67
Barrage (n=8)	Mean ± SD	0.05 ± 0.05	0.22 ± 0.14	0.28 ± 0.2
	Range	0.03–0.14	0.09–0.38	0.11–0.5
Thabela Thabeng (n=17)	Mean ± SD	0.01 ± 0.03	0.04 ± 0.06	0.06 ± 0.06
	Range	0.01–0.13	0.02–0.18	0.02–0.2

2.3.2 Polybrominated diphenyl ethers (PBDEs)

Concentrations of PBDEs were greatest in fish from Barrage (Table 2.5), and these were significantly higher than the other two sites ($p<0.05$). Fish from Thabela Thabeng also had concentrations of PBDEs that were significantly greater than those from Vischgat. Fish

samples at Vischgat had equal proportions of the PBDE 28, -47, and -99 congeners and these were also the only congeners present in the fish from this site (Figure 2.4). The ΣPBDEs in fish from Thabela Thabeng and Barrage was dominated by PBDE 47 (Figure 2.4). The next greatest contributor to the ΣPBDEs was PBDE 99, and -100 in Thabela Thabeng and Barrage, respectively. The PBDE 153, and -183 congeners were not detected in any of the fish at any of the sites.

Table 2.5 Mean (±SD) and minimum–maximum concentrations (ng/g wm) of PBDEs in various fish species from the three sample sites in the Vaal River: Vischgat, Barrage, and Thabela Thabeng.

		PBDE 28	PBDE 47	PBDE 100	PBDE 99	PBDE 154	PBDE 153	PBDE 183	ΣPBDE
Vischgat (n=14)	Mean ± SD Range	0.01 ± 0.01 0.003–0.05	0.01 ± 0.04 0.003–0.16	ND	0.01 ± 0.01 0.003–0.05	ND	ND	ND	0.03 ± 0.04 0.01–0.16
Barrage (n=8)	Mean ± SD Range	0.06 ± 0.06 0.02–0.19	0.34 ± 0.29 0.04–0.77	0.08 ± 0.06 0.03–0.18	0.03 ± 0.04 0.01–0.01	0.06 ± 0.04 0.03–0.13	ND	ND	0.56 ± 0.47 0.13–1.28
Thabela Thabeng (n=17)	Mean ± SD Range	ND	0.14 ± 0.38 0.02–1.62	0.03 ± 0.07 0.01–0.3	0.07 ± 0.16 0.01–0.68	0.02 ± 0.06 0.003–0.27	ND	ND	0.25 ± 0.68 0.04–2.86

ΣPBDEs = PBDE-28, -47, -99, -100, -153, -154, and -183

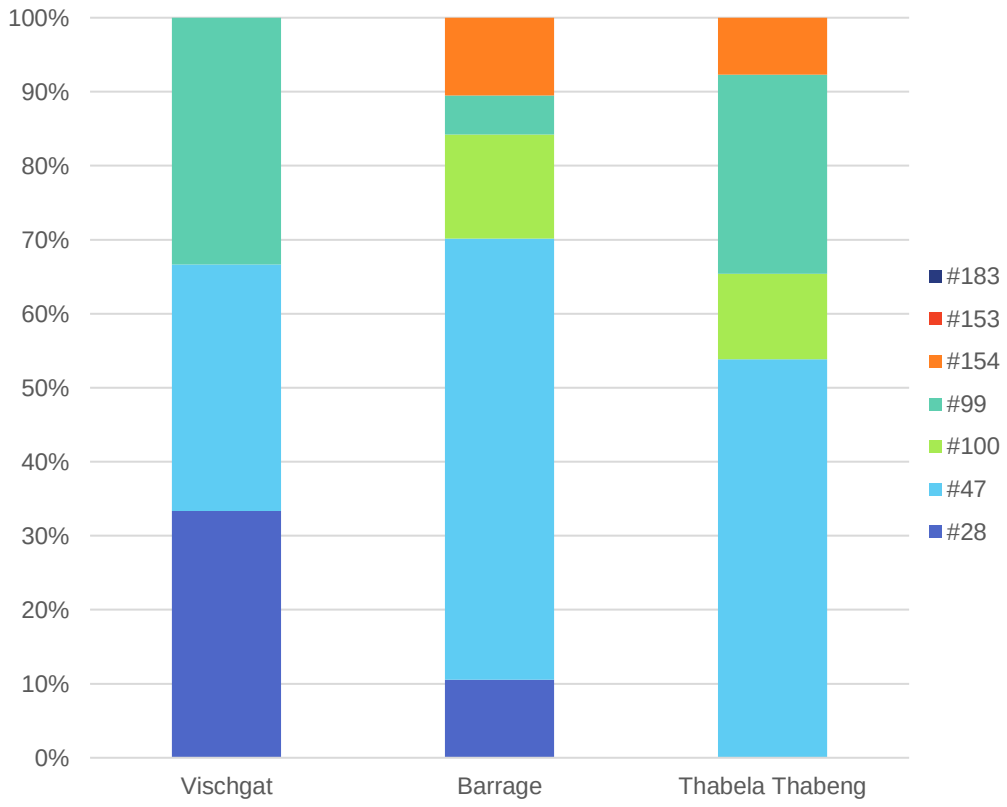


Figure 2.4 Contributions of PBDE congeners in fish sampled from the three sites in the Vaal River

2.3.3 Polychlorinated biphenyls (PCBs)

The concentrations of PCBs were significantly greatest in fish sampled from Barrage ($p<0.05$), with as much as an order of magnitude difference between the concentrations at Vischgat and

Thabela Thabeng (Table 2.6). The fish from Barrage had the most number of congeners detected (Figure 2.5). The most dominant PCB congeners from the greatest contribution were PCB 153, -138, -149, -180 (Figure 2.5). Penta-, hexa-, hepta-, octa-chlorinated biphenyls were the most commonly detected congeners.

Table 2.6. Mean (±SD) and minimum–maximum concentrations (ng/g wm) of PCB congeners in various fish species from the three sample sites in the Vaal River: Vischgat, Barrage, and Thabela Thabeng.

	Vischgat (n=14)		Barrage (n=8)		Thabela Thabeng (n=17)	
	Mean ± SD	Mean ± SD	Range	Range	Mean ± SD	Range
PCB 28	ND		0.63 ± 0.47	0.13– 1.16		ND
PCB 52	0.04 ± 0.1	0.69 ± 0.43	0.18– 1.34	0.01–0.37	0.03 ± 0.06	0.01– 0.27
PCB 49	0.03 ± 0.06	0.37 ± 0.25	0.13– 0.72	0.01–0.24	ND	
PCB 74	ND		0.28 ± 0.21	0.1– 0.59		ND
PCB 95	0.02 ± 0.05	0.41 ± 0.29	0.09– 0.8	0.01–0.21	0.06 ± 0.04	0.03– 0.16
PCB 101	0.07 ± 0.1	0.62 ± 0.47	0.09– 1.17	0.02–0.33	0.09 ± 0.07	0.04– 0.26
PCB 99	0.03 ± 0.04	0.23 ± 0.15	0.08– 0.46	0.01–0.13	0.06 ± 0.07	0.02– 0.22
PCB 105	0.01 ± 0.03	0.17 ± 0.15	0.05– 0.34	0.01–0.1	0.01 ± 0.04	0.01– 0.15
PCB 118	0.06 ± 0.08	0.41 ± 0.3	0.09– 0.81	0.02–0.27	0.08 ± 0.09	0.03– 0.33
PCB 110	0.04 ± 0.06	0.51 ± 0.4	0.09– 1.01	0.01–0.24	0.08 ± 0.06	0.04– 0.24
PCB 151	0.02 ± 0.04	0.22 ± 0.15	0.08– 0.41	0.01–0.17	0.01 ± 0.03	0.01– 0.11
PCB 149	0.05 ± 0.11	0.91 ± 0.72	0.09– 1.69	0.01–0.43	0.16 ± 0.1	0.06– 0.38
PCB 146	0.05 ± 0.05	0.28 ± 0.21	0.09– 0.53	0.02–0.17	0.04 ± 0.05	0.02– 0.19
PCB 153	0.24 ± 0.31	1.68 ± 1.4	0.09– 2.95	0.05–1.04	0.35 ± 0.31	0.08– 1.31
PCB 138	0.11 ± 0.15	0.73 ± 0.61	0.09– 1.27	0.03–0.54	0.17 ± 0.14	0.07– 0.61
PCB 187	0.07 ± 0.11	0.6 ± 0.46	0.09– 1	0.02–0.35	0.11 ± 0.1	0.05– 0.38
PCB 183	0.02 ± 0.03	0.22 ± 0.16	0.08– 0.34	0.01–0.13	0.02 ± 0.03	0.01– 0.13
PCB 128	ND		0.13 ± 0.09	0.05– 0.21		ND
PCB 174	0.02 ± 0.03	0.23 ± 0.18	0.06– 0.36	0.01–0.13	ND	
PCB 177	ND		0.17 ± 0.14	0.05– 0.3		ND
PCB 171	ND		0.06 ± 0.04	0.04– 0.1		ND
PCB 156	ND		0.06 ± 0.04	0.04– 0.11		ND
PCB 180	0.1 ± 0.13	0.72 ± 0.54	0.09– 1.17	0.04–0.51	0.15 ± 0.14	0.06– 0.57
PCB 170	0.02 ± 0.06	0.3 ± 0.21	0.09– 0.48	0.01–0.22	0.05 ± 0.07	0.02– 0.25
PCB 199	0.02 ± 0.03	0.18 ± 0.14	0.06– 0.31	0.01–0.12	0.02 ± 0.03	0.01– 0.12
PCB 196/203	0.02 ± 0.05	0.2 ± 0.13	0.08– 0.32	0.01–0.18	0.03 ± 0.05	0.01– 0.17
PCB 194	ND		0.02 ± 0.01	0.01– 0.05		ND
PCB 206	0.02 ± 0.03	0.1 ± 0.06	0.05– 0.18	0.01–0.13	0.02 ± 0.03	0.01– 0.11
PCB 209	ND		ND			ND
ΣPCBs	1.1 ± 1.5	11 ± 8.2	2.2– 20.15	0.34–5.8	1.5 ± 1.3	0.59– 5.6

ΣPCBs = PCB-28, -52, -49, -74, -95, -101, -105, -110, -118, -128, -138, -146, -149, -151, -153, -156, -170, -171, -174, -177, -180, -183, -187, -194, -195, -196/203, -199, -206, and -209

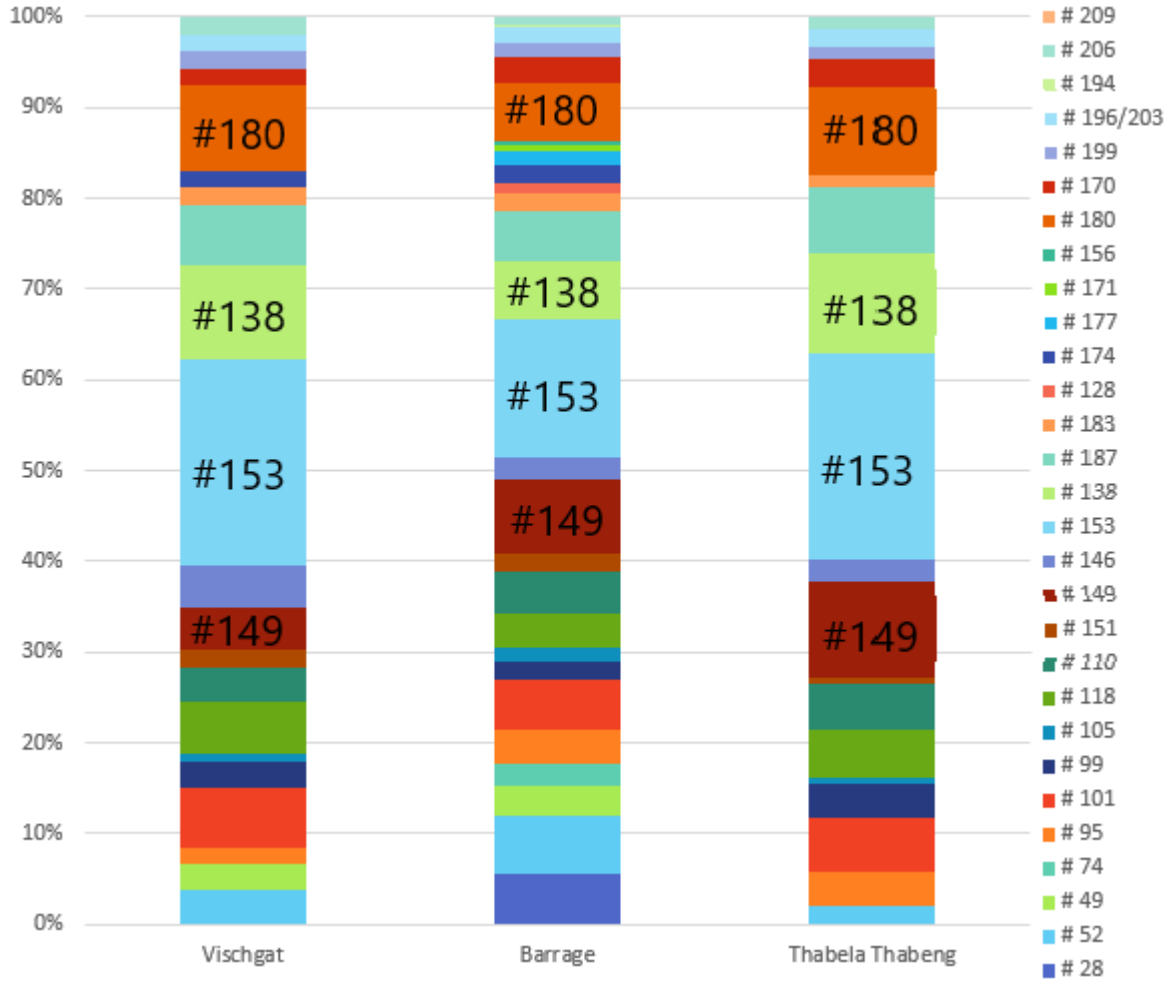


Figure 2.5 Contributions of PCBs in fish sampled from the three sites in the Vaal River

2.4 Discussion

Very low concentrations of Σchlors were detected in fish in the Vaal River. This is likely because the Vaal is a highly industrialised area and therefore it is unlikely that this banned OCP was used abundantly in the region (Batterman *et al.*, 2008). Oxychlordane, the primary metabolite of chlordane (Batterman *et al.*, 2008), is generally absent which supports the lack of use of the OCP in the area. The low concentrations present across the catchment may be attributed to air deposition, some localised application of the pesticide, or historical use. Without having analysed chlordane, itself, current use of the pesticide could not be determined. However, with the low concentrations of the breakdown products, this is unlikely.

Similarly, low concentrations were quantified from fish from the Olifants River (Verhaert *et al.*, 2017), and Tanzania (Polder *et al.*, 2014) (Table 2.7). There were higher concentrations reported from fish in Lake Awassa, Ethiopia (Deribe *et al.*, 2014) and in fish sourced from rivers in the vicinity of the Kruger National Park (Gerber *et al.*, 2016) , although this was in a high

trophic species, *H. vittatus*, and higher levels are expected due to bioaccumulation. Much higher concentrations were quantified in *C. gariepinus* from dams in the North West and Gauteng provinces (Barnhoorn *et al.*, 2015). A study in the Klip River, one of the tributaries of the Vaal River, documented concentrations of chlordanes much higher than what was reported in this study (Pheiffer *et al.*, 2018c). However, the number of chlordane compounds analysed in that study were greater than what was targeted in the current study. Higher concentrations were also quantified at Barrage in *L. capensis* sampled in an earlier study (Wepener *et al.*, 2011).

HCB was seldom quantified in the Vaal, likely because it is an insecticide used in agriculture and this study sampled from the industrialised stretch of the river (Shatalov *et al.*, 2004). Like chlordanes, it is probable that the little HCH that was detected at the Barrage site was due to localised use, atmospheric deposition, or historical use. Similar concentrations were reported in *Oreochromis* sp from Tanzania (Polder *et al.*, 2014), *H. vittatus* from Olifants, Letaba, and Luvuvhu Rivers (Gerber *et al.*, 2016), and fishes from the Democratic Republic of Congo (DRC) (Verhaert *et al.*, 2013) (Table 2.7). Concentration in *L. capensis* from Barrage sampled previous to this study were much greater than what was reported in this study (Wepener *et al.*, 2011). *Clarias gariepinus* from the Klip River also had higher concentrations (Pheiffer *et al.*, 2018c).

There were detectable concentrations of DDx at all of the sample sites and within all of the species sampled. DDT is known to breakdown into DDE and DDD. In aerobic environments *p,p'*-DDE is formed, and this metabolite is highly persistent and bioaccumulative in lipids (Fisher, 1999; Bornman *et al.*, 2010). In anaerobic environments, DDD is formed (Fisher, 1999). Using ratios of the different DDTs and metabolites it can be determined if the contamination is due to recent or historic use (Doong *et al.*, 2002; Zhou *et al.*, 2006). In the Vaal system the majority of the quantified DDx was made up of the metabolites. The only instances of specifically DDT was in *L. capensis* at the Vischgat site. However, their contribution to the ΣDDx was very low 0.1–0.5%, which indicates historical use of the pesticide. This is expected because this pesticide had been restricted for use in malaria regions only (Stockholm Convention, 2019). The metabolite, *p,p'*-DDE, was the most dominant DDx found in tissue from the Vaal River, and in other studies in South Africa (Barnhoorn *et al.*, 2015; Pheiffer *et al.*, 2018c) which is expected because it is the most persistent and bioaccumulative form (Fisher, 1999; Bornman *et al.*, 2010). Notwithstanding, DDT and metabolites had also been detected in other inland water bodies in Gauteng and North-West provinces, neither of which are malaria areas (Barnhoorn *et al.*, 2015; Pheiffer *et al.*, 2018c).

The concentrations recorded from the Vaal were lower than the other regions in South Africa (Barnhoorn *et al.*, 2015; Gerber *et al.*, 2016; Verhaert *et al.*, 2017; Pheiffer *et al.*, 2018c), and slightly less than the previous study conducted at the Barrage (Wepener *et al.*, 2011) (Table 2.7). The general low concentrations of DDx across the sites point towards low DDT input in the region, and seems to be decreasing with time—especially the reduction in DDx at Barrage between the 2011 study and this one. It could also be a result of DDx in the system being somewhat unavailable due to sedimentation in the area (Chutter, 1969) covering up the DDx. Concentrations from Lake Koka, Ethiopia (Deribe *et al.*, 2011), and Tanzania (Polder *et al.*, 2014) are similar to or slightly higher than reported in this study, while fish from Lake Awassa (Deribe *et al.*, 2014), Ethiopia had approximately an order of magnitude higher levels. Fish from the Congo River in the DRC (Verhaert *et al.*, 2013) had lower concentrations than reported in this study, even though the use of DDT for malaria vector control had been reintroduced to the area since 2008 (WHO, 2011). It would be expected that a region with active spraying of DDT would have higher concentrations.

HCHs were detected in most species at the sample sites, at fairly low concentrations. Lindane or γ-HCH is the only HCH isomer with significant insecticidal activity, however during manufacturing tons of other HCHs are produced as so-called waste isomers (Vijgen *et al.*, 2006). Lindane degrades quickly to form the highly stable and most persistent of the isomers β-HCH (Willett *et al.*, 1998). Therefore, if lindane is present it indicates recent application of the pesticide, whereas the dominance of β-HCH indicates a historical input of the pesticide. Across the sampling regions there seemed to be slight differences in the fingerprints. At Vischgat, only one individual *L. capensis* had quantifiable concentrations of both analysed HCHs—the γ-HCH was only slightly higher than β-HCH. This implies recent use of the pesticide because lindane degrades rapidly into β-HCH. At the Thabela Thabeng site *L. aeneus* and *L capensis* only had presence of γ-HCH, and *C. gariepinus* only had β-HCH. This likely indicates a mixture of fresh and a degraded form of the pesticide, or application of technical grade HCH which contains approximately 60–70% α-, 5–12% β-, and 10–15% γ-HCH (Iwata *et al.*, 1995; Willett *et al.*, 1998; Jackovitz and Hebert, 2015; Yuan *et al.*, 2017). The fish at Barrage had greater proportions of γ- compared to β-HCH and in the case of *C. carpio* the ΣHCH was comprised solely of γ-HCH which is also an indication that the banned lindane is being used, even though these are low concentrations. These low concentrations could be due to deposition by long range transport (Vijgen *et al.*, 2006), or limited local use. This OCP is known to be very volatile, and a study documented that only 10.4% of the applied ΣHCH in soils remained after 100 days due to volatilisation of the HCH (Kaushik, 1991).

Concentrations of HCHs in the current study are greater than those reported in the Olifants River (Verhaert *et al.*, 2017) (Table 2.7). Gerber *et al.* (2016) reported higher concentrations of HCHs in the Letaba and Luvuvhu Rivers than those in the Vaal River. Again, as for the DDx, this may be attributed to the use of a higher trophic level fish, *H. vittatus*, that were used in the Gerber *et al.* (2016) study. The Barnhoorn *et al.* (2015) study documented concentrations in fish approximately two to three orders of magnitude higher than the current study. *C. gariepinus* from the Klip River also had higher concentrations in the range of one to two orders of magnitude (Pheiffer *et al.*, 2018c). The concentrations in the current study were comparable to concentrations reported in the DRC (Verhaert *et al.*, 2013) and Tanzania (Polder *et al.*, 2014).

The PCBs contributed the greatest of all the contaminants targeted; this is due to the area being highly industrialised (Nieuwoudt *et al.*, 2009; Pheiffer *et al.*, 2014). The congeners that make up the greatest proportion to the ΣPCBs were PCB-153, -138, 180, -187, -149, -99, -101, -118, and -110 (Figure 2.5). Only three of the dioxin-like (DL) PCBs were analysed—PCB-105, -118, and -156– these are the most toxic PCBs and are known for a variety of adverse effects (Van den Berg *et al.*, 2006). Of these three, PCB-105 and -118 contributed moderately to the ΣPCBs, while PCB-156 only contributed to the ΣPCBs at Barrage, and this was 0.46–1%. Overall the higher chlorinated PCBs were most commonly detected, these are also the most persistent congeners (Ren *et al.,* 2016; Dhakal *et al.,* 2017).

The concentrations in the current study had higher concentrations than those reported by Verhaert, *et al* (2013, 2017) in the Olifants River and Congo River, Polder *et al.* (2014) in Tanzania, and both studies in Ethiopia by Deribe et al (2011, 2014) (Table 2.7). Concentrations at the Barrage were higher in the previous study (Wepener *et al.*, 2011) and a study in the Port Elizabeth harbour (Kampire *et al.*, 2015).

Considering the highly industrial region, and all the urban runoff the Vaal catchment receives, the concentrations of PBDEs were low. PBDE-49 was by far the most dominant congener present, approximately 50% or greater, followed by BDE-100, -154, and -183. This is a common BDE profile due to the use of these penta-BDEs as flame retardants (Verhaert *et al.*, 2013). A similar congener profile was also documented in sediment from Durban harbour (La Guardia *et al.*, 2013).

Even though these concentrations seem low, they are in a similar range, even slightly higher than other studies (Verhaert *et al.*, 2013; Polder *et al.*, 2014; Verhaert *et al.*, 2017) (Table 2.7). This includes the previous study at the Barrage site (Wepener *et al.*, 2011), which up to now

had higher concentrations for the pesticides and PCBs than the current study. Another study in the Vaal, with sampling conducted at the Barrage had concentrations that were lower than these reported in this study (Chokwe *et al.*, 2015). However, there are limited studies done, particularly in South Africa on PBDEs in tissues.

Table 2.7 Concentrations of OCPs, PCBs, and PBDEs analysed in this study and others from South Africa, and Africa

Region	Species	ΣChlors		HCB		ΣDDx		ΣHCHs		ΣPCBs		ΣPBDE		Reference
		ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	
Vischgat (This study)		0.02 ± 0.03	3.0 ± 11	ND		0.71 ± 0.69	120 ± 230	0.06 ± 0.18	15 ± 55	1.05 ± 1.5	200 ± 470	0.03 ± 0.04	4.7 ± 13	This study
		<LOD–0.13	0.1–41			0.24–2.9	3.6–880	0.01–0.67	0.24–210	0.34–5.8	6.4–1800	0.01–0.16	0.17–48	
Thabela Thabeng (This study)		0.02 ± 0.02	2.1 ± 4.6	ND		0.25 ± 0.44	75 ± 64	0.02 ± 0.02	3.1 ± 5.2	0.59 ± 0.85	120 ± 99	0.04 ± 0.04	22 ± 71	
		0.02–0.38	0.47–16			0.25–3.7	4.1–240	0.02–0.19	0.47–17.41	0.59–5.5	12–402	0.04–2.9	0.82–300	
Barrage (This study)		0.2 ± 0.18	8.6 ± 12	0.11 ± 0.19	3.3 ± 5.8	3.8 ± 3.2	210 ± 190	0.28 ± 0.19	13 ± 6.7	11 ± 8.2	630 ± 630	0.56 ± 0.47	30 ± 32	
		0.08–0.52	1.6–36	0.03–0.56	0.5–16	0.49–9.6	8.2–640	0.11–0.52	2.3–22	2.2–20	45–2100	0.13–1.3	2.6–104	
Olifants River SA	*C. gariepinus*	0.1±0.1				16 ± 13		<LOQ		0.3±0.4		<LOD		Verhaert *et al.* (2017)
		<LOQ–0.2				1–6		<LOQ		<LOQ–2.3				
	Labeo rosae	<LOQ				5.1 ± 3.2		<LOQ		<LOQ		<LOD		
		<LOQ				1.4–7.8		<LOQ						
	Labeo congoro	<LOQ				1.2 ± 0.5		<LOQ		0.1±0.01		<LOD		
		<LOQ				0.4–1.4		<LOQ		<LOQ–0.5				
	Labeobarbus marequensis	0.3±0.1				1.6 ± 0.7		0.1–0.2		<LOQ		<LOD		
		<LOQ–0.2				<LOQ–2.5		0.2 ± 0.1						
	S. zambezensis	0.4±0.04				18±3.3		<LOQ–0.8		1.2±1.5		<LOD		
		<LOQ–0.1				8.5–43		0.4 ± 0.3		0.17–3				
	Schilbe intermedius	0.2±0.2				11 ± 5.1		1.1–5.6		0.9±0.5		<LOD		
		<LOQ–0.1				4.8–14		0.02±0.01		0.5–1.4				
	H. vittatus	<LOQ				5.2±0.9		<LOQ		<LOQ		<LOD		
						3.4–8.1								
	O. mossambicus	<LOQ–0.1				5.1±4.7		<LOQ–0.9		<LOQ		<LOD		
Vaal River, SA	*L. capensis*		21±4.7		250±250		280±84		80±22		630±130		23±5.8	Wepener et al. (2011)

Region	Species	ΣChlors		HCB		ΣDDx		ΣHCHs		ΣPCBs		ΣPBDE		Reference
		ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	
Hartbeespoort Dam SA						130 ± 25		75±18						Barnhoorn et al. (2015)
						100–160		<LOD–230						
Roodeplaat Dam, SA		0				150±88		200±53						
		<LOQ–230				<LOD–350		<LOD–490						
Rietvlei Dam, SA		83±41				340±260		55±7.0						
		<LOD–160				<LOD–760		<LOD–63						
Klip River, SA	*C. gariepinus*	42±39		20.1±12.5		130±120		8±1.8						Pheiffer et al. (2018b)
		<LOD–170		8.24–65		14–890		4.9–11						
Olifants River SA (high flow: Sept 2010)	*H. vittatus*		25±12		<LOD		500±200		88 ± 14					Gerber et al. (2016)
			17–79				120–1400		19–110					
Olifants River SA (low flow 2010)			380±140		2.6±1.5		9000±3200		310±110					
			16–1500		3.4–16		900–3400		27–1100					
Letaba River SA (low flow 2010)			350±60		9.9±1.9		9400±2400		310±36					
			160–790		5.9–19		2700–1800		210–500					
Luvuvhu River SA (low flow 2010)			510±180		26±11		32000±8000		500±220					
			47–3800		5.0–190		2700–11000		470–3800					
Luvuvhu River SA (highflow 2011)			240±43		7.4±1.7		4200±1700		430±71					
			12–1100		0.9–26		110–22000		12–1100					
Lake Awassa, Ethiopia	*Oreochromis niloticus*	0.85±0				19±28.91				2.4 ± 2.8				Deribe et al. (2014)
		0.85–0.85				1.7–102.5				0.38–4.3				
	C. gariepinus	1.2 ± 0				29 ± 27				1.0 ± 1.2				
		1.2–1.2				5.4–100				0.35–27				
	Barbus intermedius	2.2 ± 2.0				56 ± 86				2.8 ± 7.4				
		0.75–3.6				13–410				0.35–27				

Region	Species	ΣChlors		HCB		ΣDDx		ΣHCHs		ΣPCBs		ΣPBDE		Reference
		ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	
Lake Victoria, Tanzania	*Oreochromis* sp	<LOD	0.5	0.01	2.5	0.19	35	<LOD	<LOD	0.08	12	0.12	34	Polder et al. (2014)
			<LOD–4.1	<LOD–0.02	1.4–4.0	0.02–0.73	7.2–103			<LOD–0.47	<LOD–68	<LOD–0.55	<LOD–170	
Lake Tanganyika, Tanzania		0.03	0.9	0.04	1.2	9.48	270	0.03	1.1	0.57	17.2	0.14	4.1	
		0.02–0.04	<LOD–3.7	0.03–0.05	1.1–1.3	6.34–12.19	210–319	0.02–0.04	0.8–1.5	0.34–0.84	14–21	0.09–0.27	2.8–6.7	
Lake Nyasa, Tanzania		0.02	0.2	0.3	21	0.26	13.2	0.02	1.3	0.02	0.7	0.1	6.5	
		0.01–0.04	<LOD–0.7	0.2–0.04	1.2–2.9	0.29–0.34	11–19	0.01–0.04	0.9–2.1	<LOD–0.05	<LOD–1.8	<LOD–0.15	<LOD	
Lake Babati, Tanzania		<LOD	0.1	0.04	1.2	1.6	43	<LOD	<LOD	<LOD	0.1	0.06	1.5	
			<LOD–0.8	0.03–0.07	0.6–2.8	1.43–4.09	14–150			<LOD–0.05	<LOD–1.1	<LOD–0.15	<LOD–6.8	
Lake Koka, Ethiopia	*B. intermedius*					6.2 ± 5.8				0.05 ± 0.05				Deribe et al. (2011)
						0.05–21				ND–0.14				
	C. carpio					4.5 ± 2.2				0.17 ± 0.08				
						2.1–11				ND–0.22				
	C. gariepinus					15 ± 17				0.33 ± 0.5				
						1.8–73				ND–2.0				
	O. niloticus					6.9 ± 2.7				0.05 ± 0.24				
						2.3–12				ND–0.63				

Region	Species	ΣChlors		HCB		ΣDDx		ΣHCHs		ΣPCBs		ΣPBDE		Reference
		ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g wm	ng/g wm	ng/g wm	ng/g lm	ng/g wm	
Congo River, Democratic Republic of the Congo	*Marcusenius sp*			0.02		0.2		0.19		<LOQ		0.11		Verhaert et al. (2013)
				<LOQ–0.06		<LOQ–0.96		0.12–0.44		<LOQ–2.4		0.5–0.21		
	Schilbe marmoratus			0.05		0.37		0.12		3.9		0.06		
				<LOQ–0.08		0.25–0.53		<LOQ–0.21		2.0–28		<LOQ–0.21		
	Synodontis alberti			0.8		0.09		0.16		1.5		1.2		
				<LOQ–0.13		<LOQ–0.45		<LOQ–0.35		<LOQ–66		0.26–1.6		
	Brycinus imberti			0.7		0.18		0.15		1.9		0.38		
				0.02–0.09		0.09–0.28		0.03–0.4		1.4–50		0.05–0.78		
Port Elizabeth Harbour, SA	*Mytilus galloprovincialis*									14–21				Kampire *et al.* (2015)
Vaal River, SA	*Cyprinus sp*												3.0–12	Chokwe *et al.* (2015)

SA = South Africa

2.5 Conclusion

Concentrations of the targeted pollutants were greatest at the Barrage site. When this site was compared to a similar study conducted previously in the region (Wepener *et al.*, 2011) the concentrations were lower in the recent study. It is possible that these compounds are degrading over time, or are being released from already depleted sources—particularly in the case of PCBs which leach from discontinued stored oils. The PBDEs have remained somewhat constant. The Stockholm Convention has only recently listed tetra-, penta-, hexa-, hepta-, and deca-DBEs under Annex A in 2017 (Stockholm Convention, 2017), which was after these samples were collected.

The region had low concentrations of OCPs which is expected because the area does not have much agricultural activities. However, there is a high occurrence of DDx, although mostly all in the metabolite forms. Due to the occurrence of DDx in other inland regions of the country it is speculated that there is active use of the pesticide.

Performing effect-based assays such as biomarkers of exposure would help determine whether the concentrations of the various contaminants targeted in this study, and the vast other compounds that are present in the environment could cause detrimental effects to the exposed organism.

3 Organochlorine pesticides in aquatic biota tissue from Phongola Floodplain

3.1 Introduction:

Persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), and organochlorine pesticides (OCPs) are common contaminants of the environment (Kanzari *et al.*, 2014). These compounds have been banned by the Stockholm Convention for use or production because of the many detrimental effects they pose. Because of this South Africa has ratified this convention (Stockholm Convention, 2019). However, because of the widespread and large volume use, combined with their persistence, these compounds, and/or their metabolites are still ubiquitous in the environment where they adhere to particulate matter (Quinn *et al.*, 2011). They persist because they are capable of resisting photolytic, chemical, and biological degradation (Wong *et al.*, 2005).

South Africa has more than 3000 registered pesticides, and is in the top four importers of pesticides in the sub-Saharan Africa (DAFF, 2010). In 2002 it was reported that approximately 10 million litres of liquid insecticides were produced for crop protection, with 43% of this consisting of organophosphates—however, this data is limited (Quinn *et al.*, 2011). OCPs including aldrin, dieldrin, endrin, chlordane, heptachlor, dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB), and α-, β-, and γ-hexachlorocyclohexane (HCH) have been banned for use by the Stockholm Convention, with the exception of DDT. DDT can be used with special considerations such as the control of the malaria vector, *Anopheles* mosquito. HCH, HCB, and DDT have been the most widely used OCPs worldwide during the 1970–2000s (El-Shahawi *et al.*, 2010; Tsygankov *et al.*, 2015). In South Africa HCHs, aldrin, heptachlor, and endosulfan have also been frequently used for agricultural purposes (Buah-Kwofie and Humphries, 2017). Because of their persistence and ubiquitous use in the environment this group of compounds is particularly important for toxicological studies, added to the fact that they are sequestered in lipid-rich tissues—resulting in detrimental effects spreading through the food web (Çok *et al.*, 2012). Some of these effects include damage to the central nervous system, specifically in the case of HCB (Willett *et al.*, 1998), and effects to the male reproductive systems (Vos *et al.*, 2000; Edwards *et al.*, 2006), amongst others.

DDT was first produced in 1945 with the intention of eradicating disease carrying insects, and for crop protection, and extensively used from 1960–1970. However, during this period it was determined that although it benefitted agriculture and ridded insect pests this came at a cost.

Many non-target organisms showed toxic effects from exposure—this was especially seen with birds of prey who had trouble reproducing, and suffered a decline in population (El-Shahawi *et al.*, 2010). Although DDT is restricted, it is still used in Africa, South Asia, and Central and South America for the prevention of malaria (Kumar *et al.*, 2005; El-Shahawi *et al.*, 2010). In South Africa, approximately 603 metric tons of DDT was used between 2001 and 2014, and Mozambique that neighbours KZN to the north, used 1352 tons of DDT, however, there was no DDT use between 2001–2004, and 2012 and 2013 (van den Berg *et al.*, 2017). DDT is applied through indoor residual spraying (IRS), where residential dwellings are sprayed before the peak malaria transmission period (in the summer, rainy period), usually occurring during December, and if needed a follow-up spraying occurs in March (Brooke *et al.*, 2013; WHO, 2015). The DDT then enters the environment through dust, air, and through potential spillages during application (Barnhoorn *et al.*, 2009). Even though it is restricted, it cannot be overlooked that the pesticide is made available on the market, where it can be acquired for illegal use (Verhaert *et al.*, 2017). Long range transport results in OCP residues in soils, water, sediment, and organisms in areas where these compounds have not actively been applied (El-Shahawi *et al.*, 2010; Hung *et al.*, 2016). Once in the environment it can persist for up to 15 years, where it breaks down into metabolites, dichlorodiphenylchloroethane (DDE) and dichlorodiphenyldichloroethane (DDD). The former being the most persistent and toxic (Barnhoorn *et al.*, 2009).

HCH was commercially produced since the 1950s by the process of photochlorination of benzene for use as a pesticide. This results in a mixture of HCH isomers, which predominantly consists of 60–70% α-, 5–12% β-, and 10–15% γ-HCH, which is technical grade HCH (Iwata *et al.*, 1995; Willett *et al.*, 1998; Jackovitz and Hebert, 2015; Yuan *et al.*, 2017). Lindane (γ-HCH) is the only isomer that has significant insecticidal properties, but the production of it is inefficient—for every ton of γ-HCH obtained, between 6 and 10 tons of other “waste” isomers are produced (Vijgen *et al.*, 2006). In 2009 α-, β-, and γ-HCH isomers were included in the Stockholm Convention because they are persistent, bioaccumulative, and toxic (UNEP, 2009).

These compounds are especially prevalent in aquatic ecosystems, and they enter the systems through runoff and atmospheric deposition (Tang *et al.*, 2013). The compounds are lipophilic, and they tend to partition to carbon particles in sediments, where they are more stable (Doong *et al.*, 2002). From the sediments they can transfer to biota via food or through dermal or gill exposure. In organisms these compounds partition to the lipid stores. Concentrations in organisms are often greater than those in the environment, and concentrations are often greater in higher trophic species (Jones and De Voogt, 1999; El-Shahawi *et al.*, 2010; Tang *et al.*, 2013). Once the organism is exposed, the compounds are suspected to cause a wide

range of detrimental effects, including disruption of the endocrine, immune, and reproductive systems, reduced survival and growth of offspring, and cancers (Damgaard *et al.*, 2006; El-Shahawi *et al.*, 2010). These compounds pose a great risk to ecosystem and human health because of their toxicity and persistence, specifically because of their impact on non-target species (Willett *et al.*, 1998; Zhou *et al.*, 2011; Wang *et al.*, 2012). Fish are long lived organisms, and are representative of large sections of river. They are also fairly high in the food web, and because of these factors they make good indicators of pollution (Yohannes *et al.*, 2013).

The Phongola River has a catchment of approximately 7000 km^2, and flows from northern KwaZulu-Natal towards Mozambique where it joins the Maputu River (Lankford *et al.*, 2011). The Pongolapoort Dam was constructed in 1973 to supply irrigation water for agriculture in the area that is mainly sugarcane and cotton (Van Vuuren, 2009). Below the dam is a floodplain consisting of many pans that are dependent on flooding events (Heeg and Breen, 1982). DDT has actively been sprayed in the region since 1946 to control the malaria vector (Sereda and Meinhardt, 2005). Many studies have detected OCPs, originating from agricultural practices and malaria control, in organisms from the region. These studies focussed on possible health risks associated with it (Bouwman *et al.*, 1990; McHugh *et al.*, 2011; Wepener *et al.*, 2012; McHugh *et al.*, 2013; Wolmarans, 2015).

The aim of this study is to determine the concentrations of OCPs in crustaceans and fish muscle tissue from the Phongola River. It is expected that these organisms will have high concentrations of OCPs, specifically DDTs because of the dominant agricultural practices, and IRS for malaria vector control in the area.

3.2 Materials and methods:

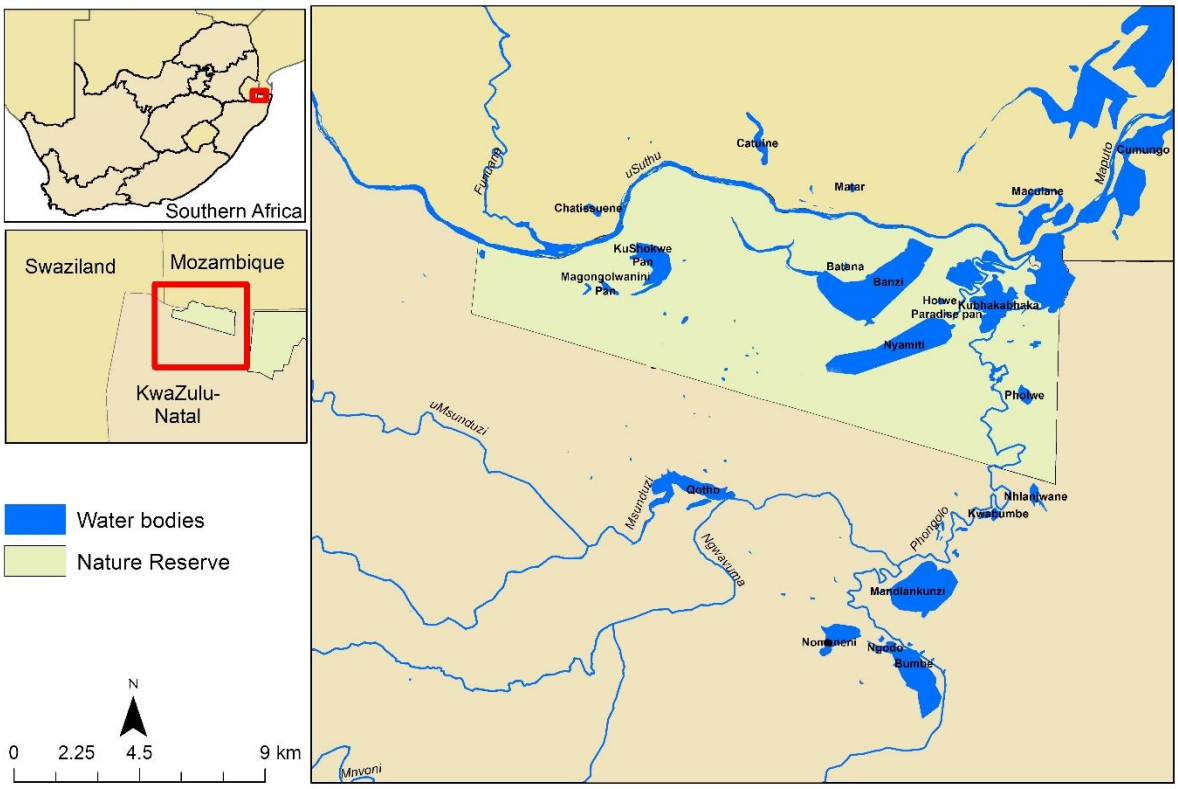


Figure 3.1. Phongola region including the Ndumo Game Reserve and pans.

3.2.1 Sample collection

The samples were collected in the Phongola system (Figure 3.1) in April and September 2013. These were omnivores: *Oreochromis mossambicus, Coptodon rendalli, and Clarias gariepinus*; detritivore: *Synodontis zambezensis*, and the predators: *Hydrocynus vittatus*, and *Anguilla bicolor*. The invasive red claw crayfish, *Cherax quadricarinatus*, was also sampled. Muscle tissue was taken from the fish and wrapped in aluminium foil and frozen until analysis. The samples were analysed in Japan at the Laboratory of Toxicology, Hokkaido University, and the target compounds were: α-, β-, γ-, and δ-HCH, HCB, DDT and its metabolites (*p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDD, *p,p'*-DDE, and *o,p'*-DDD) collectively termed DDx, cyclodienes: aldrin, dieldrin, and endrin, and chlordanes (chlors): oxychlordane (OxC), cis-nonachlor (CN), trans-nonachlor (TN), heptachlor, *cis*-heptachlor epoxide (cHept), *trans*-heptachlor epoxide (tHept), *cis*-chlordane, *trans*-chlordane.

3.2.2 Extraction and analyses

The organochlorine standard mix was obtained from Dr Ehrenstorfer GmbH (Germany). All solvents used were of analytical grades (Kanto Chemical Corp, Tokyo, Japan). Sodium

sulphate was obtained from Kanto Chemical Corp. Florisil (60–100 mesh, Kanto Chemical Corp) was activated at 180°C for 8 hrs. Extraction thimbles (25 x 100 mm, Whatman, England) were pre-extracted for 1 h with hexane/acetone (3/1; v/v) and dried at 100°C for 1 h.

Approximately 10 g of wet fish muscle was homogenised with Na_2SO_4, until the tissue was dry. The sample was transferred into the Soxhlet thimble, and spiked with the internal standard (PCB 77), followed by extraction with 150 mL 3:1 hexane: acetone (v/v*)* for 6 hours. The extracts were concentrated, and solvent exchanged to hexane. Lipid determination was performed gravimetrically with an aliquot of the sample, and the remaining sample was cleaned in a glass column packed with 6 g 5% deactivated Florisil. The target compounds were eluted with 120 mL 3:7 DCM:hexane (v/v*)*, and the eluent concentrated to near dryness. The sample was resuspended in 100 µL *n*-decane containing surrogate standard, tetrachloro-*m*-xylene.

Analysis of OCPs was conducted using a gas chromatograph coupled to a ^{63}Ni electron capture detector (GC-ECD Shimadzu GC-2014, Kyoto, Japan). The compounds were separated on an ENV-8MS capillary column (30 m x 0.25 mm x 0.25 µm film thickness). With helium as the carrier gas, set to 1.0 mL/min, constant flow, and nitrogen as the make-up gas at a flow rate of 45 mL/min. The 1 µL of sample was injected in splitless mode. The GC oven temperature was set at 100°C at the start time (hold 1 min), ramp at 12°C/min to 180°C, 4°C/min ramp to 240°C, and finally to 270°C at 10°C/min with 5 min hold. The injector and detector temperatures were set at 250°C and 320°C respectively.

3.2.3 Quality assurance/control

The OCPs were identified by comparing their retention time with reference to the corresponding standard. The concentrations of the target analytes were quantified comparing the peak area of the sample to that of the standard peak area. The correlation coefficients (r^2) for the calibration curves were all greater than 0.995. For each set of 10 samples, a procedural blank and spiked blank were run to check for interference and cross-contamination. The mean recovery of OCPs for the spiked blanks was 90 ± 11%. Spiking experiments using fortified samples, *Oreochromis niloticus* at 5 ng/g of the composite standards showed recovery ranged from 70% to 110% for all OCPs. To further test the precision and accuracy of the analytical method, the standard reference material SRM 1947 (Lake Michigan Fish Tissue) was analysed using the same procedures. Acceptable recoveries were obtained, and ranged from 75% to 115% with relative standard deviation (RSD) less than 12%. Limits of detection based on 3:1 signal to noise ratio (S/N) were between 0.05 and 0.1 ng/g for all OCPs. Samples recoveries were between 92–180%, therefore recovery adjustment was not performed.

Significance was set at $p<0.05$. For concentrations below the limit of detection (<LOD) a surrogate value of detection frequency x LOD was used (Verhaert *et al.*, 2013). All results are expressed as ng/g wet mass (wm). GraphPad Prism 6 was utilised for statistical analyses. Mann-Whitney U tests were used to determine significance between medians in data, with significance set at $p<0.05$.

3.3 Results

3.3.1 Hexachlorocyclohexane (HCH)

In the April sampling period the greatest ΣHCH concentration was in *C. quadricarinatus* (Table 3.1). This concentration was also significantly higher ($p<0.05$), approximately 2 orders of magnitude greater than any of the other species sampled in this season. *C. gariepinus* had the lowest ΣHCH concentration, composed solely of γ-HCH. This was the dominant isomer in all of the species sample (Figure 3.2), and the most toxic HCH isomer. β-HCH was the next most dominant isomer, however it was not detected in all the species.

Table 3.1 Mean (±SD) and minimum–maximum concentrations (ng/g wm) of HCH isomers in species sampled during April in Phongola.

Sample name		α-HCH	β-HCH	γ-HCH	δ-HCH	ΣHCHs
C. rendalli (n=17)	Mean ± SD Range	<LOD	<LOD	0.12±0.1 <LOD–0.3	0.01±0.02 <LOD–0.06	0.13±0.1 <LOD–0.3
C. gariepinus (n=25)	Mean ± SD Range	<LOD	<LOD	0.01±0.05 <LOD–0.26	<LOD	0.01±0.05 <LOD–0.26
O. mossambicus (n=23)	Mean ± SD Range	0.1±0.28 <LOD–1.14	0.04±0.09 <LOD–0.38	0.02±0.06 <LOD–0.29	0±0.01 <LOD–0.03	0.15±0.31 <LOD–1.14
S. zambezensis (n=22)	Mean ± SD Range	0.07±0.24 <LOD–1.14	0.02±0.08 <LOD–0.38	0.07±0.14 <LOD–0.41	0.01±0.02 <LOD–0.09	0.14±0.26 <LOD–1.14
H. vittatus (n=9)	Mean ± SD Range	<LOD	<LOD	0.07±0.08 <LOD–0.26	<LOD	0.07±0.08 <LOD–0.26
C. quadricarinatus (n=13)	Mean ± SD Range	1.8±3.7 <LOD–14	29±65 <LOD–230	29±17 1.8–57	8.9±10 <LOD–33	68±79 1.8–310

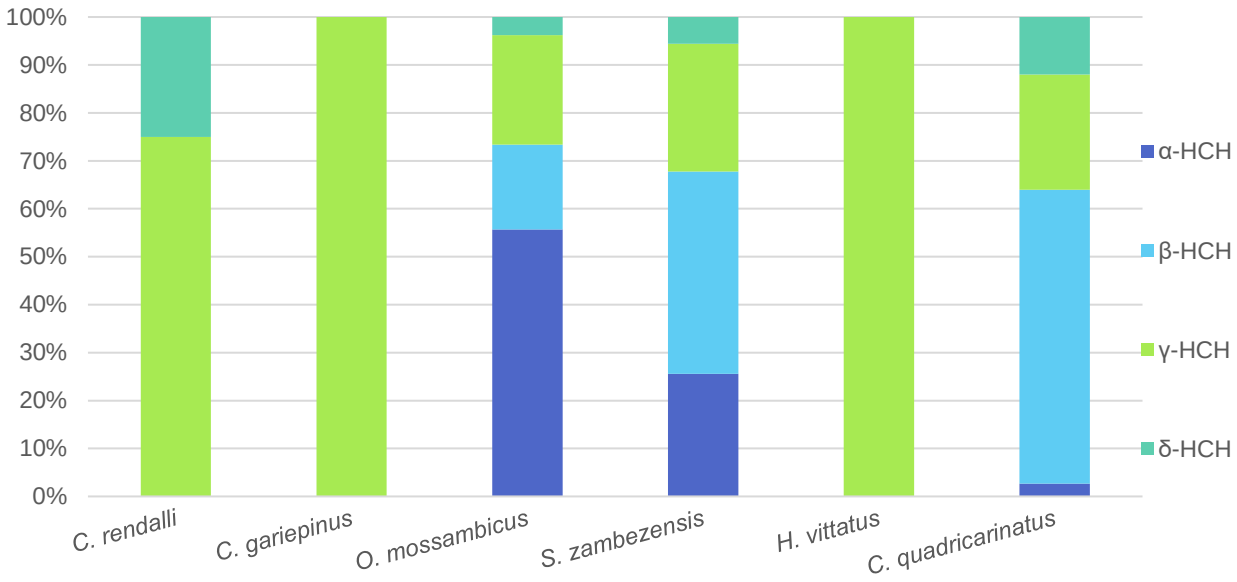


Figure 3.2 Ratios of the HCH isomers analysed in sampled species in the Phongola system from April.

Concentrations of ΣHCHs were again greatest in *C. quadricarinatus,* during the September sampling event and slightly lower, but not significantly (p>0.05) than those in the April sample season (Table 3.1 and 2). However, there were no significant differences between the ΣHCHs in the fish between April and September, but there were significant differences between fish species. The concentrations in *C. quadricarinatus* were significantly greater than *S. zambezensis, O. mossambicus,* and *H. vittatus. A. bicolor* had concentrations that were significantly greater than *S. zambezensis,* and *H. vittatus.* Generally, γ-HCH had the greatest proportion of the isomers (Figure 3.3), except in *S. zambezensis*, where α-HCH made the greatest contribution, and γ-HCH only a small proportion. *O. mossambicus* had an almost equal contribution of all isomers (Figure 3.3).

Table 3.2 Mean (±SD) and minimum–maximum concentrations (ng/g wm) of HCH isomers in species sampled during September in Phongola.

Sample name		α-HCH	β-HCH	γ-HCH	δ-HCH	ΣHCHs
C. rendalli (n=19)	Mean ± SD Range	<LOD	<LOD	0.16±0.12 <LOD–0.42	0.01±0.02 <LOD–0.08	0.17±0.14 <LOD–0.48
S. zambezensis (n=20)	Mean ± SD Range	0.04±0.08 <LOD–0.24	0.01±0.02 <LOD–0.08	0.001±0.004 <LOD–0.02	<LOD	0.05±0.07 <LOD–0.24
O. mossambicus (n=40)	Mean ± SD Range	0.02±0.02 <LOD–0.05	0.03±0.02 <LOD–0.06	0.02±0.03 <LOD–0.08	0.02±0.02 <LOD–0.05	0.09±0.07 <LOD–0.22
H. vittatus (n=19)	Mean ± SD Range	<LOD	<LOD	<LOD	<LOD	<LOD
A. bicolor (n=4)	Mean ± SD Range	0.55±1.1 <LOD–2.19	0.34±0.44 <LOD–0.92	21±10 10–34	13±8.3 3.4–23	35±18 14–57
C. quadricarinatus (n=7)	Mean ± SD Range	5.0±7.1 <LOD–17	2.3±6.2 <LOD–16	34±15 5.4–45	13±4.2 4.2–17	54±26 9.7–89

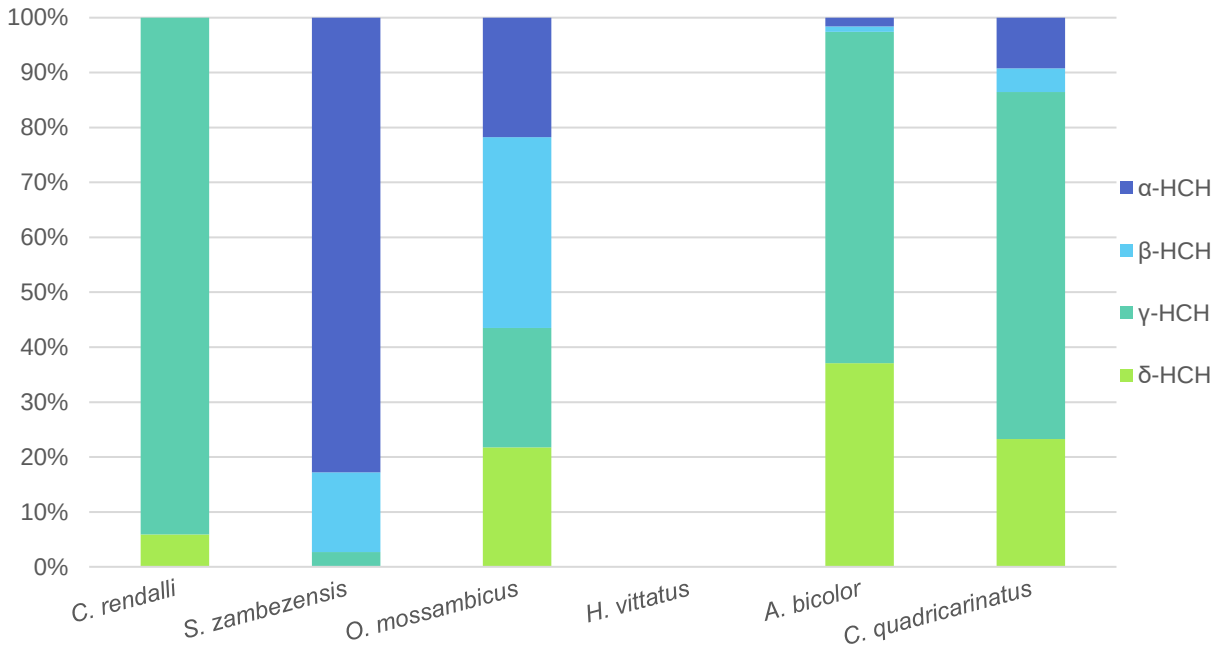


Figure 3.3 Ratios of the HCH isomers analysed in sampled species in the Phongola system from September

3.3.2 Hexachlorobenzene (HCB)

HCB was only detected in *H. vittatus* in the April sampling event (Table 3.3). In the September sampling HCB was more frequently detected. HCH was not detected in *H. vittatus,* or *C. gariepinus*. There were more detections of HCB in the September event, however, there were no detections in *H. vittatus.* The highest mean concentration in the September sampling was in *A. bicolor* (0.86 ng/g wm).

Table 3.3 Mean (±SD) and minimum–maximum concentrations (ng/g wm) of HCB in species sampled during April and September in Phongola.

Species		ΣHCB April	ΣHCB September
C. rendalli	Mean ± SD Range	<LOD	0.01±0.02 <LOD–0.07
C. gariepinus	Mean ± SD Range	<LOD	NA
O. mossambicus	Mean ± SD Range	<LOD	<LOD
S. zambezensis	Mean ± SD Range	<LOD	0.02±0.07 <LOD–0.31
H. vittatus	Mean ± SD Range	0.35±0.75 <LOD–2.14	<LOD
C. quadricarinatus	Mean ± SD Range	<LOD	<LOD
A. bicolor	Mean ± SD Range	NA	0.86±1.05 <LOD–2.13

NA=not analysed

3.3.3 Chlordanes (Chlors)

Concentrations of Σchlors in organisms from the April sampling event were typically low (Table 3.4). *Cherax quadricarinatus* had the highest Σchlors (3.8 ± 3.9 ng/g wm) while there were no detections in *H. vittatus.* Concentrations in *C*. *quadricarinatus* were significantly greater ($p<0.05$) than those in *C. rendalli, C. gariepinus, H. vittatus* and *S. zambezensis*. There were no clear trends in the ratio composition of the compounds contributing to the Σchlors (Figure 3.4). *Trans*-nonachlor was dominant in *C. rendalli* and *O. mossambicus*, CN and to a slightly lower percent TN were dominant in *S. zambezensis.* In *C. gariepinus* only cHept was quantified, and *C. quadricarinatus* had the highest ratio of heptachlor present (Figure 3.4). *Oreochromis mossambicus* and *S. zambezensis* had the most frequent number of quantified chlors present.

Table 3.4 Mean (±SD) and minimum–maximum concentrations (ng/g wm) of chlordanes in species sampled during April in Phongola.

Sample name		Heptachlor	Oxy-chlordane	*Cis*-hept-epoxide	*Trans*-Hept-Epoxide	*Trans*-Chlordane	*Trans*-nonachlor	*Cis*-Chlordane	*Cis*-Nonachlor	ΣChlors
C. rendalli (n=17)	Mean ± SD Range	<LOD	0.03±0.08 <LOD–0.31	0.01±0.03 <LOD–0.08	<LOD	0.03±0.07 <LOD–0.28	0.13±0.29 <LOD–0.94	0.04±0.11 <LOD–0.42	<LOD	0.24±0.43 <LOD–1.43
C. gariepinus (n=25)	Mean ± SD Range	<LOD	<LOD	0.003±0.01 <LOD–0.07	<LOD	<LOD	<LOD	<LOD	<LOD	0.003±0.01 <LOD–0.07
O. mossambicus (n=23)	Mean ± SD Range	<LOD	0.004±0.01 <LOD–0.05	<LOD±0.01 <LOD–0.05	<LOD±0.01 <LOD–0.03	0.01±0.02 <LOD–0.1	0.02±0.05 <LOD–0.18	0.01±0.03 <LOD–0.11	0.01±0.03 <LOD–0.13	0.05±0.1 <LOD–0.41
. zambezensis (n=22)	Mean ± SD Range	0.001±0.01 <LOD–0.02	0±0.01 <LOD–0.05	0.01±0.04 <LOD–0.17	0±0.01 <LOD–0.03	0.02±0.06 <LOD–0.26	0.05±0.11 <LOD–0.38	0.02±0.07 <LOD–0.3	0.06±0.09 <LOD–0.3	0.15±0.21 <LOD–0.69
H. vittatus (n=9)	Mean ± SD Range	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
C. quadricarinatus (n=13)	Mean ± SD Range	1.6±2.7 <LOD–8.1	<LOD	0.06±0.2 <LOD–0.73	<LOD	0.88±0.82 0.2–3.5	0.55±0.96 <LOD–2.4	0.12±0.44 <LOD–1.6	0.51±1.0 <LOD–3.4	3.8±3.9 0.2–12

Table 3.5 Mean (±SD) and minimum–maximum concentrations (ng/g wm) of HCB in species sampled during September in Phongola.

Sample name		Heptachlor	Oxy-chlordane	*Cis*-hept-epoxide	*Trans*-Hept-Epoxide	*Trans*-Chlordane	*Trans*-nonachlor	*Cis*-Chlordane	*Cis*-Nonachlor	ΣChlors
C. rendalli (n=19)	Mean ± SD Range	<LOD	0.09±0.13 <LOD–0.51	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.09±0.13 <LOD–0.51
S. zambezensis (n=20)	Mean ± SD Range	<LOD	0.001±0.01 <LOD–0.03	0.01±0.04 <LOD–0.14	0.01±0.01 <LOD–0.04	0.02±0.07 <LOD–0.33	0.01±0.02 <LOD–0.07	0.01±0.03 <LOD–0.11	0.01±0.02 <LOD–0.08	0.06±0.12 <LOD–0.47
O. mossambicus (n=40)	Mean ± SD Range	0.01±0.03 <LOD–0.09	0.004±0.01 <LOD–0.05	0.01±0.01 <LOD–0.06	0.02±0.02 <LOD–0.06	0.01±0.02 <LOD–0.07	0.02±0.02 <LOD–0.06	0.02±0.03 <LOD–0.07	0.01±0.02 <LOD–0.05	0.09±0.08 <LOD–0.28
H. vittatus (n=19)	Mean ± SD Range	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
A. bicolor (n=4)	Mean ± SD Range	3.0±1.8 1.5–5.6	3.4±6.1 <LOD–13	0.28±0.55 <LOD–1.11	<LOD	<LOD	4.7±6.6 <LOD–14	1.6±2.3 <LOD–5.0	<LOD	13±16 2.1–36
C. quadricarinatus (n=7)	Mean ± SD Range	2±3 <LOD–6.4	<LOD	<LOD	<LOD	1.04±1.4 <LOD–4.2	3.6±2.1 1.8–7.1	<LOD	0.13±0.35 <LOD–0.91	6.8±5.8 2.0–18

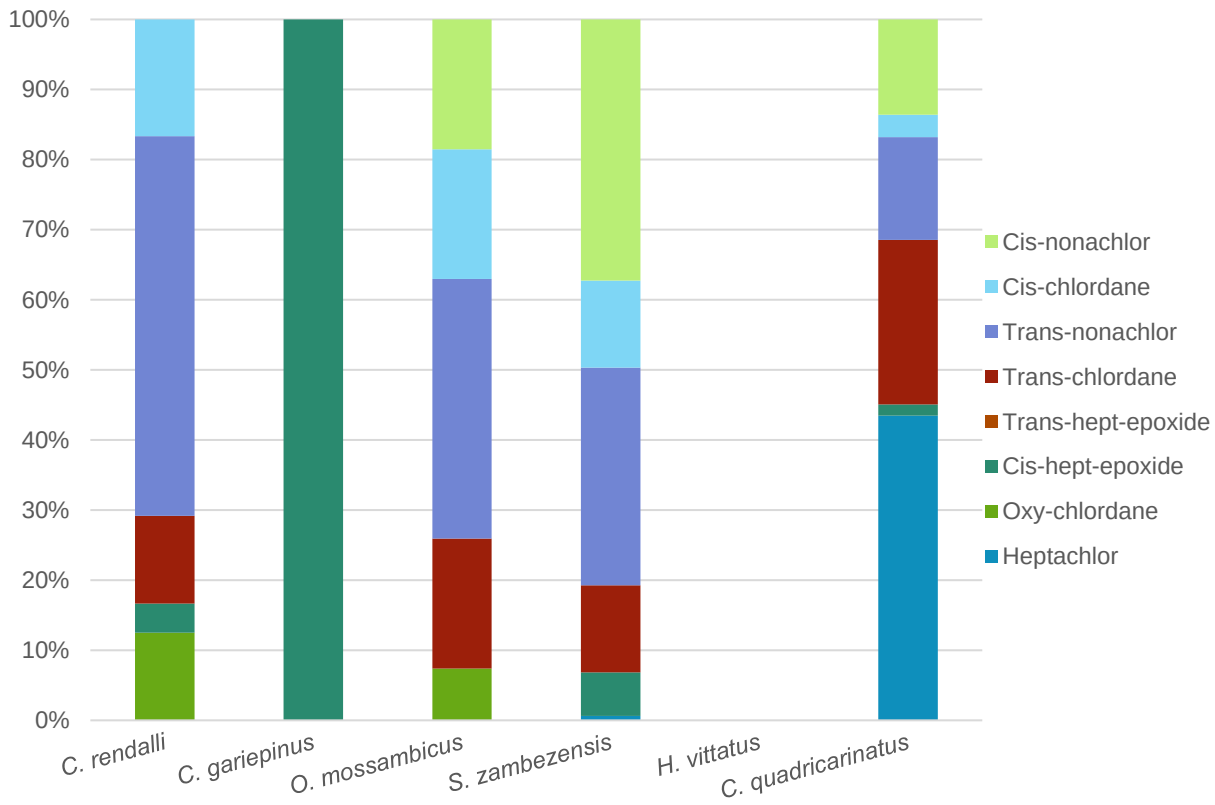


Figure 3.4 Chlordane contributions in the species analysed in the April sampling events from Phongola.

In the September sampling event the highest concentrations of chlors were in *A. bicolor* and *C. quadricarinatus* (Table 3.5). There were no significant differences ($p>0.05$) between seasons at species level. However, the concentrations of chlors in *C. quadricarinatus* were significantly greater than *O. mossambicus, S. zambezensis, H. vittatus* and *C. rendalli. A. bicolor* contained concentrations of chlors that were significantly greater than *C. rendalli, H. vittatus,* and *S. zambezensis*. Similar to the April sampling event, the September results showed variability in the dominant compound contributing to the Σchlors (Figure 3.5). The Σchlors in *C. rendalli* was completely comprised of oxychlordane, *S. zambezensis* was dominated by *trans*-chlordane, while *O. mossambicus* had equal contributions of *trans*-heptachlor epoxide, *trans*-nonachlor, and *cis*-chlordane. *Trans*-nonachlor was the dominant compound in *A. bicolor* and *C. quadricarinatus.*

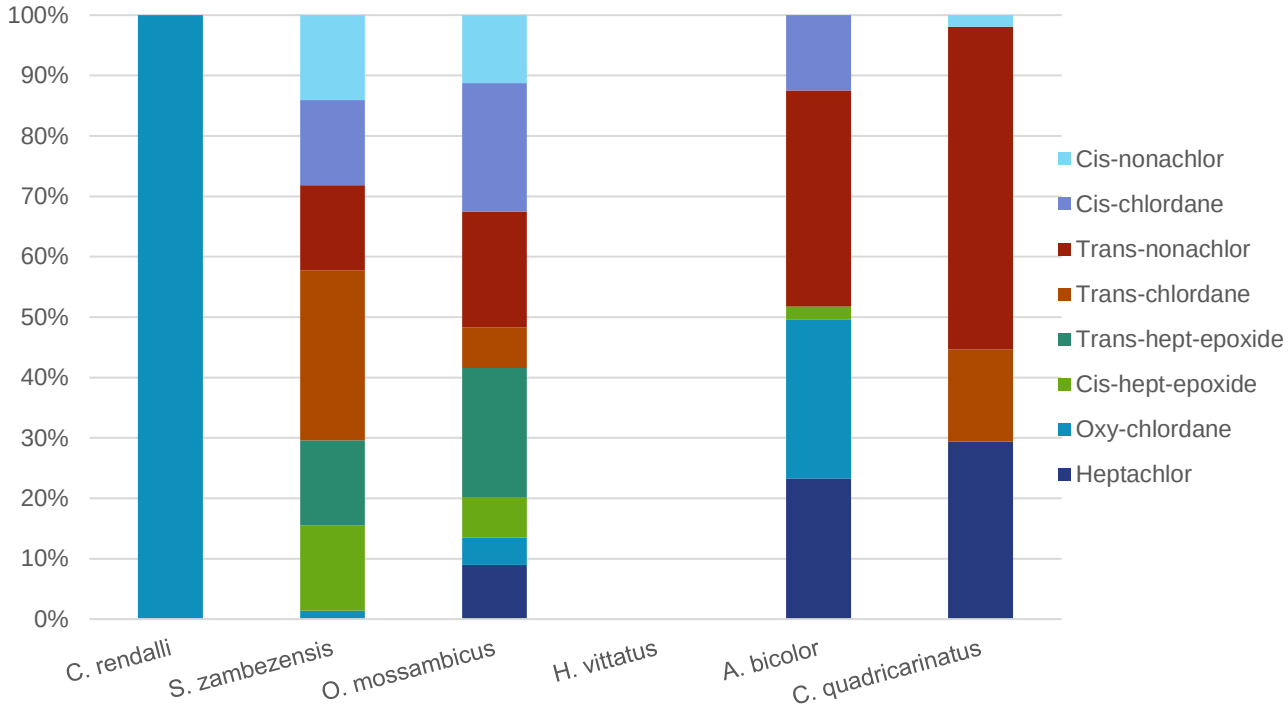


Figure 3.5 Chlordane contributions in the species analysed in the September sampling events from Phongola.

3.3.4 Cyclodienes (drins)

Concentrations of Σdrins were greatest in *C. quadricarinatus* and there were no detections in *C. gariepinus,* or *H. vittatus* (Table 3.6). The concentrations of Σdrins in *C. quadricarinatus* were significantly greater than *C. rendalli, O. mossambicus, S. zambezensis,* and *H. vittatus. C. quadricarinatus* was the only species to have detectable concentrations of all the drins analysed. The dominant drin in *C. rendalli* and *O. mossambicus* was aldrin, while dieldrin was dominant in *S. zambezensis,* and endrin was dominant in *C. quadricarinatus* (Figure 3.6).

Table 3.6 Concentrations (ng/g wm) of cyclodienes in species sampled from Phongola in April 2013, expressed as ng/g wm.

Sample name		Aldrin	Dieldrin	Endrin	ΣDrins
C. rendalli (n=17)	Mean ± SD Range	0.01±0.04 <LOD–0.14	<LOD	<LOD	0.01±0.04 <LOD–0.14
C. gariepinus (n=25)	Mean ± SD Range	<LOD	<LOD	<LOD	<LOD
O. mossambicus (n=23)	Mean ± SD Range	0.01±0.01 <LOD–0.03	<LOD	0.003±0.01 <LOD–0.06	0.01±0.02 <LOD–0.09
S. zambezensis (n=22)	Mean ± SD Range	<LOD	0.07±0.12 <LOD–0.42	0.01±0.03 <LOD–0.14	0.07±0.12 <LOD–0.42
H. vittatus (n=9)	Mean ± SD Range	<LOD	<LOD	<LOD	<LOD
C. quadricarinatus (n=13)	Mean ± SD Range	1.1±1.2 <LOD–3.4	5.7±2.8 3.0–14	7.8±2.5 3.8–12	15±5.5 8.3–30

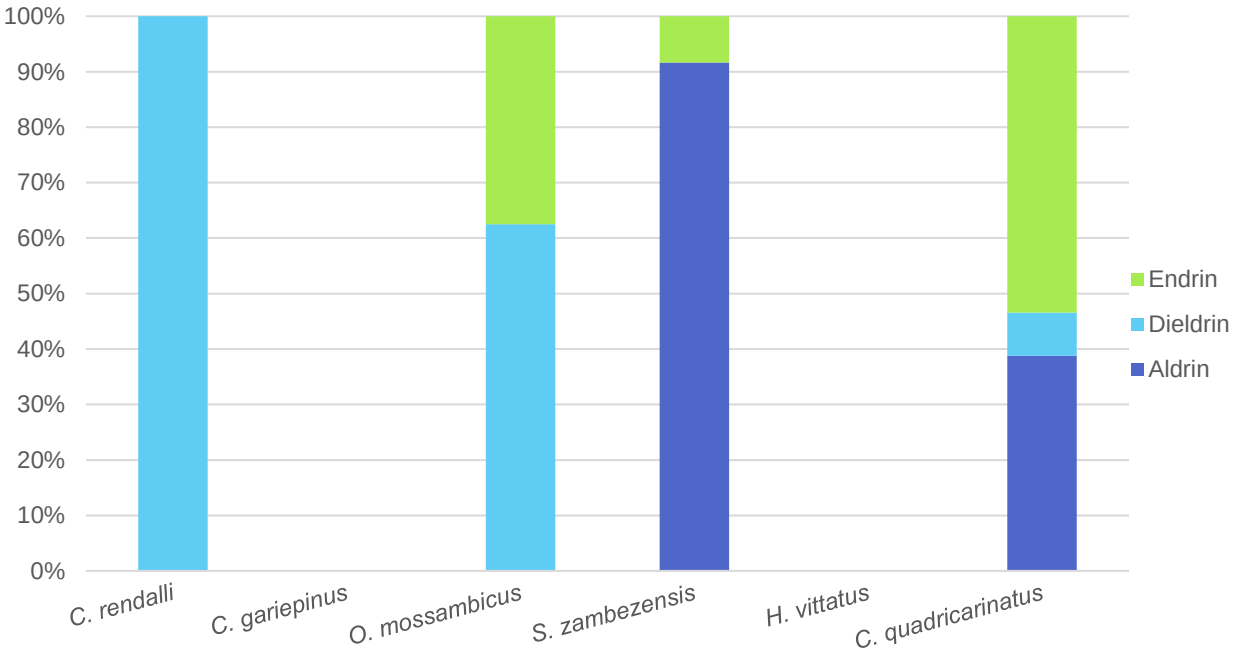


Figure 3.6 Cyclodiene contributions in the species analysed in the April sampling events from Phongola.

The highest concentrations of Σdrins from the September sampling period were in *C. quadricarinatus* followed by *A. bicolor* (Table 3.7). There were no significant differences between sampling events, and concentrations were generally similar, except in *C. quadricarinatus* which had slightly higher mean concentrations in the September sampling event. However, the maximum concentration recorded in April was greater (Table 3.6). There was no clear trend in the composition of the Σdrins. In *C. rendalli* and *S. zambezensis* the Σdrins were solely comprised of aldrin and endrin respectively (Figure 3.7). *O. mossambicus* had equal proportions of aldrin and endrin. *A. bicolor* had a dominance of dieldrin and slightly lower percentage of endrin, while the opposite was true for *C. quadricarinatus.*

Table 3.7. Mean (±SD) and minimum–maximum concentrations (ng/g wm) of cyclodienes in species sampled during September in Phongola.

Sample name		Aldrin	Dieldrin	Endrin	ΣDrins
C. rendalli (n=19)	Mean ± SD Range	0.01±0.04 <LOD–0.17	<LOD	<LOD	0.01±0.04 <LOD–0.17
S. zambezensis (n=20)	Mean ± SD Range	<LOD	<LOD	0.04±0.17 <LOD–0.78	0.04±0.17 <LOD–0.78
O. mossambicus (n=40)	Mean ± SD Range	0.003±0.01 <LOD–0.05	0.003±0.01 <LOD–0.06	<LOD	0.01±0.02 <LOD–0.06
H. vittatus (n=19)	Mean ± SD Range	<LOD	<LOD	<LOD	<LOD
A. bicolor (n=4)	Mean ± SD Range	1.1±1.3 <LOD–2.9	8.4±10 <LOD–23	6.0±5.1 2.0–13	15±15 3.3–38
C. quadricarinatus (n=7)	Mean ± SD Range	0.7±1.1 <LOD–2.9	8.0±4.2 <LOD–13	12±5.1 3.6–20	21±5.8 9.3–28

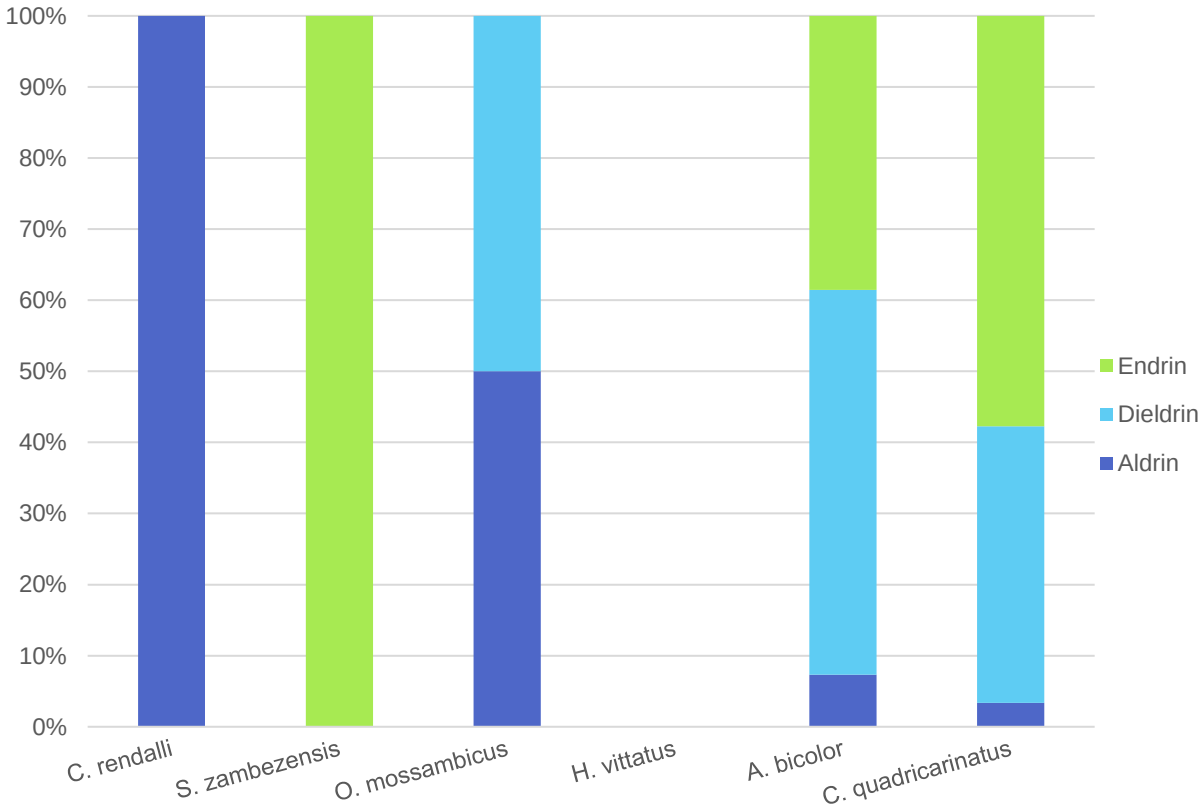


Figure 3.7 Cyclodiene contributions in the species analysed in the September sampling event from Phongola.

3.3.5 Dichlorodiphenyltrichloroethane (DDx)

The highest mean concentrations of ΣDDx from the April sampling event were in *C. quadricarinatus,* and the lowest were in *C. gariepinus* (Table 3.8). Concentrations in *C. rendalli* were significantly greater than those in *C. gariepinus. Cherax quadricarinatus* had concentrations that were significantly greater than *C. gariepinus, O. mossambicus,* and *S. zambezensis.* There were variations of which was the dominant compound to the ΣDDx: *C. rendalli* and *H. vittatus* had a dominance of ***p,p'***-DDT, *C. gariepinus, O. mossambicus,* and *S. zambezensis* a dominance of ***p,p'***-DDE, and *C. quadricarinatus* a dominance of ***o,p'***-DDT (Figure 3.8).

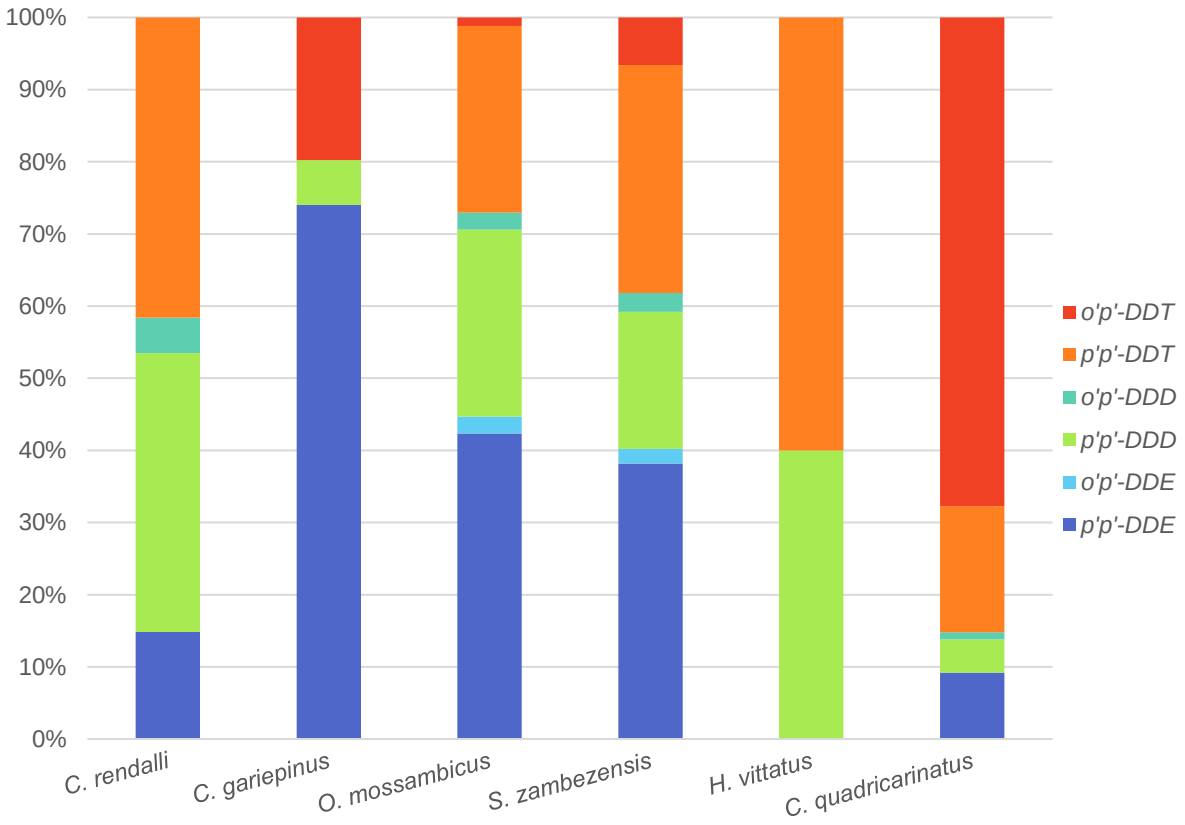


Figure 3.8 DDx contributions in the species analysed in the April sampling events from Phongola.

Table 3.8 Mean (±SD) and minimum–maximum concentrations (ng/g wm) of DDx in species sampled during April in Phongola.

Sample name		*p,p'*-DDE	*o,p'*-DDE	*p,p'*-DDD	*o,p'*-DDD	*p,p'*-DDT	*o,p'*-DDT	ΣDDx
C. rendalli (n=17)	Mean ± SD Range	0.15±0.44 <LOD–1.8	<LOD	0.39±0.48 <LOD–1.6	0.05±0.15 <LOD–0.59	0.42±0.7 <LOD–2.7	<LOD	0.87±1.0 <LOD–3.2
C. gariepinus (n=25)	Mean ± SD Range	0.44±0.67 <LOD–3.13	<LOD	0.04±0.13 <LOD–0.53	<LOD	<LOD	0.12±0.59 <LOD–3.0	0.16±0.7 <LOD–3.5
O. mossambicus (n=23)	Mean ± SD Range	0.18±0.23 <LOD–0.73	0.01±0.03 <LOD–0.11	0.11±0.08 <LOD–0.32	0.01±0.03 <LOD–0.1	0.11±0.1 <LOD–0.45	0.01±0.02 <LOD–0.11	0.24±0.2 <LOD–0.79
S. zambezensis (n=22)	Mean ± SD Range	2.0±3.1 <LOD–12	0.11±0.17 <LOD–0.46	1.0±1.4 <LOD–4.8	0.14±0.2 <LOD–0.59	1.7±2.7 <LOD–12	0.35±0.55 <LOD–2.1	3.3±4.9 <LOD–19
H. vittatus (n=9)	Mean ± SD Range	<LOD	<LOD	0.34±0.43 <LOD–1.2	<LOD	0.52±0.66 <LOD–1.6	<LOD	0.86±1.1 <LOD–2.8
C. quadricarinatus (n=13)	Mean ± SD Range	2.2±1.2 <LOD–4.3	<LOD	1.1±2.9 <LOD–9.4	0.23±0.44 <LOD–1.1	4.2±1.4 1.5–6.02	16±58 <LOD–210	22±59 2.3–220

Table 3.9 Mean (±SD) and minimum–maximum concentrations (ng/g wm) of DDx in species sampled during September in Phongola.

Sample name		*p,p'*-DDE	*o,p'*-DDE	*p,p'*-DDD	*o,p'*-DDD	*p,p'*-DDT	*o,p'*-DDT	ΣDDx
C. rendalli (n=19)	Mean ± SD Range	<LOD	<LOD	0.11 ± 0.2 <LOD–0.63	0.01 ± 0.05 <LOD–0.2	0.04 ± 0.08 <LOD–0.25	<LOD	0.16 ± 0.29 <LOD–0.84
S. zambezensis (n=20)	Mean ± SD Range	0.2 ± 0.42 <LOD–1.38	0.03 ± 0.05 <LOD–0.16	0.02 ± 0.07 <LOD–0.29	0.002 ± 0.01 <LOD–0.03	0.03 ± 0.06 <LOD–0.22	0.05 ± 0.08 <LOD–0.22	0.33 ± 0.55 <LOD–1.7
O. mossambicus (n=40)	Mean ± SD Range	0.16 ± 0.14 <LOD–0.51	<LOD	0.12 ± 0.07 <LOD–0.3	0.01 ± 0.01 <LOD–0.04	0.06 ± 0.04 <LOD–0.14	0.01 ± 0.01 <LOD–0.05	0.36 ± 0.23 <LOD–0.9
H. vittatus (n=19)	Mean ± SD Range	1.4 ± 1.3 0.41–4.6	<LOD	0.27 ± 0.22 0.02–0.8	0.01 ± 0.06 <LOD–0.28	0.22 ± 0.12 0.08–0.51	<LOD	1.9 ± 1.6 0.67–5.9
A. bicolor (n=4)	Mean ± SD Range	530 ± 330 140–950	1.9 ± 3.8 <LOD–7.5	120 ± 84 33–240	5.8 ± 4.2 0.58–11	230 ± 250 45–600	180 ± 270 <LOD–570	1 100 ± 920 220–2 400
C. quadricarinatus (n=7)	Mean ± SD Range	2.0 ± 1.4 <LOD–3.9	0.9 ± 2.4 <LOD–6.3	0.17 ± 0.44 <LOD–1.16	0.74 ± 1.6 <LOD–4.21	10 ± 13 <LOD–38	<LOD	14 ± 12 4.4–38

Concentrations of ΣDDx from the September sampling event were greatest in *A. bicolor.* This was two orders of magnitude greater than the next highest mean concentration, *C. quadricarinatus*, however this was not significant (p>0.05). The lowest mean concentrations were in *C. rendalli* (Table 3.9). There were no significant differences between sampling events, but the concentrations in the April sampling event were slightly higher than the September event. Unfortunately, no *A. bicolor* was sampled in April making comparisons between the two periods impossible. Composition of the ΣDDx differed between species: *C. rendalli* was dominated by *p,p'*-DDD, *S. zambezensis, H. vittatus, O. mossambicus,* and *A. bicolor* were dominated by *p,p'*-DDE, while *C. quadricarinatus* was dominated by *p,p'*-DDT (Figure 3.9).

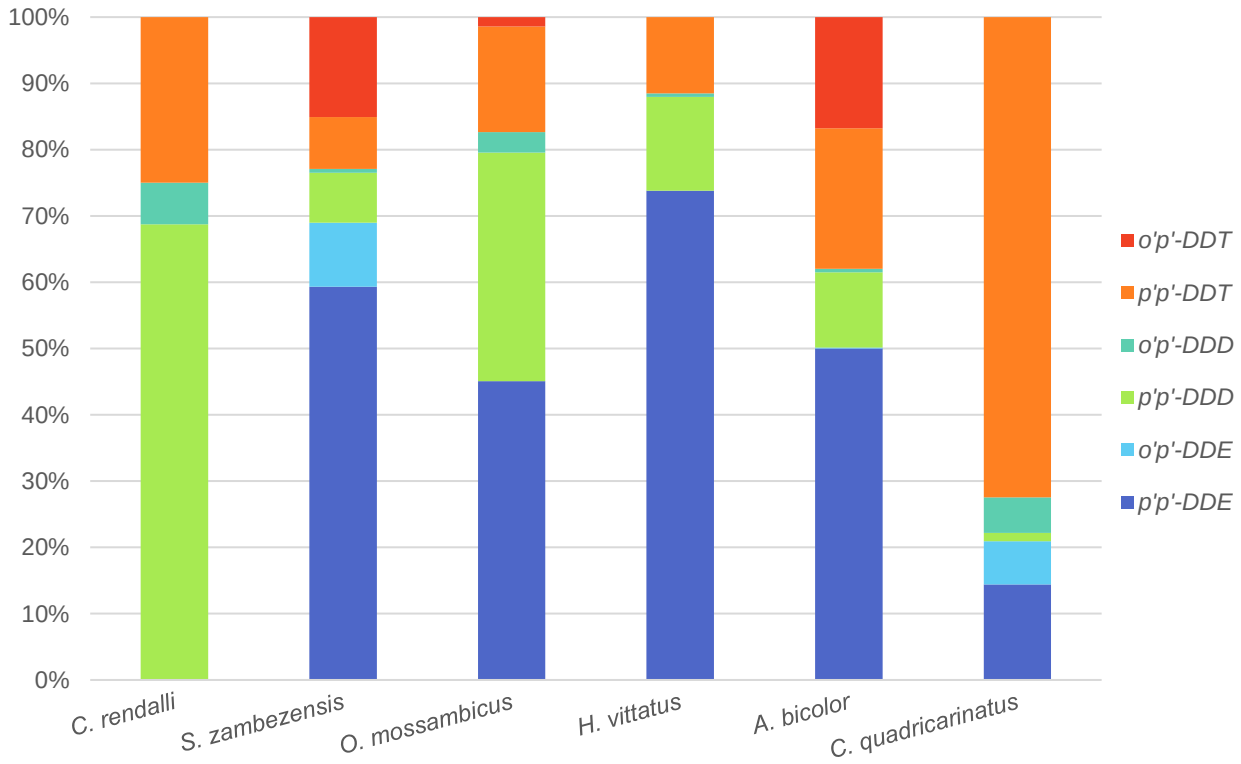


Figure 3.9 DDx contributions in the species analysed in the September sampling events from Phongola

3.3.6 ΣOCPs between seasons

The ΣOCPs were greatest in all species, except *H. vittatus*, in the April sampling period (Table 3.10). In the case of *O. mossambicus* and *C. gariepinus* this difference was very minor. However, when individual OCP classes were compared there were variations in the sample period that had higher concentrations, such as *C. rendalli* that had higher ΣHCHs and HCB in September, and the remaining OCPs were greater in April. The ΣOCPs in the September samples of *C. gariepinus* were solely comprised of ΣDDx.

Table 3.10 Summary of mean (±SD) and minimum–maximum concentrations (ng/g wm) OCP classes, and ΣOCPs analysed in the species sampled across the two sampling periods.

Species		ΣHCHs		HCB		ΣChlors		ΣDrins		ΣDDx		ΣOCPs	
		April	September	April	September	April	September	April	September	April	September	April	September
C. rendalli	Mean ± SD	0.13±0.1	0.17±0.14	ND	0.01±0.02	0.24±0.43	0.09±0.13	0.01±0.04	0.01±0.04	1.02±1.1	0.16±0.29	1.4±1.7	0.44±0.62
	Range	0–0.3	0–0.48		0–0.07	0–1.4	0–0.51	0–0.14	0–0.17	0–3.2	0–0.84	0–5.06	0–2.07
C. gariepinus	Mean ± SD	0.01±0.05	ND	ND	ND	0.003±0.02	ND	ND	ND	0.56±0.95	0.5 ± 0.12	0.65±1.0	0.5 ± 0.12
	Range	0–0.26				0–0.07				0–3.5	0–0.53	0–3.8	0–0.53
O. mossambicus	Mean ± SD	0.15±0.31	0.09±0.07	ND	ND	0.05±0.1	0.09±0.08	0.01±0.02	0.01±0.02	0.42±0.34	0.36±0.23	0.63±0.77	0.54±0.39
	Range	0–1.1	0–0.22			0–0.41	0–0.28	0–0.09	0–0.06	0.03–1.1	0–0.9	0.03–2.7	0–1.5
S. zambezensis	Mean ± SD	0.14±0.26	0.05±0.07	ND	0.02±0.07	0.15±0.21	0.06±0.12	0.07±0.12	0.04±0.17	5.3±8.0	0.33±0.55	5.7±8.5	0.5±0.98
	Range	0–1.1	0–0.24		0–0.31	0–0.69	0–0.47	0–0.42	0–0.78	0–31	0–1.7	0–33	0–3.5
H. vittatus	Mean ± SD	0.07±0.1	ND	0.35±0.75	ND	ND	ND	ND	ND	0.86±1.1	1.9±1.6	1.3±1.9	1.9±1.6
	Range	0–0.26		0–2.14						0–2.8	0.67–5.9	0–5.2	0.67–5.9
C. quadricarinatus	Mean ± SD	68±79	54±26	ND	ND	3.8±3.9	6.8±5.8	15±5.5	21±5.8	24±59	14±12	110±150	95±49
	Range	1.8–310	9.7–89			0.2–12	2.0–18	8.3–30	9.3–28	2.3–220	4.4–38	13–570	25–170
A. bicolor	Mean ± SD	NA	35±18	NA	0.86±1.05	NA	13±16	NA	15±15	NA	1100±920	NA	1100±970
	Range		14–57		0–2.13		2.1–36		3.3–38		217–2400		240–2500

September results of *C. gariepinus* taken from Volschenk *et al.* (2019) for comparative purposes, these samples were collected at the same time and location as the other samples from the September period.

3.4 Discussion

3.4.1 Hexachlorocyclohexanes (HCHs)

There were little changes between the April and September sampling events, and the concentrations in the sampled organisms remained relatively consistent (Table 3.1 and Table 3.2). In this highly agricultural region HCH would have been applied on both crops and livestock for its insecticidal properties since the 1940s (Willett *et al.*, 1998), until 2009 when South Africa ratified the Stockholm Convention (DEA, 2011). The pesticide would settle into aquatic systems via runoff (Tang *et al.*, 2013). Because of their persistence, toxicity, and tendency to bioaccumulate, their presence of these compounds in fish tissue indicates health risks (UNEP, 2009).

The ratio of the HCH isomers in the environment can indicate the formulation type (lindane or technical grade HCH), and transport pathway of HCHs. This can be achieved by comparing the abundance of α–HCH, which most abundant isomer in technical HCH, to the other metabolites and unintentionally produced "waste" isomers that will also be present. Therefore, different ratios of the isomers can explain if the HCH pattern is derived from technical HCH or lindane. It is expected that technical grade HCH would have a ratio of 4–7 α-/γ-HCH, while below 4 indicates lindane (Iwata *et al.*, 1995). The larger the ratio, the more weathered the source of technical HCH, because γ-HCH is degraded faster than α-HCH by light and microorganisms in soils and sediments (Malik *et al.*, 2007; Liu *et al.*, 2010). Conversely, the closer the ratio is to one, the more recent the lindane application. Both the volatile α-, and γ-HCH isomers can degrade into β-HCH, which is the most persistent and stable isomer (Willett *et al.*, 1998). A dominance of β-HCH indicates historical inputs of technical HCH.

For the April sampling event most instances had a ratio of <1 indicating a fairly recent application of lindane. Only *O. mossambicus* had a ratio that tended towards technical HCH (α-/γ-HCH = 6.0). *O. mossambicus, S. zambezensis,* and *C. quadricarinatus* also contained the highly persistent β-HCH which is a trait of historical use of HCH. The September event also had a ratio <1 indicating the use of lindane. *S. zambezensis* was the only sample species that had a ratio indicative of weathered HCH (α-/γ-HCH = 31). There was some presence of β-HCH in *O. mossambicus, S. zambezensis, A. bicolor* and *C. quadricarinatus.* This indicates that the HCHs have broken down into this stable form from historical use and these are accumulating in the exposed organisms.

Wolmarans (2015) collected frog samples from Phongola in April and October 2013 for a similar study. In these samples lindane (γ-HCH) was the most dominant isomer, however the persistent β-HCH was not detected, which was a common isomer in the current study. The mean ΣHCHs reported in the frog tissue were similar to what was recorded in the current study from Phongola, with the exception of *C. quadricarinatus* and *A. bicolor* which were significantly greater (Table 3.11). Lindane was also detected in *C. gariepinus* in a study conducted in the upper reaches of Klip River, Gauteng—largely affected by urban and industrial inputs (Pheiffer *et al.*, 2018c). Here, lindane was the dominant isomer, and there were also no detections of β-HCH. The mean concentrations of HCHs reported in the Klip River were similar to those in this study (Table 3.11). A study in Hartbeespoort, Roodeplaat, and Rietvlei Dams, reported ΣHCHs in *C. gariepinus* (Barnhoorn *et al.*, 2015). These dams, located in the North West and Gauteng provinces, in central South Africa, are severely polluted, and hyper-eutrophic impoundments (Van Ginkel, 2011). These concentrations were higher than those reported in the current study (Barnhoorn *et al.*, 2015). Concentrations in *L. capensis* from the Barrage section in the Vaal River (Wepener *et al.*, 2011), were within the range of the HCH concentrations in the Phongola system. *Hydrocynus vittatus* sampled in 2010 and 2011 from the Olifants, Letaba, and Luvuvhu Rivers within the Kruger National Park (which are impacted anthropogenically, and are situated in the north of South Africa bordering Mozambique) had concentrations within the range of the current study (Gerber *et al.*, 2016). These authors also reported that the ΣHCHs were dominated by γ-HCH, and a proportion of β-HCH and other isomers. The trends of all these studies indicate a mixture of technical HCH and "recent application" lindane within the vicinity of the sampling areas, although it has been banned since 2009. A study conducted in the iSimangaliso Wetland Park sediments, approximately 100 km east of Phongola, however detected that α-, and β-HCH were the dominant isomers, while γ-HCH was much lower (Buah-Kwofie and Humphries, 2017). Even though α- was much higher than γ-HCH the ratio of α-/γ-HCH indicates that technical HCH and/or lindane have recently entered the system in the iSimangaliso Wetland Park. Within the sediments of the industrial region of eThekwini, south of Phongola, no HCHs were detected (Vogt *et al.*, 2018).

3.4.2 Hexachlorobenzene (HCB)

Very little HCB was detected in fish in either sampling period. This indicates that this compound has not been used extensively in the area or it is broken down more rapidly. It has been detected in fish from the Vaal River (Wepener *et al.*, 2011), and Klip River (Pheiffer *et al.*, 2018c) at higher concentrations (Table 3.11). There were, however, no HCBs detected in frogs from Phongola (Wolmarans, 2015), yet higher concentrations were detected in *H. vittatus*

in the same region by Wepener *et al.* (2012). Similar concentrations were found in *Oreochromis* from Tanzania (Polder *et al.*, 2014) and fish from the DRC (Verhaert *et al.*, 2013).

3.4.3 Chlordane

Chlordanes were widely detected in species in the Phongola system. This can be attributed to a wide use of the pesticides in the region (Vijgen *et al.*, 2006; Wepener *et al.*, 2011; Wepener *et al.*, 2012). Chlordane has been used on vegetables, grains, maize, potatoes, sugarcane, fruits, nuts, cotton, and to control termites. In South Africa it was restricted to stem treatments of citrus and vineyards, and pest control treatment of buildings from 1993 (Batterman *et al.*, 2008). In 2000 it was withdrawn as an agricultural remedy, and in 2005 completely banned (DAFF, 2008). Technical chlordane is comprised of *cis*- and *trans*-chlordane with a ratio of approximately 0.77 between them. If the ratio is <0.77 it indicates a recent application of chlordane (Eitzer *et al.*, 2001). Oxychlordane is the primary metabolite of chlordane and nonachlor (Bondy *et al.*, 2000; ATSD, 2007; Batterman *et al.*, 2008).

In the cases where this ratio could be calculated (when both isomers were present), the ratio based on mean concentrations ranged between 0–1.4, indicating a mix in recent and historic application, although this compound has been banned under the Stockholm Convention. Oxychlordane was infrequently detected in Phongola. Recent application patterns were also detected in Durban sediments (Batterman *et al.*, 2008; Vogt *et al.*, 2018). A study in the iSimangaliso Wetland Park, approximately 100 km east of Phongola, also reported some chlordanes (heptachlor, heptachlor epoxide, and methoxychlor) in sediments (Buah-Kwofie and Humphries, 2017).

The concentrations reported in this study are on average greater than the concentrations detected in Phongola from *H. vittatus* collected a few years previously (Wepener *et al.*, 2012) (Table 3.11) for most of the compounds except for chlors that had not been detected in *H. vittatus* of the current study. In Phongola 2014, Σchlors only consisted of α-chlordane in *Xenopus muelleri*, and within the same range as what was detected in the current study (Wolmarans, 2015). Similar concentrations were reported in fish from Ethiopia (Deribe *et al.*, 2014), and Tanzania (Polder *et al.*, 2014). The Σchlors reported in this study were much lower than in *C. gariepinus* from the Klip River (Pheiffer *et al.*, 2018c), *H. vittatus* from the Olifants, Letaba, and Luvuvhu Rivers (Gerber *et al.*, 2016), *C. gariepinus* from Hartbeespoort, Roodeplaat, and Reitvlei Dams (Barnhoorn *et al.*, 2015). Concentrations of Σchlors from the Barrage region in the Vaal River had similar concentration to the Phongola samples (Table

3.11). The current study detected similar, to slightly higher concentrations of ΣChlors in comparison with fish sampled in the Olifants River 2012 (Verhaert *et al.*, 2017) (Table 3.11).

3.4.4 Cyclodienes

Most species did not have quantifiable concentrations of the compounds, and when detected the concentrations were low, with the exception of *C. quadricarinatus* in both seasons (April and September), and *A. bicolor.* A ratio of dieldrin/aldrin of approximately 2 indicates that the source of the cyclodiens is historical (Buah-Kwofie and Humphries, 2017). This is because aldrin degrades to the more stable and persistent dieldrin. This is expected because this group of compounds had been banned since the 1980s (Fisher, 2011). In the few instances where the cyclodiens were detected it was evident that it was from weathered sources (Table 3.6 and Table 3.7). Sediments in the iSimangaliso Wetland Park had patterns of recent-application of cyclodiens. ΣCyclodienes (with the addition of endrin ketone) were at least an order of magnitude higher than what was detected in this study (Buah-Kwofie and Humphries, 2017).

Only aldrin was detected in the Phongola region in 2014 in *X. muelleri* (Wolmarans, 2015), and at a slightly lower concentrations than recorded in this study (Table 3.11). Dieldrin, but not aldrin and endrin, was detected in *C. gariepinus* from the Klip River (Pheiffer *et al.*, 2018c). These compounds were also detected in the Roodeplaat and Rietvlei Dams from *C. gariepinus* (Barnhoorn *et al.*, 2015). Both of these studies had high concentrations, and only *A. bicolor* and *C. quadricarinatus* had similar concentrations.

3.4.5 Dichlorodiphenyltrichloroethane (DDx)

The DDx concentrations were quantifiable in all species analysed. DDT metabolises to form DDE and DDD, and other breakdown products. By calculating DDT/(DDE+DDD) it is possible to determine if the DDx signature in the samples originated from fresh (ratio >1), or aged (ratio <1) sources (Doong *et al.*, 2002; Zhou *et al.*, 2006). The ratio in general from the Phongola was <1 in both seasons. There were a few species that had a ratio over one (1–5.7). These were *H. vittatus* and *C. quadricarinatus* in the April sample event, and *C. quadricarinatus* from the September event. This indicates that the sources were mostly from aged DDT, with the exception of *C. quadricarinatus* and *H. vittatus* (April). This is unusual considering reintroduction of DDT spraying in 2000 because of increased infection of, and mortality due to malaria in the region (Quinn *et al.*, 2011). It is typically sprayed from January to March (Bouwman *et al.*, 1990), and it is therefore expected that the April event would have trends of fresh DDT.

Similar concentrations of DDx have been reported in frogs in the Phongola system sampled in 2013 and 2014 (Wolmarans, 2015) (Table 3.11). A study conducted on *H. vittatus* in the Phongolapoort Dam in 2009 detected much higher concentrations of DDx in the system, in cases almost two orders of magnitude greater than what has been analysed in the species in this study (Wepener *et al.*, 2012). Much higher concentrations were reported from *C. gariepinus* in the Klip River (Pheiffer *et al.*, 2018c), dams in Gauteng and North West (Barnhoorn *et al.*, 2015). Similar, to slightly higher concentrations were reported from fish in the Olifants River (Verhaert *et al.*, 2017), and in *H. vittatus* from the Olifants, Letaba, and Luvuvhu Rivers (Gerber *et al.*, 2016)—these rivers are situated in Limpopo where DDT is utilised, fish from Ethiopia (Deribe *et al.*, 2011; Deribe *et al.*, 2014), fish from Tanzania (Polder *et al.*, 2014), and from the DRC (Verhaert *et al.*, 2013).

Table 3.11 Concentrations of contaminants found in tissues from studies conducted in South Africa and other parts of Africa, in both ng/g wm and lipid mass (lm)

Region	Species	ΣDDx		ΣHCH		Σchlors		ΣHCB		Σdrins		Reference
		ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	
Phongola 2013	*C. quadricarinatus* April	22±58 2.3–220	3800±10000 140–38000	68±79 1.8–310	8500±8100 220–25000	3.8±3.9 0.2–12	460±480 19–1700	<LOD		15±5.5 8.3–30	1900±800 850–3100	This study
	C. quadricarinatus September	14±12 4.4–38	1200±970 260–2600	54±26 9.7–89	4700±1900 1000–7200	6.8±5.8 2.0–18	720±790 170–2300	<LOD		21±5.8 9.3–28	1900±890 990–3600	
	H. vittatus April	0.86±1.08 <LOD–2.82	44±52 <LOD–130	0.01±0.08 <LOD–0.3	7.4±12 <LOD–35	<LOD		0.35±0.75 0–2.14	15±30 <LOD–79	<LOD		
	H. vittatus September	0.36±0.23 <LOD–0.9	1100±1800 81–6800	<LOD		<LOD		<LOD		<LOD		
	S. zambezensis April	3.28±4.9 <LOD–19.18	80±150 <LOD–690	0.14±0.26 <LOD–1.1	1.2±1.8 <LOD–5.4	0.2±0.2 <LOD–0.69	1.9±2.7 <LOD–8.5	<LOD		0.07±0.12 <LOD–0.42	0.9±1.6 <LOD–5.7	
	S. zambezensis September	0.33±0.55 <LOD–1.7	19±41 <LOD–170	0.05±0.07 <LOD–0.24	1.9±3.6 0–14	0.06±0.12 <LOD–0.47	2.7±6.0 <LOD–27	0.02±0.07 <LOD–0.31	0.13±0.37 <LOD–1.6	0.04±0.17 <LOD–0.78	0.87±3.9 <LOD–17	
	O. mossambicus April	0.24±0.2 <LOD–0.79	210±170 16–610	0.15±0.31 <LOD–1.1	220±650 <LOD–2900	0.05±0.1 <LOD–0.41	13±27 <LOD–97	<LOD		0.01±0.02 <LOD–0.09	4.1±11 <LOD–43	
	O. mossambicus September	0.36±0.23 <LOD–0.9	320±450 <LOD–1700	0.09±0.07 <LOD–0.22	120±260 <LOD–1200	0.09±0.08 <LOD–0.28	110±210 <LOD–740	<LOD		<LOD <LOD	0.84±2.6 <LOD–9.5	
	C. gariepinus April	0.16±0.7 <LOD–3.5	120±140 <LOD–540	0.01±0.05 <LOD–0.26	16±82 <LOD–410	0.003±0.015 <LOD–0.07	0.49±2.5 <LOD–12	<LOD		<LOD		
	C. rendalli April	0.87±1.0 <LOD–3.2	100±120 <LOD–430	0.13±0.1 <LOD–0.3	28±47 <LOD–180	0.24±0.43 <LOD–1.43	35±75 <LOD–240	<LOD		0.01±0.04 <LOD–0.14	9.7±31 <LOD–120	
	C. rendalli September	0.16±0.29 <LOD–0.84	51±110 <LOD–350	0.17±0.14 <LOD–0.48	46±34 <LOD–110	0.09±0.13 <LOD–0.51	25.99±37.52 0–117.96	0.01±0.02 0–0.07	9.1±1.4 8.1–10	0.01±0.04 <LOD–0.17	1.4±6.3 <LOD–27	
	A. bicolor September	1100±920 220–2400	6600±3200 3400–11000	35±18.01 14–57	290±170 70–460	13±16 2.1–36	78.15±64.76 25–170	0.86±1.1 <LOD–2.1	4.1±4.9 <LOD–9.8	15±15 3.3–38	98±62 43–170	

Region	Species	ΣDDx		ΣHCH		Σchlors		ΣHCB		Σdrins		Reference
		ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	
Olifants River	*C. gariepinus*	26 ± 31 2.6–61		<LOQ		0.1±0.1 <LOQ–0.2		NA		NA		Verhaert *et al.* (2017)
	Labeo rosae	5.1 ± 3.2 1.4–7.8		<LOQ		<LOQ		NA		NA		
	Labeo congoro	1.2 ± 0.5 0.4–1.4		<LOQ		<LOQ		NA		NA		
	Labeobarbus marequensis	1.6 ± 0.7 <LOQ–2.5		0.2 ± 0.1 0.1–0.2		0.3±0.1 <LOQ–0.2		NA		NA		
	S. zambezensis	18±3.3 8.5–43		0.4 ± 0.3 <LOQ–0.8		0.4±0.04 <LOQ–0.1		NA		NA		
	Schilbe intermedius	11 ± 5.1 4.8–14		0.02±0.01 1.1–5.6		0.2±0.2 <LOQ–0.1		NA		NA		
	H. vittatus	5.2±0.9 3.4–8.1		<LOQ		<LOQ		NA		NA		
	O. mossambicus	5.1±4.7 1.1–10		0.3±0.5 <LOQ–0.9		0.1±0.02 <LOQ–0.1		NA		NA		
Barrage, Vaal River	*L. capensis*	280±84		80±22		21±4.7		250±250		NA		Wepener *et al.* (2011)
Hartbeespoort Dam	*C. gariepinus*	130 ± 25 100–160		75±18 <LOD–230		ND		NA		ND		Barnhoorn *et al.* (2015)
Roodeplaat Dam		150±88 <LOD–350		200±53 <LOD–490		290±0 <LOQ–290		NA		150±35 <LOQ –470		
Rietvlei Dam		340±260 <LOD–760		55±7.0 <LOD–63		83±41 <LOD–160		NA		77±36 <LOD–100		
Klip River	*C. gariepinus*	130±120 14–890		8.0±1.8 4.9–11		42±39 <LOD–170		20.1±12.5 8.24–65		15±9.6 4.4–43		Pheiffer *et al.* (2018c)

Region	Species	ΣDDx		ΣHCH		Σchlors		ΣHCB		Σdrins		Reference
		ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	
Pongolapoort Dam	*H. vittatus Feb–09*		5400±1200		7.7±2.6		3.5±1.2		82±41	NA		Wepener *et al.* (2012)
	H. vittatus Sep–09		6400±2600		16±11		3.8±1.6		360±230	NA		
Olifants River (high flow 2010)	*H. vittatus*		500±200 120–1400		88 ± 14 19–110		25±12 17–79		<LOD	NA		Gerber *et al.* (2016)
Olifants River (low flow 2010)			9000±3200 900–3400		310±110 27–1100		380±140 16–1500		2.6±1.5 3.4–16	NA		
Letaba River (low flow 2010)			9400±2400 2700–1800		310±36 210–500		350±60 160–790		9.9±1.9 5.9–19	NA		
Luvuvhu River (low flow 2010)			32000±8000 2700–11000		500±220 470–3800		510±180 47–3800		26±11 5.0–190	NA		
Luvuvhu River (highflow 2011)			4200±1700 110–22000		430±71 12–1100		240±43 12–1100		7.4±1.7 0.9–26	NA		
Phongola	*A. garmani*	2.1±0.5 0.9–7.1		0.3±0.1 <LOD–1.7		<LOD		ND		ND		Wolmarans (2015)
	C. xerampelina	9.3±2.8 3.2–33		0.2±0.1 0.1–0.5		ND		ND		ND		
	P. anchietae	0.6±0.4 <LOD–3.3		0.8±0.2 0.4–3.8		ND		ND		ND		
	X. meuleri	1.8±0.4 0.7–4.		1.3±0.2 0.9–2.3		0.2±0.14 <LOD–1.2		ND		0.1±0.04 <LOD–0.6		

Region	Species	ΣDDx		ΣHCH		Σchlors		ΣHCB		Σdrins		Reference
		ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	
Lake Awassa, Ethiopia	*Oreochromis niloticus*	19±28.91 1.7–102.5		NA		0.85±0 0.85–0.85		NA		NA		Deribe *et al.* (2014)
	C. gariepinus	29 ± 27 5.4–100		NA		1.2 ± 0 1.2–1.2		NA		NA		
	Barbus intermedius	56 ± 86 13–410		NA		2.2 ± 2.0 0.75–3.6		NA		NA		
Lake Victoria, Tanzania	*Oreochromis sp*	0.19 0.02–0.73	35 7.2–103	<LOD	<LOD	<LOD	0.5 <LOD–4.1	0.01 <LOD–0.02	2.5 1.4–4.0	NA		Polder *et al.* (2014)
Lake Tanganyika, Tanzania		9.5 6.3–12	270 210–320	0.03 0.02–0.04	1.1 0.8–1.5	0.03 0.02–0.04	0.9 <LOD–3.7	0.04 0.03–0.05	1.2 1.1–1.3	NA		
Lake Nyasa, Tanzania		0.26 0.29–0.34	13.2 11–19	0.02 0.01–0.04	1.3 0.9–2.1	0.02 0.01–0.04	0.2 <LOD–0.7	0.3 0.2–0.04	21 1.2–2.9	NA		
Lake Babati, Tanzania		1.6 1.4–4.1	43 14–150	<LOD	<LOD	<LOD	0.1 <LOD–0.8	0.04 0.03–0.07	1.2 0.6–2.8	NA		
Lake Koka, Ethiopia	*B. intermedius*	6.2 ± 5.8 0.05–21		NA		NA		NA		NA		Deribe *et al.* (2011)
	C. carpio	4.5 ± 2.2 2.1–11		NA		NA		NA		NA		
	C. gariepinus	15 ± 17 1.8–73		NA		NA		NA		NA		
	O. niloticus	6.9 ± 2.7 2.3–12		NA		NA		NA		NA		

Region	Species	ΣDDx		ΣHCH		Σchlors		ΣHCB		Σdrins		Reference
		ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	ng/g wm	ng/g lm	
Congo River, Democratic Republic of the Congo	*Marcusenius sp*	0.2 <LOD–0.96		0.19 0.12–0.44		NA		0.02 <LOD–0.06		NA		Verhaert *et al.* (2013)
	Schilbe marmoratus	0.37 0.25–0.53		0.12 <LOD–0.21		NA		0.05 <LOD–0.08		NA		
	Synodontis alberti	0.09 <LOD–0.45		0.16 <LOD–0.3		NA		0.8 <LOD–0.13		NA		
	Brycinus imberti	0.18 0.09–0.28		0.15 0.03–0.4		NA		0.7 0.02–0.09		NA		

NA: not analysed

3.5 Conclusion:

Organic contaminants have been detected in muscle tissue of fish inhabiting the Phongola River. This poses a risk not only to the fish but also to higher trophic level organisms who feed on these organisms, like birds, crocodiles, and even humans. It is unusual however that a high trophic position fish, *H. vittatus*, had some of the lowest concentrations, and a lower trophic feeder, *C. quadricarinatus,* had such high concentrations. In previous studies conducted in the Phongola system *C. gariepinus* and *H. vittatus* were analysed for abnormalities, histologically, although there were many alterations in the tissues, the health of the fish was not compromised (McHugh *et al.*, 2011; McHugh *et al.*, 2013). Fish from this system do not seem to be showing any serious detrimental effects due to long term exposure. However, possible increases in both the concentrations, and number of stressors could eventually lead to adverse effects, especially considering that these compounds bioaccumulate and biomagnify. To determine if the fish exposed to these compounds might be experiencing detrimental effects a fish health assessment could be conducted. In this study we utilised biomarkers to determine the effects of these compounds on the sampled fish, this will be discussed in the next chapter. Because this system is often used for subsistence fishing, resulting in humans also being at risk through their diet apart from being exposed to the compounds during application, i.e. in the case of the residual indoor spraying of DDT.

4 Biomarker responses from organisms in the Vaal River and Phongola Floodplain

4.1 Introduction

Animals are frequently used to give an indication of the health of the environment—and they are termed bioindicators. Animals are useful indicators of environmental health because they are often exposed to a wide range of stressors present in the environment, like severe fluctuations of temperature, ultraviolet light, pH, oxygen, and food availability during all stages of their life cycle (Benedetti *et al.*, 2015; Hackenberger *et al.*, 2015; Lee *et al.*, 2015). Anthropogenic pollutants that are present in the environment, are also capable of inducing stress responses in organisms, and examples of these pollutants include metals, polycyclic aromatic hydrocarbons (PAHs), organohalogenated xenobiotics, pesticides, and flame retardants. In recent years emerging pollutants, such as microplastics, endocrine disrupting compounds, personal care products, pharmaceuticals, and nanoparticles have been documented in environmental matrices, and these are also known to result in stress responses (Fabbri, 2015). Bioindicators are useful because they provide a time-integrated evaluation of environmental contaminant bioavailability since they are capable of accumulating the chemicals in tissues (Schmitt and Dethloff, 2000; Van der Oost *et al.*, 2003; Newman, 2010). Effects begin at the molecular level and then progress to the biochemical, subcellular, cellular, tissue, organ, whole organism, and population levels. Biomarker responses—any measurable change in behaviour, physiology, cellular, biochemical, or molecular—can be used to determine biological and/or biochemical effects after an exposure to toxicants or environmental stressors. Thus, utilising molecular biomarkers can be an early warning tool before effects are seen at the higher levels of biological organisation (Van der Oost *et al.*, 2003; Newman, 2010; Lee *et al.*, 2015). They are useful for pre-emptive risk assessment for organisms, and in turn human health (Benedetti *et al.*, 2015). Additionally, biomarkers are robust indicators of exposure and effect in organisms (Depledge, 1994; Van der Oost *et al.*, 2003).

Exposure to pollutants are known to produce many detrimental effects because they interact with several cellular regions which induce activation of detoxification or biotransformation pathways, or the alteration on cellular mechanisms (Benedetti *et al.*, 2015). Because there is a wide range of regions in the body that can be affected by toxicants a suite of biomarkers can be utilised to determine what effects are manifesting. The biomarkers are often grouped according to the responses they measure: biomarkers of exposure (e.g. acetylcholinesterase

and cytochrome P450), biomarkers of effect (e.g. superoxide dismutase, catalase, malondialdehyde, protein carbonyls, and energetics), and susceptibility (Van der Oost *et al.*, 2003). In this study only the biomarkers of exposure and effect were determined.

4.1.1 Biomarkers of exposure

Acetylcholinesterase (AChE) is found in nervous tissues, muscle, and in the liver of organisms (Van der Oost *et al.*, 2003). It is responsible for the termination of impulse transmission of nerve firing, by catalysing the hydrolysis of acetylcholine into choline and acetic acid (Cajaraville *et al.*, 2000; Viarengo *et al.*, 2007; Linde-Arias *et al.*, 2008). This is crucial for signal transmission. This type of signal transmission occurs in controlling functions such as movement, respiration, hormonal function, feeding, prey-hunter relationships, and reproduction (Labenia *et al.*, 2007; Solé *et al.*, 2010; Peakall, 2012). Inhibition of AChE results in excessive stimulation of cholinergic nerves, which in turn leads to tremors, convulsions, and death (Üner *et al.*, 2006). Its inhibition has been directly linked with the toxic mechanisms of action of organophosphorus and carbamate pesticides, even at low concentrations (Cajaraville *et al.*, 2000; Van der Oost *et al.*, 2003; Andreescu and Marty, 2006; Üner *et al.*, 2006). Other compounds such as metals (Gill *et al.*, 1991; Zinkl *et al.*, 1991; Guilhermino *et al.*, 1998; Frasco *et al.*, 2005; Roy *et al.*, 2006), organochlorines, antifoulants (López-Galindo *et al.*, 2010), herbicides, surfactants (Guilhermino *et al.*, 2000), detergents (Guilhermino *et al.*, 1998; Jifa *et al.*, 2005; Feng *et al.*, 2008), polychlorinated biphenyls (PCBs) (Buet *et al.*, 2006), PAHs (Kang and Fang, 1997; Vieira *et al.*, 2008), polybrominated diphenylether (PBDEs) (Vidal-Liñán *et al.*, 2016), pharmaceuticals (Nunes *et al.*, 2006), and microplastics (Oliveira *et al.*, 2013), and complex mixtures of contaminants (Payne *et al.*, 1996; Moreira *et al.*, 2004) can also cause inhibition of the AChE. These xenobiotics can either reversibly or irreversibly bind to the catalytic site of the AChE enzyme (Cajaraville *et al.*, 2000; Viarengo *et al.*, 2007). This causes inactivation of the enzyme and results in the accumulation of acetylcholine, which results in neurotoxic effects like hyperstimulation of nicotinic and muscarinic receptors, and disrupted neurotransmission (Čolović *et al.*, 2013).

Organisms are capable of metabolising various compounds, including drugs, some endogenous compounds, and xenobiotics like halogenated aromatic hydrocarbons (HAHs) through the cytochrome P450 (CYP450) pathway (Stegeman and Lech, 1991; Van der Oost *et al.*, 2003). As a biomarker, its importance is in its role to biotransform xenobiotics like polychlorinated dibenzo-*p*-dioxins and polychlorinated furans (PCDD/Fs), dioxin-like PCBs, and some PAHs—collectively termed dioxin-like compounds (DLCs). DLCs bind to the aryl hydrocarbon receptor (AhR) that is present in vertebrate cells' cytoplasm, to form a complex.

This complex moves into the nucleus where it binds with a specific DNA sequence, called the dioxin response element (DRE). This activates the transcription of genes, including the CYP450 genes (Aarts *et al.*, 1995; Denison *et al.*, 2004; Whyte *et al.*, 2004). The CYP enzyme converts the xenobiotic to a more water soluble form and this facilitates the excretion of the compound from the cell, and in turn detoxification of the body occurs (Cajaraville *et al.*, 2000; Parkinson and Ogilvie, 2001; Newman, 2010). This process can however be slow, and may result in uncoupling of electron transfer and oxygen reduction from substrate oxidation, which can cause reactive oxygen species (ROSs) to be released (Benedetti *et al.*, 2015). The metabolised compound can also be more toxic than the parent compounds, and become genotoxic by adducting to the DNA, such as in the case of PAHs causing oxidative carcinogenicity (Irwin *et al.*, 1997; Benedetti *et al.*, 2015). For example, the PAH benzo-*a*-pyrene is biotransformed by sequential oxygenation and hydration that is catalysed by the CYP450 and epoxide hydroxylase to form several isomeric dihydrodiol-epoxide structures (Stegeman and Lech, 1991). Benzanthracene, diethylnitrosamine, aflatoxin B_1, and 2-acetylaminofluorine are other compounds that become more toxic after biotransformation (Stegeman and Lech, 1991).

4.1.2 Biomarkers of effect

The toxicity of many xenobiotics is associated with oxidative stress, which is the imbalance between antioxidants and free radicals, and are referred to as ROSs, which are highly reactive, and unstable (Abdollahi *et al.*, 2004). When ROSs are in the body they are capable of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids—which can lead to cell damage and the disruption of homeostasis (Halliwell, 1994; Lobo *et al.*, 2010). Organisms however, do have antioxidant enzymes that breakdown the ROSs. Firstly, superoxide dismutase (SOD) catalyses the breakdown of the ROS, superoxide, into oxygen and hydrogen peroxide (Bannister *et al.*, 1987; Zelko *et al.*, 2002; Lobo *et al.*, 2010). This is followed by catalase (CAT) which decomposes the harmful hydrogen peroxide into water and oxygen (Gaetani *et al.*, 1996; Chelikani *et al.*, 2004; Lobo *et al.*, 2010). However, pollutants can reduce the efficiency of the antioxidant enzymes, and/or enhance the formation of ROSs (Benedetti *et al.*, 2015). Free radicals often react with polyunsaturated fatty acids in cell membranes, and this results in lipid peroxidation (Livingstone, 2001). Malondialdehyde (MDA) is the resulting breakdown product formed when lipid membranes degrade (Livingstone, 2001; Üner *et al.*, 2006). Lipid peroxides can react with DNA and form DNA adducts (Livingstone, 2001), disrupt cell membrane functionality, and can affect the antioxidant system. Damage of lipids had been linked to PAHs (Choi and Oris, 2000; Kaloyianni *et al.*, 2009), and Aroclor 1254 (Wafford and Thomas, 1988) exposure. Measuring MDA reflects the severity of the lipid

peroxidation in an organism. Oxidation of amino acids results in the formation of protein carbonyls (PC). Compounds such as deltamethrin, endosulfan, and paraquat have been determined to cause PCs (Parvez and Raisuddin, 2005). An increase in PC results in cellular and tissue damage, decreased enzymatic function, and delayed protein regeneration, and once PCs have been formed they cannot be reversed (Almroth *et al.*, 2005).

Cellular energy allocation details the energy production, storage, and use within the tissue of the target organism (De Coen and Janssen, 1997). The energy availability of organisms fluctuates with seasons, food availability, flow rate, trophic level, and life stage (Gourley and Kennedy, 2009; Hackenberger *et al.*, 2015). Exposure to pollutants can also cause decreases in the nutrient stores of the organisms, which cause reduced energy stores and changes in cellular energy allocation (Hackenberger *et al.*, 2015).

Dichlorodiphenyltrichloroethane (DDT) had been sprayed in the South African provinces of KwaZulu-Natal (KZN) and Limpopo, as well as the neighbouring Mozambique, north of KZN, for malaria vector control. It had been used since 1946, and was briefly replaced with deltamethrin between 1995 and 2000, however mosquitos developed a resistance to deltamethrin and DDT was reinstated (Sharp and Le Sueur, 1996; Sereda and Meinhardt, 2005). DDT, and metabolites had been reported in fish from the Phongola floodplain (Bouwman *et al.*, 1990; Wepener *et al.*, 2012; Wolmarans, 2015). Other pollutants are also still quantified in fish from the region, despite many being banned by the Stockholm Convention (Stockholm Convention, 2017): chlordane, hexachlorocyclohexanes (HCHs), hexachlorobenzenes (HCBs) (Wepener *et al.*, 2012; Wolmarans, 2015), PCBs, and PBDEs (Wepener *et al.*, 2012), and metals (Fisher, 2011; Tate, 2014) have also been detected in fish in the area.

4.1.3 Study areas

The section of the Vaal River chosen for sampling in this study flows through a highly industrial region, known as the Vaal Triangle, and receives water from numerous tributaries which flow through similarly impacted regions in Gauteng. These industries include iron and steel works, a petrochemical plant, coal powered electricity generation plants, gold mines, and a coal-based synthetic chemical manufacturing plant. Additionally, three large metropolitan areas border this region of the Vaal River. The anthropogenic activities are known to release a variety of pollutants and these have been quantified in matrices from the Vaal River, its tributaries, and the Vaal Triangle. These include PCBs (Wepener *et al.*, 2011), PAHs (Moja *et al.*, 2013; Pheiffer *et al.*, 2018b), PCDD/Fs (Nieuwoudt *et al.*, 2009; Quinn *et al.*, 2009; Nieuwoudt *et al.*,

2011), and brominated flame retardants (BFR) like brominated diphenyl ethers (BDEs) (Polder *et al.*, 2008; Wepener *et al.*, 2011; Vogt *et al.*, 2015). Additionally, there have been reports of pesticides within environmental matrices in the Vaal Triangle (Quinn *et al.*, 2009; Wepener *et al.*, 2011; Pheiffer *et al.*, 2018c).

The Phongola Floodplain is located in the northern area of KwaZulu-Natal close to the borders of Mozambique and Swaziland. The area around the floodplain is rural with many informal settlements that lack infrastructure and facilities. Agriculture is prevalent in the area, with numerous sugarcane and subtropical fruit plantations, subsistence farming is also common in the area (McHugh *et al.*, 2011). There are, however, some industrial activities in the area including a saw mill, paper mill (Venter *et al.*, 2010), and a gold mine (Tate, 2014). DDT is also utilised in and around the Phongola area as a malaria preventative measure (Sereda and Meinhardt, 2005). Many pollutants relating to these activities have been determined in the Phongola system, these include pesticides, PCBs, metals, and flame retardants (Bouwman *et al.*, 1990; McHugh *et al.*, 2011; Wepener *et al.*, 2012; McHugh *et al.*, 2013).

The aims of this chapter are to link the biological effects to concentrations of selected xenobiotics in the sampled fish by i) determining whether the biomarker data reflect trends based on the chemical exposure data, ii) investigating for temporal, spatial, or species trends, iii) explore whether the fish sampled from the Vaal River experience more detrimental effects based on the greater xenobiotic exposure present in this system compared to the Phongola floodplain, and iv) determine the toxicant-induced stress levels of different fish species by means of the Integrated Biomarker Index (IBR).

4.2 Methods:

4.2.1 Sample collection:

Fish (*Oreochromis mossambicus, Clarias gariepinus*, *Synodontis zambezensis*, *Hydrocynus vittatus*) and invertebrate (*Cherax quadricarinatus*) samples were collected from the Phongola River in the high flow season (April 2013) and the low flow season (September–November 2013) and. Multiple sites were selected from around the Phongola floodplain, namely in the Phongola River, Ngwavuma River, and the Nyamithi Pan.

Fish (*C. gariepinus, Labeobarbus aeneus, Cyprinus carpio*, and *Labeo capensis*) were also collected from three sites in the Vaal River in downstream sequence: Vischgat, Barrage and Thabela Thabeng. These samples were collected in October 2014.

The fish were collected from both systems by utilising electro-shock devices, and fyke nets. Lateral muscle and liver tissue were dissected, and preserved in Hendrickson's buffer (40 nM tris-HCl, 10 nM β-mercaptoethanol, 1 mM 0.04% bovine serum albumin (BSA), and 1 nM EDTA) and stored on ice until return to the laboratory where they were stored at -80°C.

4.2.2 Sample preparation:

Approximately 0.05 g of muscle was homogenised in 250 µL tris-sucrose buffer (25 mM tris-HCl and 250 nM sucrose) for the determination of AChE and MDA. The sample was centrifuged at 9 500 g for 10 minutes. For CAT, SOD, CYP450 and PC analysis 0.1 g of liver was homogenised in 1 mL general homogenising buffer (GHB) containing: 0.1 M potassium phosphate buffer (PPB: 0.09 M K_2HPO_4 and the pH adjusted to 7.4 with 0.9 M potassium dihydrogen phosphate), 1.15% KCl, 1 nM EDTA, 0.1 nM phenylmethanesulphonyl fluoride, and 20% glycerol. This sample was centrifuged at 10 000 g for 10 minutes. Lastly, 0.2 g muscle tissue was homogenised in 400 µL electron transport system (ETS) buffer (0.1 M tris-HCl, 0.2% Triton X-100, 15% polyvinylpyrrolidone, 153 µM $MgSO_4$) for CEA analysis.

Biomarker activity is calculated based on the amount of protein in the sample. Protein content of each sample was determined using the Bradford protein assay (Bradford, 1976). Proteins in the sample bind to the active ingredient in the Bradford's reagent (Coomassie Brilliant Blue) and the optical density (OD) was determined (Berthold multi-mode microplate reader (LB941) at 590 nm. The absorbance was compared to the absorbance of a protein standard curve at concentrations between 0–2 500 µg/mL.

4.2.3 Acetylcholine esterase

The method followed is adapted from the procedure by Ellman *et al.* (1961). The following were added into the first 3 columns of a clear 96 well microtitre plate: 210 µL PPB, 10 µL *s*-acetylthiocholine iodide (30 mM), an 10 µL Ellman's reagent (10 mM 2,2'-dinitro-5,5'dithio-dibenzoic acid (DNTP) in methanol) and was mixed by gently tapping the plate. The plate was incubated for 5 minutes at 37°C, after which 5 µL of sample was added to the wells in triplicate, and GHB was used as a blank. The kinetic reaction was determined by recording the OD at 450 nm every minute for 6 minutes using the Berthold plate reader.

4.2.4 Cytochrome P450

The CYP450 activity was determined by using the DetectX® Demethylating P450 fluorescent enzyme linked immunosorbent assay (ELISA) kit (Arbor Assays) and according to package

inserts. Sample supernatants, standards (12.5–400 µM formaldehyde), and blanks (0.1 M PPB) were added to a black, flat bottom 96-well microtitre plate. The plate was sealed and incubated for 15 minutes at 37°C. Following the incubation, 5 µL nicotinamide adenine dinucleotide phosphate (NADPH) solution was added to each well and the plate was sealed and further incubated for 30 minutes at 37°C. After incubation, stop solution was added to the wells, along with 25 µL Formaldehyde Detection Reagent. The plate was lightly tapped to mix the contents of the wells and incubated again for 30 minutes at 37°C. Incubation at this temperature was for optimal enzyme activity. The fluorescence of the wells was quantified at 510 nm with an initial excitation at 450 nm using an automated microplate reader (Bio-Tek FLx 800).

4.2.5 Superoxide dismutase

The method was adapted from Del Maestro and McDonald (1985). The sample supernatant, and DTPA/tris-buffer (50 nM tris-HCl, 1 nM diethylene triamine penta-acetic acid) were added in triplicate to a microtitre plate. The reaction was initiated by adding pyrogallol and the sample responses were read 11 times kinetically at 560 nm for 5 minutes (Berthold plate reader).

4.2.6 Catalase activity

The measure of CAT activity was based on the method described by Cohen *et al.* (1970). The sample supernatant (10 µL), a blank (10 µL PPB), and standard (102 µL PPB) were placed into a clear 96-well microtitre plate in triplicate. Hydrogen peroxide (93 µL of 34%) was added to the wells containing the blank and samples and left at room temperature for 3 minutes to incubate. The reaction was stopped by adding sulphuric acid (19 µL of 96%). Subsequently 130 µL K_2MnO_4 was added and the OD was immediately determined at 490 nm (Bio-Tek plate reader).

The colour change is based on the reaction between K_2MnO_4 and the remaining hydrogen peroxide, whereby the OD decreases. The decomposition of hydrogen peroxide by catalase is calculated as follows:

$$k = \log\left(\frac{S_0}{S_3}\right) \times \frac{2.3}{t}$$

Where: k is the first order kinetics constant; S_0 is the concentration of the substrate initially (mean of standard absorbance); S_3 is the concentration of the substrate after 3 minutes (standard absorbance subtracted by the mean sample absorbance); 2.3 is the first order kinetic

conversion factor; and t is the time interval (3 minutes). This value is normalised based on the protein content, and reported as µM H_2O_2.min^{-1}.mg $protein^{-1}$.

4.2.7 Malondialdehyde content

The methods to determine MDA content as an indication of lipid peroxidation were developed by Ohkawa *et al.* (1979) and adapted by (Üner *et al.*, 2006). 1,1,3,3-tetramethoxypropane was prepared as a standard with seven concentrations ranging from 0–3 nM. Sample supernatant, standards, and blank (tris-sucrose buffer) were added into separate microtubes. The following were added into each of the tubes: 50 µL sodium dodecyl sulphate (8.1% in deionised water), 375 µL acetic acid (20%), 375 µL thiobarbituric acid (0.8%), and 175 µL deionised water. The tubes were incubated in a water bath at 95°C for 30 minutes and subsequently allowed to cool to room temperature. Deionised water (250 µL) and 1 250 µL *n*-butanol:pyridine solution (1:15) were added into each of the tubes. After 10 minute centrifugation at 2 700 g 245 µL of the supernatant was added to the wells of a clear 96-well microtitre plate. The absorbance was measured at 540 nm (Berthold plate reader) and the MDA content was expressed in terms of the standard curve, and normalised based on the protein content as nM MDA/mg protein.

4.2.8 Protein carbonyl induction

The method to determine protein carbonyl induction was originally described by Levine *et al.* (1990) and modified by (Floor and Wetzel, 1998). A blank (hydrochloric acid (2 M)), standard series (bovine serum albumin (0–40 nM), and sample supernatant were added to microcentrifuge tubes. Into the standard and sample tubes 2,4-dinitrophenylhydrazine (10mM in 2 M HCl) was added, and allowed to react for an hour at room temperature, and vortexed at 15 minute intervals. Trichloro-acetic acid (6%) was added to the standard and sample tubes to precipitate the proteins. All tubes were centrifuged for 3 minutes at 10 000 g, and the supernatant was discarded. The pellet was washed with ethanol:ethyl ether (1:1 v/v) and vortexed, and again centrifuged. This process was repeated thrice, each time discarding the supernatant. Guanidine hydrochloride (6 M in 50% formic acid) was added to all the tubes, and incubated at 37°C for 15 minutes. Insoluble material was removed by centrifuging at 16 000 g for 5 minutes and the supernatants (100 µL) were transferred to a microtitre plate in triplicate. Absorbance was read at 390 nm (Bio-Tek FLx 800) and the protein carbonyl content of the samples were calculated using the standard curve and expressed as nM carbonyls/mg protein.

4.2.9 Cellular energy allocation

The determination of total available energy (protein, carbohydrates, and lipids) and energy consumption via the electron transport system activity were adapted from (De Coen and Janssen, 1997) and (De Coen and Janssen, 2003).

4.2.9.1 Available energy

Determination of available energy consists of three separate assays: protein, carbohydrates and lipid. Muscle tissue homogenate that was diluted five times with deionised water, was used.

To determine the protein content the Bradford method was used (Bradford, 1976), as described above.

The total carbohydrates were quantified by using a glucose content kit (Roche, CFAS 759 350) with a glucose standard (1.93 g/L) (Roche, GlucA 657 527). Samples, blank (deionised water), and standards were added to microtitre plates in triplicate. The glucose test kit reagent was added to each of the wells and left to incubate at room temperature for 30 minutes. The absorbance was measured at 540 nm. The carbohydrate content was calculated determining the ratio of the average absorbance of the samples and the standard and multiplying this with the standard concentration.

The lipid content was determined using the Bligh and Dyer (1959) method. Chloroform and sample homogenate were vortexed in a microcentrifuge tube. To this, methanol and deionised water were added to the tubes and further vortexed. The samples, a blank (chloroform) and standards (tripalmitin dilution series: 0–6 000 µL) were centrifuged at 3 000 g for 5 minutes at 4°C. The organic phase was removed and transferred to glass test tubes. Sulphuric acid (500 µL) was added to the test tubes and covered with aluminium foil and kept at 200°C for 15 minutes. The tubes were allowed to cool and 1 000 µL deionised water was used to reconstitute the samples, and 245 µL was transferred to a microtitre plate and the absorbance read at 405 nm. The lipid content was calculated by comparing the samples' absorbance to the absorbance of the tripalmitin standard curve.

The energy fractions calculated in available energy (protein, carbohydrates, and lipids) were converted to energy equivalents by multiplying their concentrations with the combustion enthalpy constants of each fractions—glycogen (17 500 mJ/mg), protein (24 000 mJ/mg), and lipids (36 500 mJ/mg) which is expressed as mJ (De Coen and Janssen, 1997). Available energy was calculated by multiplying the energy values (mJ) with the mass of the sample to

get a mJ/g. These individual energy values are summed and this is the available energy (Ea) for each individual.

4.2.9.2 Energy consumption

The electron transport system activity assay is used to quantify the energy consumption (Ec) (De Coen and Janssen, 2003). The samples were centrifuged for 10 minutes (3 000 g at 4°C) and 25 µL of the sample supernatants were added in triplicate to the microtitre plate along with 75 µL buffered substrate solution (0.3% Triton-X, 0.13 M Tris-HCl), and 25 µL NAD(P)H solution (1.7 µM NADH, 250 µM NADPH). Finally, to initialise the reaction, 50 µL 8 mM *p*-iodinitri tetrazolium chloride (INT) solution was added to the wells. The absorbance was measured kinetically at 490 nm over 5 minutes, with 30 second intervals between measurements. The energy consumption is calculated as J/g. The oxygen consumption rates calculated in energy consumption (Ec) were multiplied by the combustion enthalpy of oxygen (484 kJ/M) (De Coen and Janssen, 1997).

4.2.9.3 Cellular energy allocation

An energy budget (CEA) was calculated as the difference between the available energy and the energy consumption.

CEA = Ea - Ec

4.2.10 Physicochemical water parameters

The physicochemical water parameters (temperature, pH, dissolved oxygen, and conductivity) were measured at each sample site using a handheld multimeter (Extech DO610).

4.2.11 Statistical analysis

The data for the biomarker analyses were tested for normality using the D'Agosino and Pearson omnibus normality test. The Kruskal-Wallis test and Dunn's multiple comparison post-hoc tests were used because the data was non-parametric. Statistical significance was set at $p<0.05$. Statistical analyses were performed using GraphPad Prism version 6. Redundancy analyses (RDA) were performed using Canoco version 5.12. The RDA technique makes use of two sets of variables, a dependant and an explanatory variable set. It is a direct form of a PCA that makes it possible to focus the analysis on the particular part of the variance that is explained by external explanatory variables (Van den Brink *et al.*, 2003).

4.2.12 Integrated Biomarker Index

This index provides an integration of the chosen biomarkers to the overall health status of the studied populations. A higher IBR score indicates poorer health of the population (Arrighetti *et al.*, 2019). The IBR was calculated using the biochemical and so called "specific" biomarkers: AChE, CAT, SOD, MDA, PC, and CYP450 (Broeg and Lehtonen, 2006). The calculation of the IBR is performed by the summation of triangular star plot areas for every two neighbouring biomarkers in a data set, as follows (Arrighetti *et al.*, 2019):

$x_i' = (x_i - x)/s$

where: x_i' is the standardised value of the biomarker, x_i is the mean value of the biomarker at a specific site, x is the mean of the biomarker across all sites, and s is the standard deviation of the biomarker across all sites.

$y_i = x_i' + |xmin'|$

where: xmin' is the minimum standardised value of the biomarkers in the data set. In instances where a decline in the biomarker indicates stress (inhibition of AChE, and reduction in the energy reserves) - x_i' was used (Wang *et al.*, 2010).

$A_i = (y_i * y_{i+1})/2$

where A_i is the star plot triangular area, y_{i+1} is the y_i of the consecutive biomarker.

$IBR = \Sigma A_i/n$

where: n is the number of biomarkers used in the summation of star plot triangular areas.

The organisation of the consecutive biomarkers used to calculate the different A_i values were arranged depending on the similarity in functions (Broeg and Lehtonen, 2006).

4.3 Results

4.3.1 Phongola Floodplain

4.3.1.1 Physicochemical parameters

The physicochemical results from the Phongola River during the high and low flow periods are displayed in Table 4.1. These will be used in the multivariate analyses.

Table 4.1. Mean and standard deviation of physicochemical parameters in the Phongola River taken from two sites during the low (November 2012) and three sites high flow (April 2013) periods. These data were published by Smit *et al.* (2016).

	Low flow	High flow
Temperature (°C)	25.4 ± 0.4	24.6 ± 1.8
Electrical conductivity (mS/m)	71.5 ± 6.4	29 ± 3.0
pH	7.73 ± 0.01	7.59 ± 0.2
Dissolved oxygen (mg/L)	6.45 ± 1.9	9.0 ± 1.6

4.3.1.2 Biomarkers of exposure

Typically, the AChE and the CYP450 levels were lower in fish from the low flow compared to the high flow season (Figure 4.1 A and B), except for *O. mossambicus*, where the low flow season had higher levels of both biomarkers than the high flow season. *O. mossambicus* had significantly greater CYP450 activity in the low flow season than in the high flow season. *H. vittatus* had significantly greater activity of both enzymes in the high flow season ($p<0.05$). *S. zambezensis* had significantly greater CYP450 activity in the high flow season. The activity of these enzymes were greatest in *S. zambezensis* and *H. vittatus* during the low flow period. These species had significantly greater AChE activity compared to *C. gariepinus* and *C. quadricarinatus*. *Synodontis zambezensis* and *H. vittatus* had significantly greater ($p<0.05$) CYP450 activity than *O. mossambicus* and *C. gariepinus* that were sampled during the high flow period. *C. quadricarinatus* had the lowest AChE activity in both flow seasons than all other species and were significantly lower than all other species, with the exception of no difference between *C. quadricarinatus* low flow and *C. gariepinus* low flow.

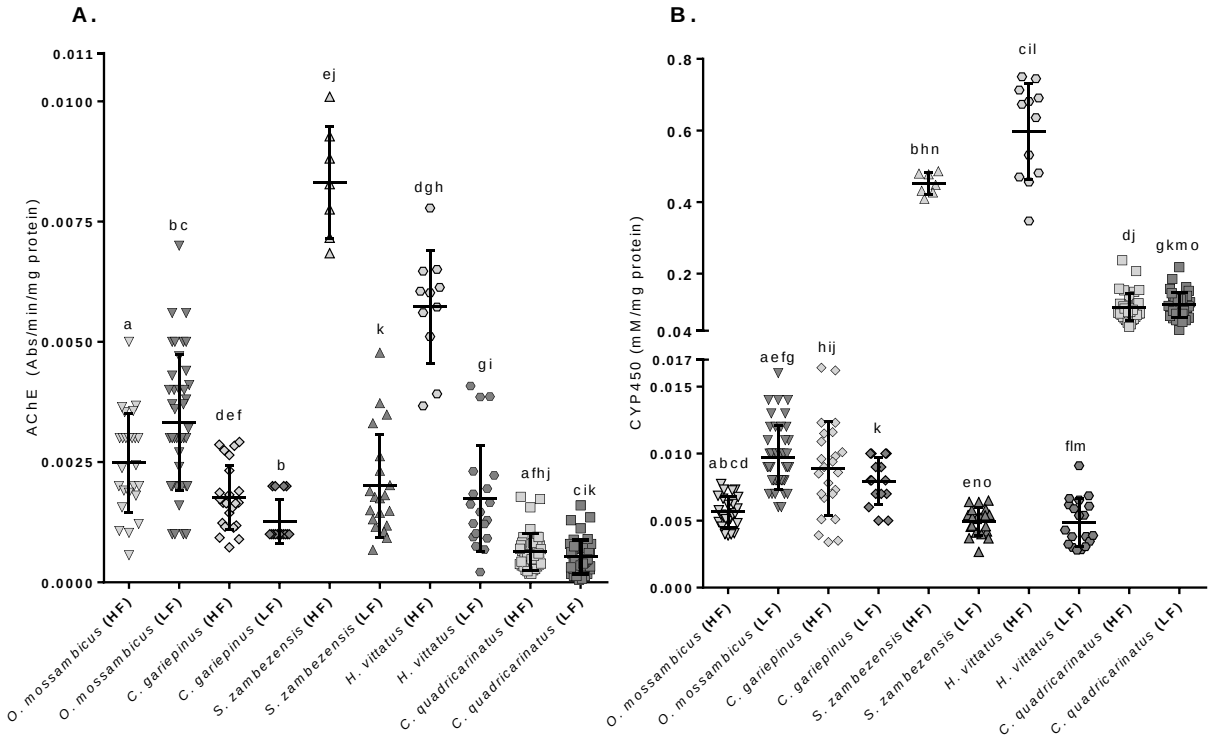


Figure 4.1 The mean (±SD) of (A.) acetylcholinesterase (AChE) activity and (B.) CYP450 measured from species sampled in Phongola, in the high (HF) and low flow (LF) seasons. Common symbols indicate significant difference.

4.3.1.3 Biomarkers oxidative stress

There tended to be more SOD and CAT activity in fish during the high flow period (Figure 4.2 A & B). The SOD of two species did not follow this trend: *C. gariepinus* had an increase of SOD activity from the high flow season to the low flow season, and *S. zambezensis* remained fairly consistent between seasons (Figure 4.2 A). *C. quadricarinatus* had higher CAT activity during the low flow period, however, this difference was negligible.

The SOD activity was significantly greater in the high flow season for *O. mossambicus* (Figure 4.2 A). The highest SOD activity was in *H. vittatus*, *S. zambezensis,* and *C. quadricarinatus.* These species also had significantly greater SOD activity than *O. mossambicus* and *C. gariepinus* in both the high- and low flow periods.

The CAT activity was significantly greater in *O. mossambicus* sampled during the high flow season compared with the low flow season (Figure 4.2 B). *C. gariepinus* collected in the high flow season had the greatest CAT activity overall, and also the greatest variability within the species. The CAT activity in *C. gariepinus* and *C. quadricarinatus* high flow were significantly greater than the CAT activity in *O. mossambicus* high flow. The CAT activity in *C. quadricarinatus* low flow was significantly greater than those of the other species sampled during this flow period. *O. mossambicus* from the low flow season also had significantly lower

CAT activity than *C. gariepinus* and *H. vittatus* from the same flow—this species had the lowest SOD and CAT activity compared with all other species in both flow seasons.

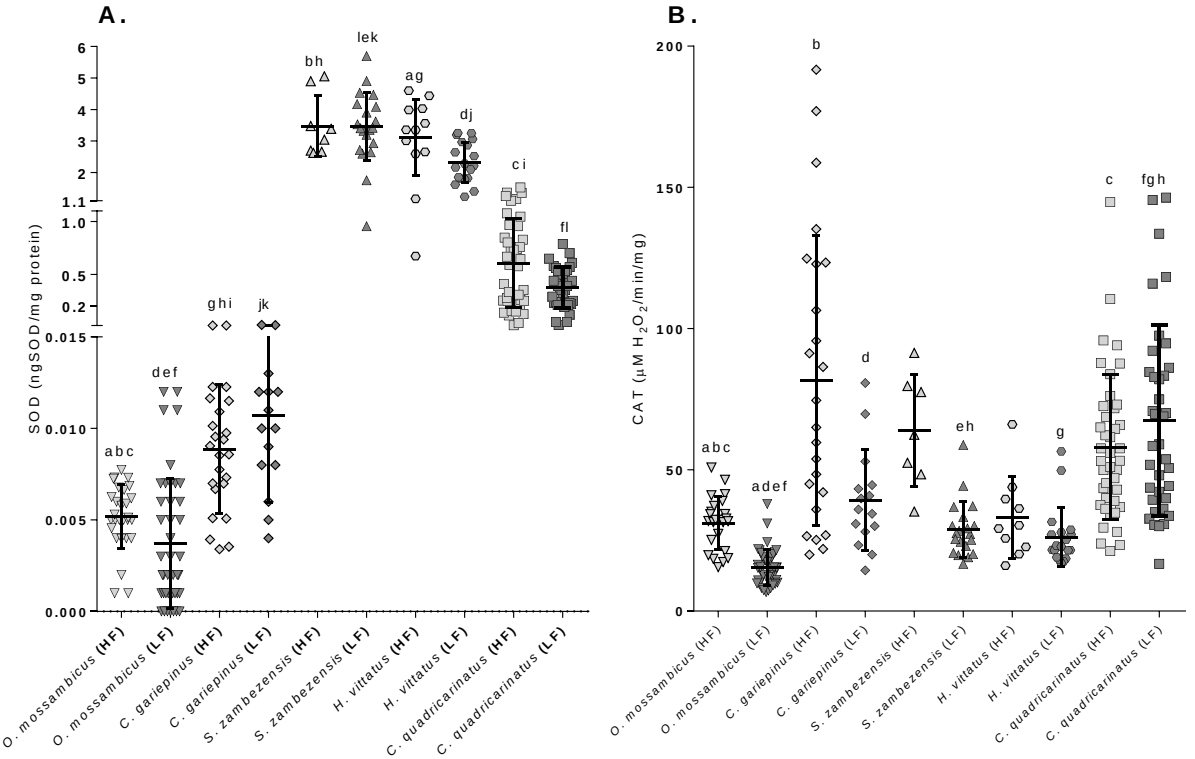


Figure 4.2 The mean (±SD) of (A.) Superoxide dismutase (SOD) activity and (B.) catalase (CAT) measured from species sampled in Phongola, in the high (HF) and low flow (LF) seasons. Common symbols indicate significant difference.

4.3.1.4 Biomarkers of oxidative stress damage

The MDA and PC activity typically followed the opposite trend of each other, and only *S. zambezensis* and *H. vittatus* had a similar trend: higher activity during the high flow season (Figure 4.3 A and B). The MDA activity was slightly higher in the low flow period for *O. mossambicus,* and *C. gariepinus* and although the change was small it was statistically significant for *C. gariepinus* ($p<0.05$) (Figure 4.3 A). The MDA was greater in the high flow compared to the low flow season for *H. vittatus, S. zambezensis,* and *C. quadricarinatus*, however none of the differences were significant. *H. vittatus* and *S. zambezensis* had the highest MDA levels in both flow periods, and were significantly greater than all other sampled species in the high flow season. These species also had significantly higher MDA activity in the low flow period than *O. mossambicus* and *C. quadricarinatus*. *C. quadricarinatus* had MDA activity that was significantly greater than the MDA activity in *C. gariepinus* during the high flow season.

Protein carbonyl content was generally greater in the high flow season than the low flow season, with the exception of *C. quadricarinatus*, which showed the opposite trend (Figure 4.3 B). The difference between seasons was significant in *O. mossambicus, C. gariepinus,* and *H. vittatus*. *C. quadricarinatus* had significantly lower PC content than all other sampled species during the high flow season, while there were no significant differences of PC content between species during the low flow period.

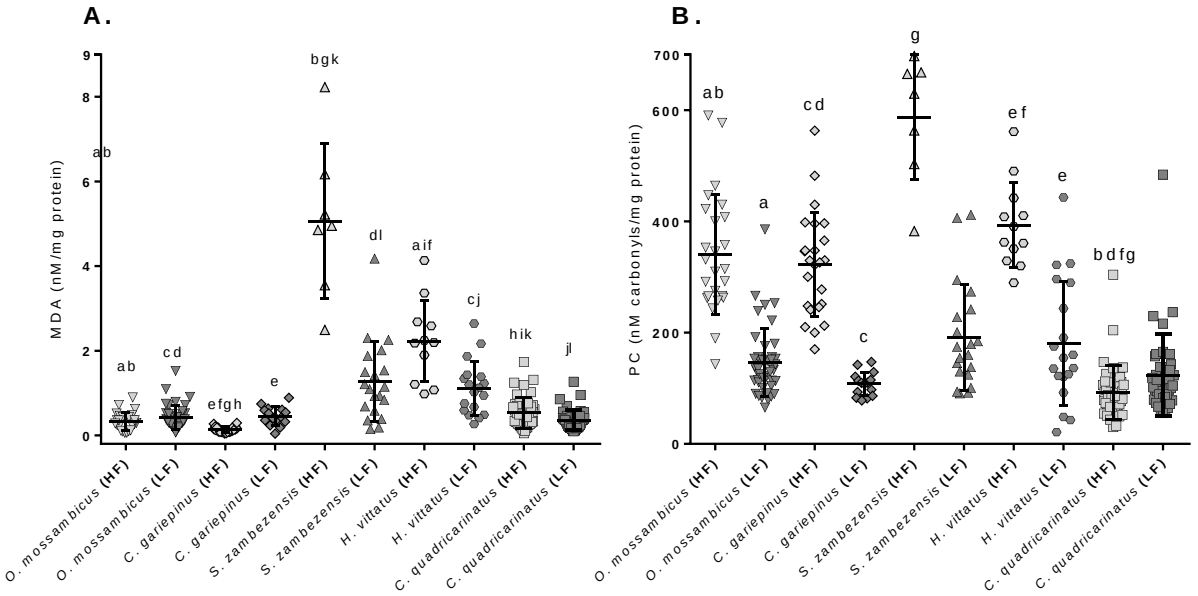


Figure 4.3 The mean (±SD) of (A.) Malondialdehyde (MDA) activity and (B.) protein carbonyls (PC) measured from species sampled in Phongola, in the high (HF) and low flow (LF) seasons. Common symbols indicate significant difference.

4.3.1.5 Biomarkers of cellular energy allocation

Carbohydrate, and lipid content were typically greater in the high flow compared to the low flow season (Figure 4.4 A, B & D). All species had greater carbohydrate content during the high flow period, although the change of carbohydrate content between seasons for *H. vittatus* and *C. quadricarinatus* was very small, and almost negligible (Figure 4.4 A). The carbohydrate content in *O. mossambicus* was significantly lower in the low flow period ($p<0.05$). *O. mossambicus* and *C. gariepinus* had the highest carbohydrate content in the high flow season of all the species sampled; there was also large variation in these values. During the low flow season *C. gariepinus, C. quadricarinatus*, and *O. mossambicus* had the greatest carbohydrate content of the sampled species. These three species had carbohydrate contents that were significantly greater than both *S. zambezensis* and *H. vittatus*.

Lipid content was generally greater during the high flow compared to the low flow season, with the exception of *S. zambezensis*, and *C. quadricarinatus*, which were slightly higher in the low flow period (Figure 4.4 B). None of the species differences between flow periods were

significant. *O. mossambicus, C. gariepinus*, and *C. quadricarinatus* had significantly higher lipid content than *S. zambezensis*, and *H. vittatus* at high flow. The same was observed during the low flow season.

Generally, protein content, energy availability, and energy consumption followed a similar trend—typically always high in the same period, which most often was the low flow period (Figure 4.4 C–E). The exception was *O. mossambicus* which had high protein and Ec during the low flow period, and higher Ea during the high flow period. *C. quadricarinatus* also deviated from the trend and had higher values during the high flow season. However, the protein content in *C. quadricarinatus* were virtually the same between seasons. *C. quadricarinatus* had the greatest protein content compared to all species, and was significantly greater than other species in both flow periods. *O. mossambicus* and *C. gariepinus* had higher protein content than *S. zambezensis* and *H. vittatus* in both flow periods, during the low flow period; the difference was significant. *S. zambezensis* in the high flow period had the overall lowest protein content.

The CEA was typically greater in the high flow period compared to the low flow season, except *C. quadricarinatus* where there was a greater CEA in the low flow period, although this difference was minor (Figure 4.4 F). None of these temporal differences were significant. *Oreochromis mossambicus* and *C. quadricarinatus* had greater CEA in the high flow period, however *C. quadricarinatus* CEA values were virtually similar between different flow seasons (Figure 4.4 F). The other species: *C. gariepinus*, *S. zambezensis*, and *H. vittatus* had higher CEA levels in the low flow period. None of the differences between seasons were significant. *Cherax quadricarinatus* had the greatest CEA levels in both flow seasons, and were significantly greater than all other species. The *O. mossambicus* that was sampled in the high flow had significantly greater CEA than those in *H. vittatus* and *S. zambezensis* that were also collected in the high flow. The CEA in *C. gariepinus* from the low flow season were significantly greater than those in *S. zambezensis* from the same season.

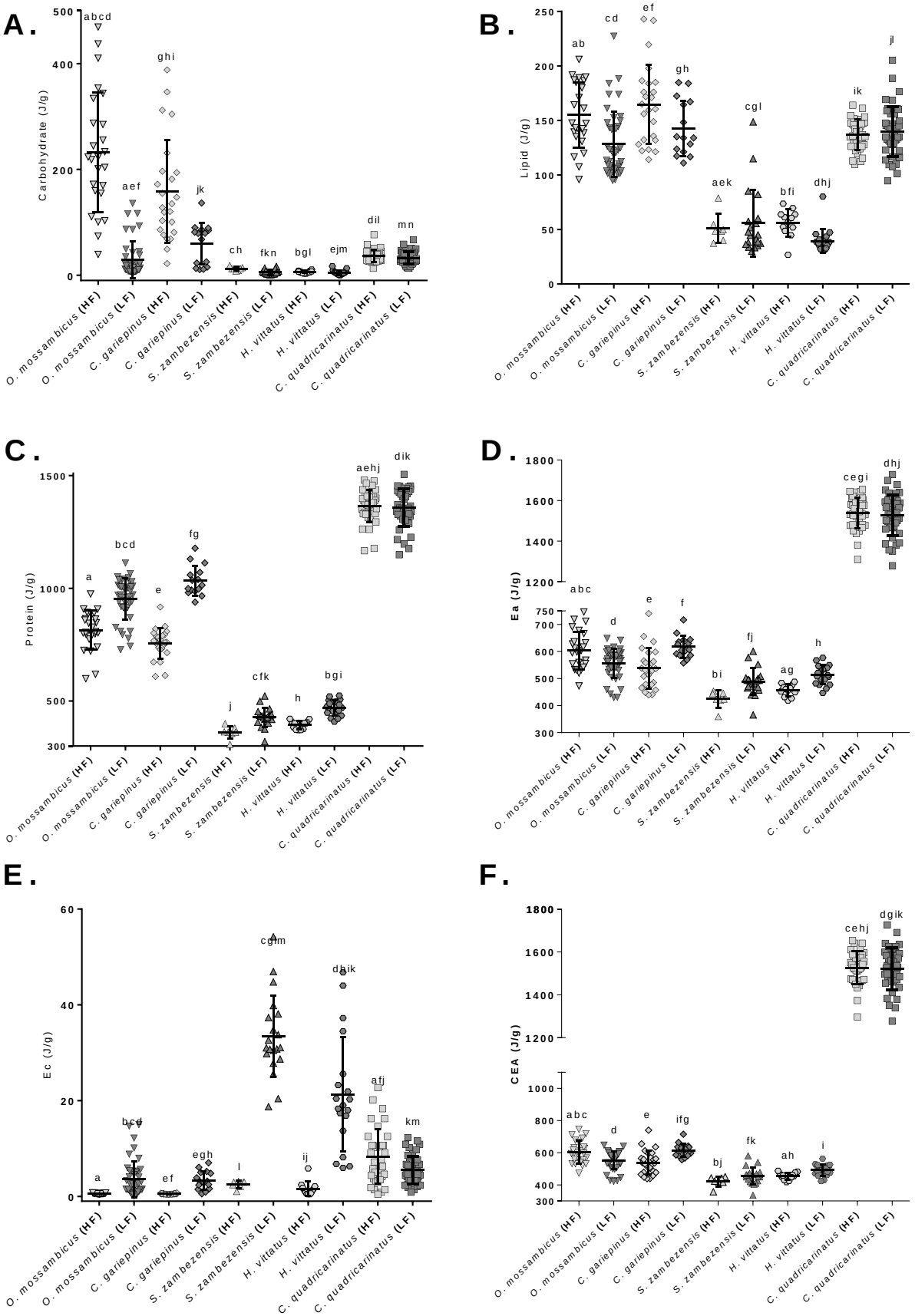


Figure 4.4 The (A.) carbohydrate, (B.) lipid, (C.) protein, (D.) energy availability (Ea), (E.) energy consumed, and (F.) cellular energy allocation (CEA) measured from species sampled in Phongola, in the high (HF) and low flow (LF) seasons. Common symbols indicate significant difference.

4.3.1.6 Relationship between biomarker responses and environmental exposure to organochlorines.

The ordination of biomarker responses in five species during two different flow periods explains 67% of the variation on the first two axes when a redundancy analysis is done (Figure 4.5). Axis one explains 42% of the variation and separates the biomarker responses in *H. vittatus* and *S. zambezensis* from *C. gariepinus* and *O. mossambicus*. The separation is driven by the biomarkers MDA, PC, SOD, and Ec on the positive side and energy reserves (lipid and carbohydrate) and CYP450 on the negative side. The physicochemical parameters: temperature and pH ordinate in the positive direction too. Temperature was a significant ($p<0.05$) explanatory variable, and accounted for 19.1% of the variation. Axis two explains 25% of the variation and separates the biomarker responses in the fish species from the freshwater crayfish, *C. quadricarinatus*. This axis is driven by total available energy (Ea) and CEA on the positive axis, and AChE on the negative axis. The Σdrins and ΣDDx were significant explanatory variables, explaining 20.5% and 6.4% of the variation, respectively. There is little influence of flow period on the biomarker responses in *C. quadricarinatus* because samples from both seasons cluster together. The biomarker responses in *O. mossambicus* and *C. gariepinus* during the low flow period are not influenced by any particular biomarker, while the high flow biomarker responses are associated with CYP450 activity and carbohydrate content. Increased dissolved oxygen (DO2) and electrical conductivity (EC) were the main explanatory environmental variables for these biomarkers.

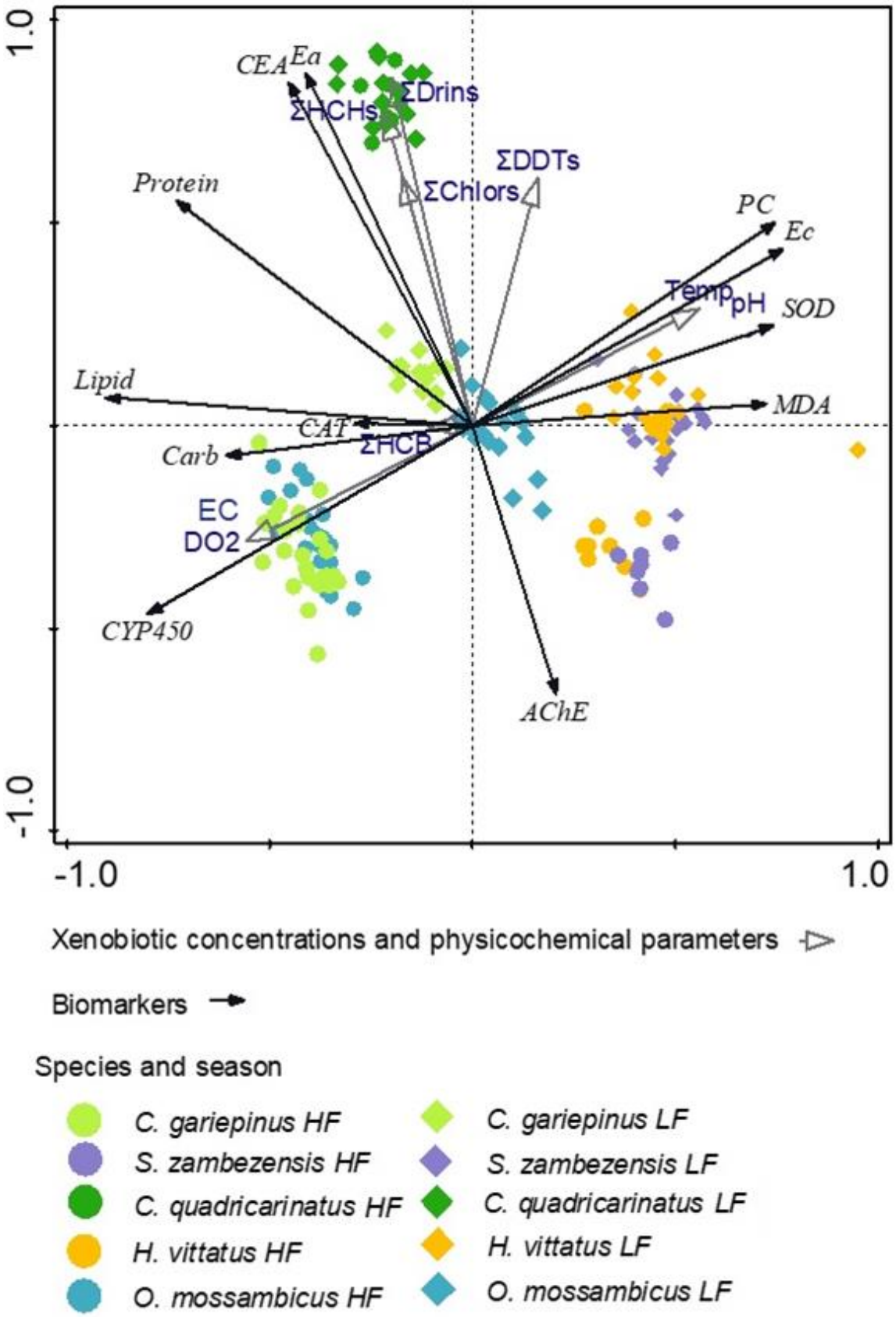


Figure 4.5 RDA of biomarker responses in individual samples of five different aquatic species sampled from the Phongola River during high and low flow periods. Organochlorine body residues and water quality parameters are superimposed as explanatory environmental variables. Axis 1 (42%) and axis 2 (25%) explain 67% of the variation in the data.

4.3.1.7 Integrated biomarker response

The nine selected biomarkers (CYP450, AChE, SOD, CAT, MDA, lipid, PC, protein, and Ec), along with the interaction of the biomarkers: SOD*CAT are represented in the star plots (Figure 4.6). These biomarkers were selected based on their biological responses and interactions. Typically, the greater IBR/n values were recorded during the high flow season with the exception of *C. gariepinus* that had a higher IBR/n score during the low flow period, and *C. quadricarinatus*'s score was the same in both flow periods (Table 4.2). Different species showed different patterns regarding which biomarker was the most influenced by the environmental stressors (both xenobiotic exposure and physicochemical parameters). In both flow periods the greatest IBR/n scores were for *H. vittatus* and *S. zambezensis*, and the biomarkers that contributed the greatest to the high flow score were protein and PC. The IBR/n scores during high flow for *C. quadricarinatus*, *O. mossambicus*, and *C. gariepinus* were greatly influenced by AChE. *Oreochromis mossambicus* and *C. gariepinus'*s scores for high flow were also highly influenced by CYP450 and protein. The IBR/n scores for the low flow period had a similar trend to the high flow period: *H. vittatus* and *S. zambezensis* had the greatest scores and *O. mossambicus* had the lowest scores. Like in the high flow period, the protein content was the greatest contributor to the IBR/n score for fish during the low flow period. Lipids, SOD, and Ec were also considerable contributors to the score. The scores in *C. quadricarinatus* and *C. gariepinus* were similar in the low flow period, and biomarkers that contributed most to the score were CYP450, CAT, and AChE. The IBR/n score in *O. mossambicus* was lowest, and protein was the dominant biomarker to this score.

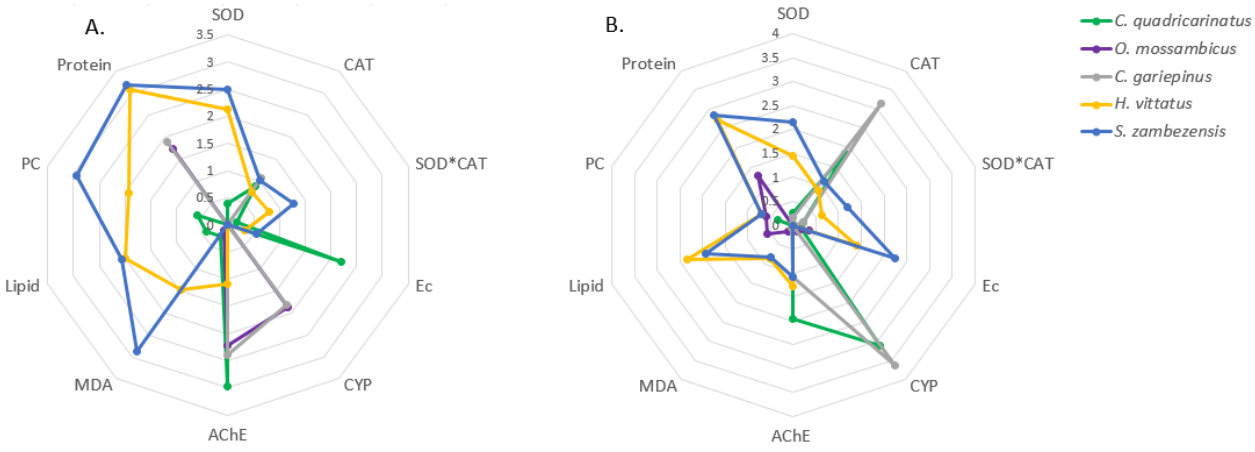


Figure 4.6 Integrated Biomarker Responses (IBR) calculated from the standardised biomarker data from the high flow (A.) and low flow (B.) seasons for Phongola species.

Table 4.2 IBR values for the species sampled from Phongola during the high- and low flow periods.

	IBR variable	*C. quadricarinatus*	*O. mossambicus*	*C. gariepinus*	*H. vittatus*	*S. zambezensis*
High flow	SOD	low flow p	0	0	2.13	2.5
	CAT	0.87	0	1.06	0.74	1.02
	SOD*CAT	0.17	0	0	0.79	1.27
	Ec	2.2	0.02	0	0.33	0.55
	CYP	0	1.89	1.83	0	0
	AChE	2.97	2.22	2.39	1.09	0
	MDA	0.25	0.12	0	1.46	2.87
	Lipid	0.42	0.02	0	2	2.06
	PC	0.61	0	0	1.93	2.93
	Protein	0	1.74	1.9	3.09	3.2
	IBR/n	**0.79**	**0.6**	**0.72**	**1.36**	**1.64**
Low flow	SOD	0.25	0	0.15	1.44	2.15
	CAT	1.85	0	3.15	0.88	1.12
	SOD*CAT	0.23	0	0.23	0.63	1.2
	Ec	0.17	0.34	0	1.39	2.24
	CYP	3.1	0.16	3.63	0	0
	AChE	1.95	0	1.09	1.28	1.09
	MDA	0	0.18	0.06	0.85	0.81
	Lipid	0	0.58	0	2.33	1.92
	PC	0.34	0.61	0	0.71	0.71
	Protein	0	1.27	0.04	2.71	2.84
	IBR/n	**0.79**	**0.31**	**0.83**	**1.22**	**1.41**

4.3.2 Vaal River

4.3.2.1 Physicochemical parameters

The physicochemical parameters for the sites in the Vaal River, are listed in Table 4.3 and these will be utilised in the multivariate statistics.

Table 4.3. In situ physicochemical parameters based on a single grab sample from the three sites in the Vaal River.

Site	pH	EC (mS/m)	Temperature (°C)	Dissolved O_2 (mg/L)	%TOC
Vischgat	8.38	11.8	15.1	8.12	0.765
Barrage	8.26	47.6	17.1	6.94	0.908
Thabela Thabeng	9.29	46.8	19.8	10.47	0.945

4.3.2.2 Biomarkers of exposure

The levels of AChE and CYP450 often responded contradictory to what was expected: an increase in AChE would also most often coincide with a decrease in CYP450, instead of increasing, but this was not always the case (Figure 4.7), however without a control it is difficult

to determine baseline levels of especially AChE, to determine if this enzyme was inhibited. *C. gariepinus* had high AChE in comparison to other samples, and lower CYP450 levels, and this trend was also true for *L. capensis* from Barrage and Vischgat, and *C. carpio* from Barrage, while *C. carpio* from Thabela Thabeng had lower AChE levels and higher CYP450 activity, in comparison to other samples.

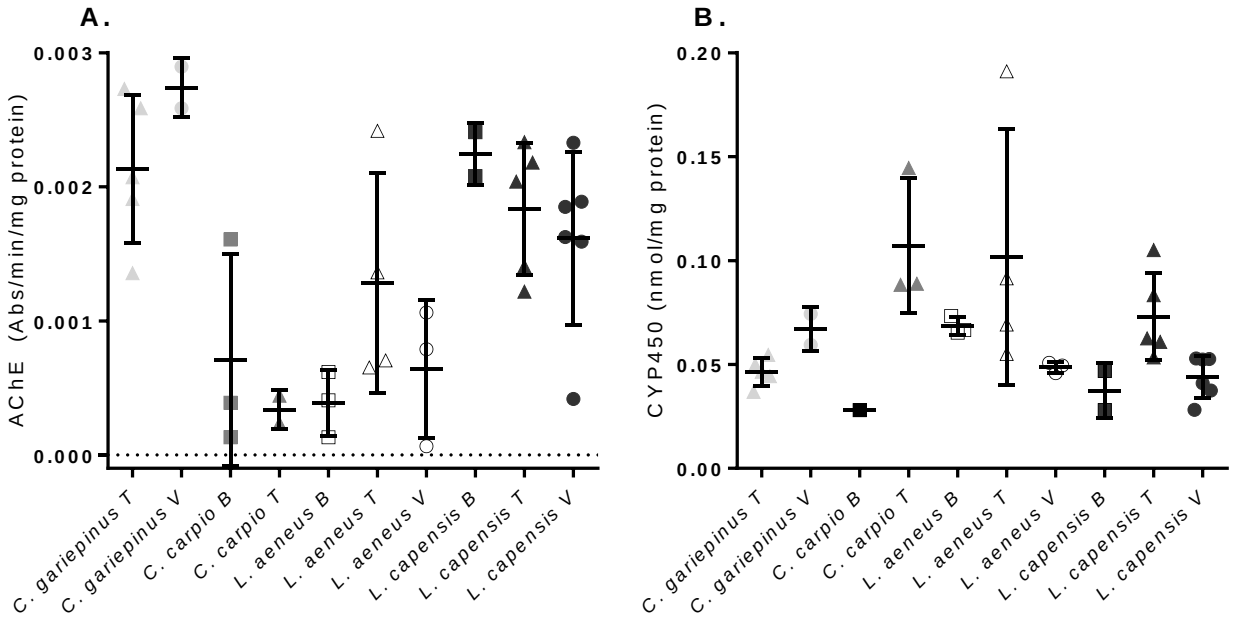


Figure 4.7 Mean ± standard deviations of (A.) acetylcholinesterase (AChE) activity and (B.) CYP450 activity measured from species sampled in the Vaal River from the three sites (T=Thabela Thabeng, V=Vischgat, B=Barrage).

4.3.2.3 Biomarkers of oxidative stress

The SOD and CAT content did not show much of a trend between the responses (Figure 4.8 A and B). However, there were many instances where the SOD values were below the values of the standard curve, and this resulted in negative responses. In these instances a value of ½ the lowest calculated SOD value were used. This was the case with all the values for *C. gariepinus* Vischgat, *C. carpio* Barrage, and *L. capensis* Vischgat. The highest levels of SOD were measured in *L. aeneus* sampled from Thabela Thabeng and *L. capensis* from Barrage. *Labeobarbus aeneus* from Thabela Thabeng also had the greatest CAT activity, while lowest CAT activity was from *L. capensis* sampled from Barrage.

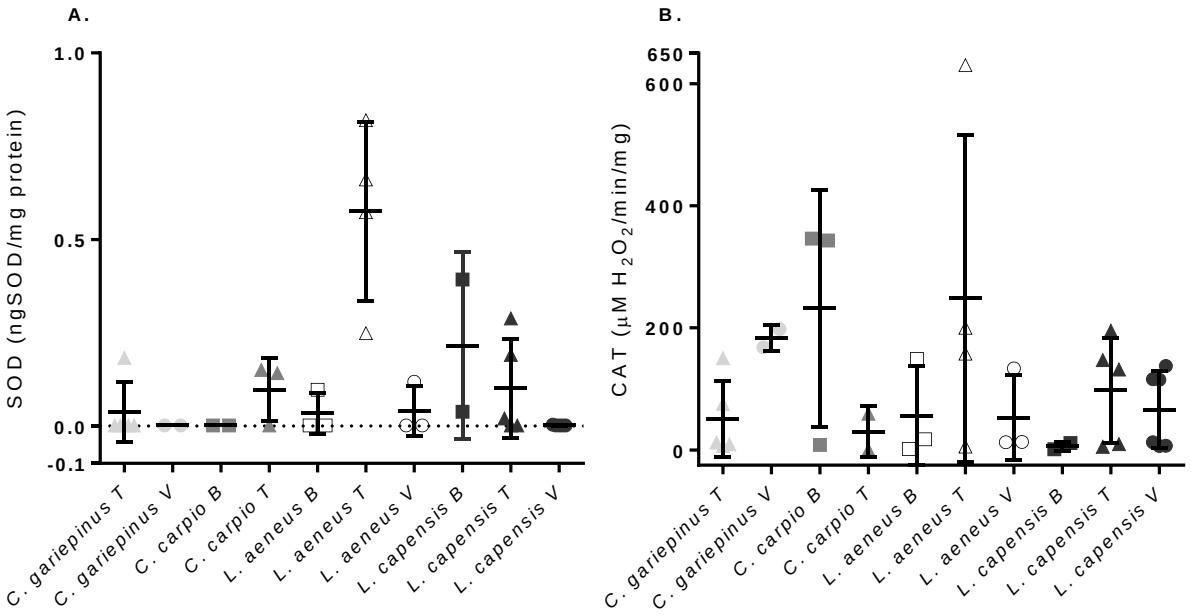


Figure 4.8 Mean ± standard deviations of (A.) superoxide dismutase (SOD) and (B.) catalase (CAT) activity measured from species sampled in the Vaal River from the three sites (T=Thabela Thabeng, V=Vischgat, B=Barrage).

4.3.2.4 Biomarkers of oxidative stress damage

The highest MDA activity was in *C. carpio* from Thabela Thabeng, while the highest PC activity was in *L. aeneus* and *C. carpio* from Thabela Thabeng (Figure 4.9). MDA and PC activity in *C. gariepinus* was greater at Vischgat compared to Thabela Thabeng. The greatest activity in *C. carpio* was at Thabela Thabeng. The MDA activity was fairly similar between the Vischgat and Thabela Thabeng for *L. aeneus*, with the activity slightly higher at Barrage. *Labeo capensis* had the highest MDA AND PC activity at Thabela Thabeng and the MDA activity was lowest at Vischgat, while PC activity was lowest at Barrage.

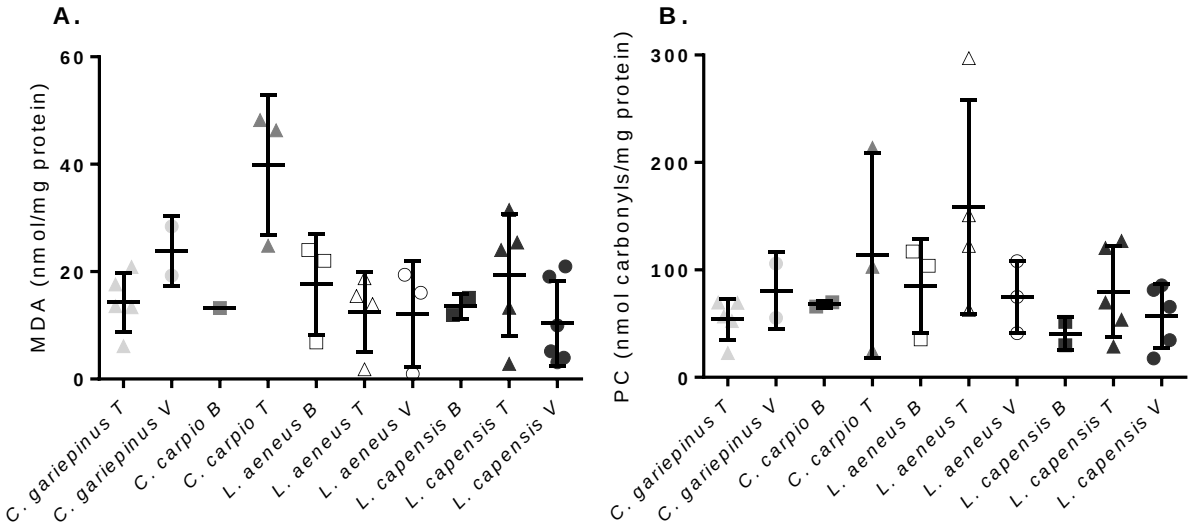


Figure 4.9 Mean ± standard deviations of (A.) malondialdehyde (MDA) and (B.) protein carbonyl (PC) activity measured from species sampled in the Vaal River from the three sites (T=Thabela Thabeng, V=Vischgat, B=Barrage).

4.3.2.5 Biomarkers of cellular energy allocation

The carbohydrate stores in *C. gariepinus* from Thabela Thabeng were slightly greater than those sampled from Vischgat (Figure 4.10 A). *Cyprinus carpio* from Barrage had higher carbohydrate stores than those from Thabela Thabeng. The carbohydrate levels in *L. aeneus* were greatest in those from Thabela Thabeng, although these also had great variation in the data. *L. aeneus* from Vischgat had the lowest carbohydrate levels in this species. *Labeo capensis* followed a similar pattern to the *L. aeneus*: high carbohydrate content at Thabela Thabeng (and high variation), and lowest carbohydrate content from Vischgat. Typically, the fish sampled from Vischgat had the lowest carbohydrate content. The lowest carbohydrate stores were in *C. gariepinus* sampled from Vischgat, and *L. aeneus* and *L. capensis* from Thabela Thabeng had the highest carbohydrate content.

Lipid content was greater in *C. gariepinus* from Vischgat than those from Thabela Thabeng (Figure 4.10 B), while *C. carpio* had higher lipid content from Thabela Thabeng than those from Barrage. *Labeobarbus aeneus* and *L. capensis* also had the highest lipid content at Thabela Thabeng. The lowest lipid content in these two species was at Vischgat. The overall highest lipid content was in *L. aeneus* from Thabela Thabeng, and the lowest lipid content was in *L. aeneus* from Vischgat.

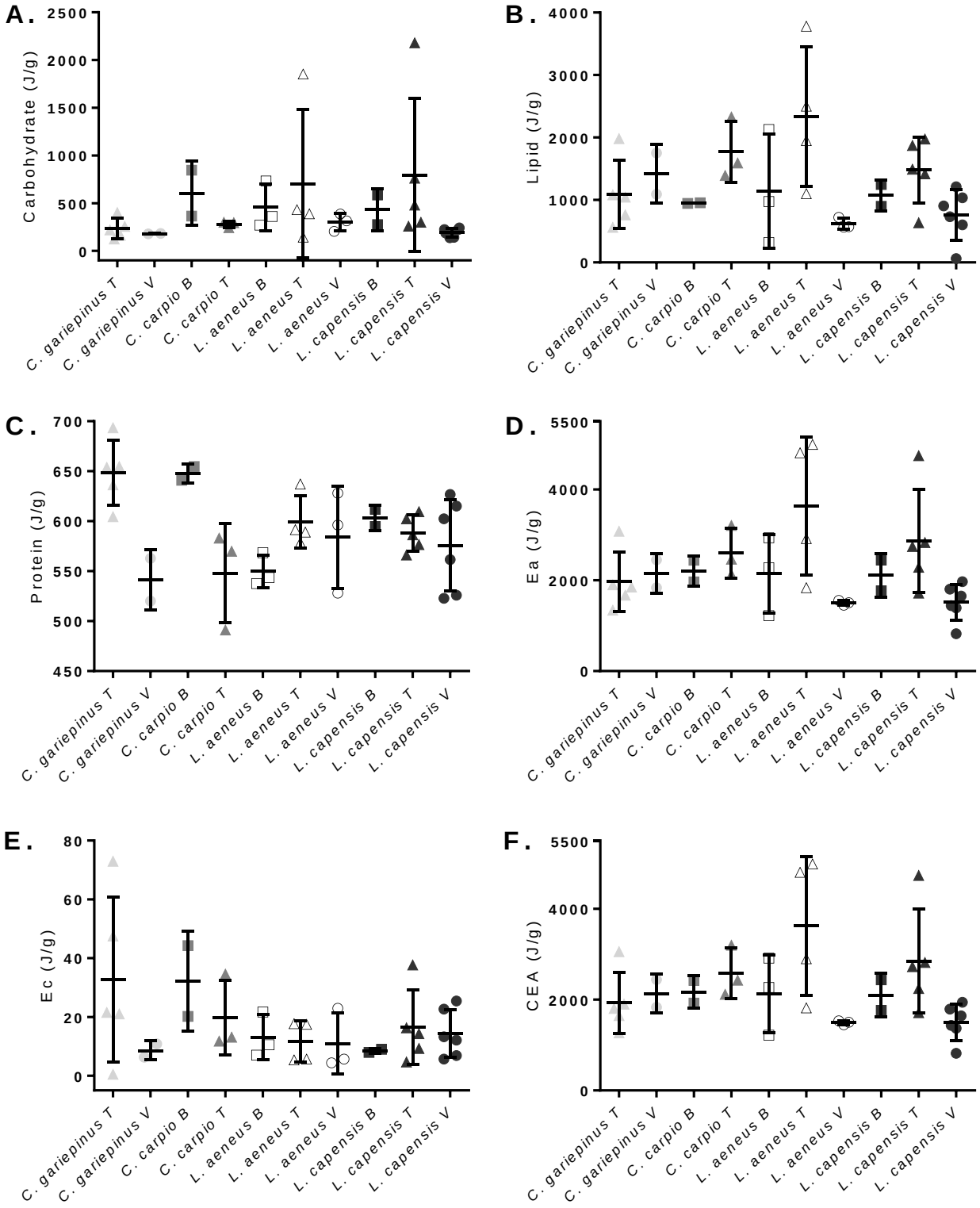


Figure 4.10 (A.) carbohydrate, (B.) lipid, (C.) protein, (D.) energy availability (Ea), (E.) energy consumed, and (F.) cellular energy allocation (CEA) measured from species sampled in the Vaal from the three sites (T=Thabela Thabeng, V=Vischgat, B=Barrage).

The protein content was greatest in *C. gariepinus* from Thabela Thabeng compared to those from Vischgat (Figure 4.10 C). *Cyprinus carpio* from Barrage had greater protein stores than

those sampled from Thabela Thabeng. *Labeobarbus aeneus* from Thabela Thabeng had the highest protein stores of this species, while those from Barrage had the lowest protein levels. *Labeo capensis* from Vischgat had the lowest protein content, while those from Barrage had the highest. Overall, *C. gariepinus* from Thabela Thabeng and *C. carpio* from Barrage had the highest protein content, while *C. gariepinus* sampled from Vischgat had the lowest measured protein content. *Labeobarbus aeneus* from Barrage, and *C. carpio* from Thabela Thabeng had similarly low protein content.

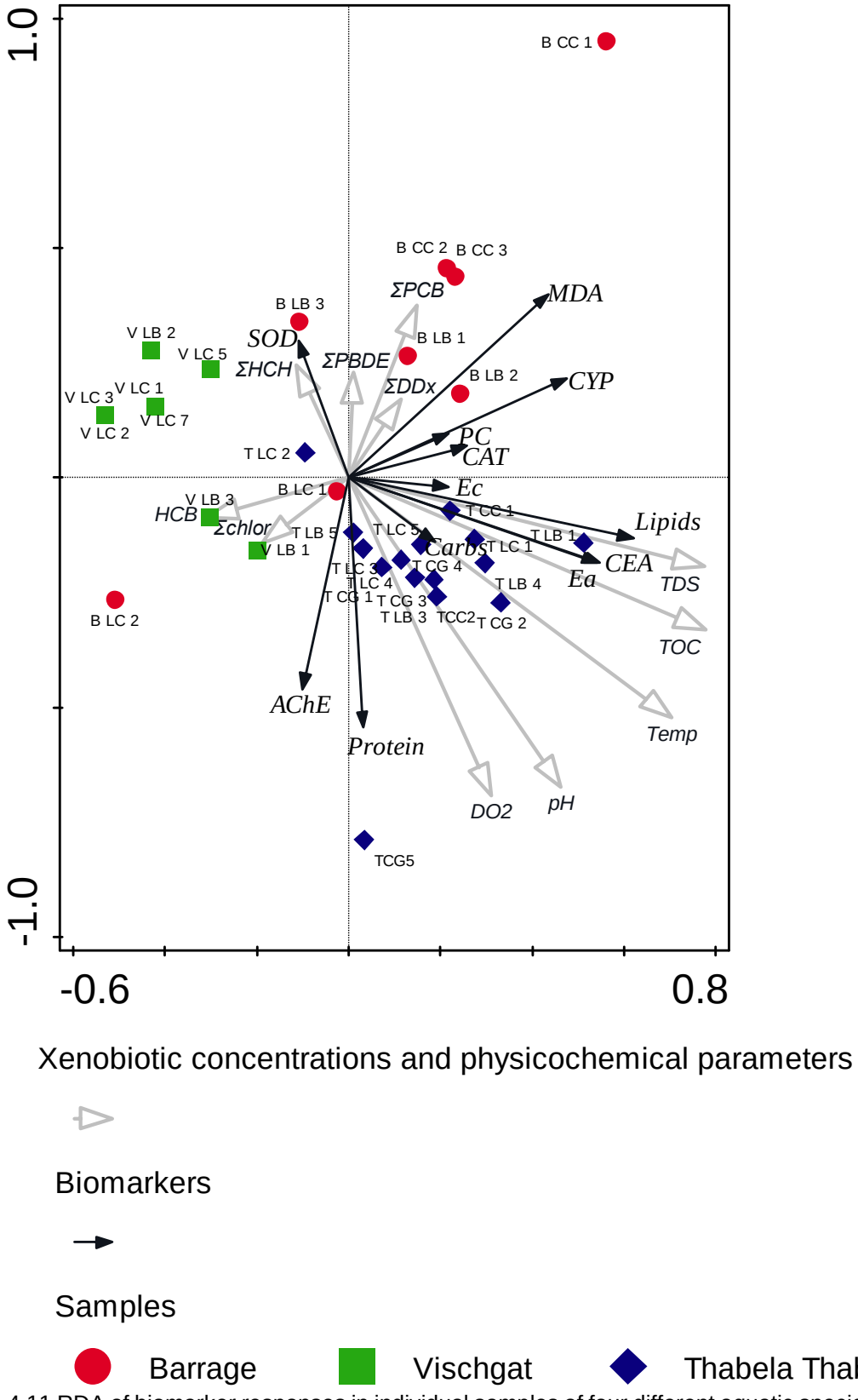
Clarias gariepinus from Thabela Thabeng had higher Ec than those from Vischgat (Figure 4.10 D). *C. carpio* from Thabela Thabeng had lower Ec than those collected from Barrage. Ec in *L. aeneus* were very similar, with the fish from Barrage having slightly higher levels than fish from the other two sites. *Labeo capensis* from Barrage had the lowest Ec, while those from Thabela Thabeng had the highest Ec. Overall, *C. gariepinus* from Thabela Thabeng and *C. carpio* from Barrage had the greatest Ec. *Labeo capensis* from Barrage and *C. gariepinus* from Vischgat had the lowest Ec.

Clarias gariepinus sampled at Thabela Thabeng had a slightly lower Ea than those from Vischgat (Figure 4.10 E). *Cyprinus carpio* from Thabela Thabeng had higher Ea than those from Barrage. The Ea of *L. aeneus* was greatest at Thabela Thabeng and lowest from Vischgat. This was similar to what was measured in *L. capensis*: highest measurements from fish from Thabela Thabeng and lowest in Vischgat fish. The Ea measurements were very similar in *C. gariepinus* from Thabela Thabeng and Vischgat, *C. carpio* from Thabela Thabeng, and *L. aeneus* and *L. capensis* from Barrage. The lowest Ea was in *L. aeneus* from Vischgat, and *L. capensis* also from Vischgat had a similarly low Ea. The highest Ea was in *L. aeneus* from Thabela Thabeng. Fish sampled from Thabela Thabeng typically had the highest Ea.

The CEA in *C. gariepinus* was greater in those collected in Vischgat than those from Thabela Thabeng (Figure 4.10 F). The CEA was greater in *C. carpio* from Thabela Thabeng than those from Barrage. *L. aeneus* from Thabela Thabeng also had the highest CEA, while the lowest CEA in *L. aeneus* was from Vischgat. A similar trend was observed for *L. capensis*: highest CEA from Thabela Thabeng and lowest from Vischgat. Overall there were similar CEA values in *C. gariepinus* from Thabela Thabeng and Vischgat, *C. carpio* from Barrage, *L. aeneus* from Barrage, and *L. capensis* from Barrage. The highest CEA was in *L. aeneus* from Thabela Thabeng, while the lowest was in *L. aeneus* and *L. capensis* from Vischgat, which had very similar CEA values.

4.3.2.6 Relationship between biomarker responses and environmental exposure to xenobiotics

The ordination of biomarker responses in four species at three sites in the Vaal River explains 21% of the variation on the first two axes in the redundancy analysis (Figure 4.11). Axis one explains 13% of the variation and separates the biomarker responses of the species sampled at Thabela Thabeng from those sampled at Vischgat. The separation is driven by the biomarkers CYP450, PC, CAT, and the energetics: Ec, lipids, CEA, Ea, and carbohydrates on the positive side. The physicochemical parameters: TDS, TOC, and temperature ordinate to this direction as well. Total organic carbon (TOC) was the only variable to have a significant effect ($p<0.05$), and explained 9.2% of the variation. At Vischgat the biomarkers of exposure and effect were low when compared to the other sites even though HCB and Σchlors were the two groups of OCPs predominating at this site. Axis two explains 8% of the variation and separates the species sampled at the Barrage from those sampled at Thabela Thabeng. The separation is driven by an increased SOD and decreased AChE activities, and the explanatory variables ΣHCHs, ΣPBDEs, ΣPCBs, ΣDDx also ordinate in this direction.



Xenobiotic concentrations and physicochemical parameters

Biomarkers

Samples

Barrage Vischgat Thabela Thabeng

Figure 4.11 RDA of biomarker responses in individual samples of four different aquatic species sampled from three sites in the Vaal River: Barrage, Vischgat, and Thabela Thabeng. Organochlorine body residues and water quality parameters are superimposed as explanatory environmental variables. Axis 1 (13%) and axis 2 (8%) explain 21% of the variation in the data. of xenobiotic concentrations and physicochemical parameters, and biomarkers from species at the Vaal. CG=*C. gariepinus*, CC=*C. carpio*, LB=*L. aeneus*, LC=*L. capensis*.

4.3.2.7 Integrated biomarker response

The nine selected biomarkers (CYP450, AChE, SOD, CAT, MDA, lipid, PC, protein, and Ec), along with the interaction of the biomarkers: SOD*CAT are represented in the star plot (Figure 4.12). The greatest IBR/n was from the samples collected from Barrage with a score of 0.49 and the contributors to the score were: MDA > CYP450 > AChE > protein > SOD > lipid > CAT > PC > SOD*CAT, and Ec was zero. Thabela Thabeng had the next highest IBR/n score of 0.26 and the contributors were: SOD > CYP450 > MDA > PC > protein > Ec, and the remaining values were zero. Vischgat had the lowest IBR/n score of 0.14 and the contributions were lipid > CAT > AChE > Ec, and all the other contributions were zero.

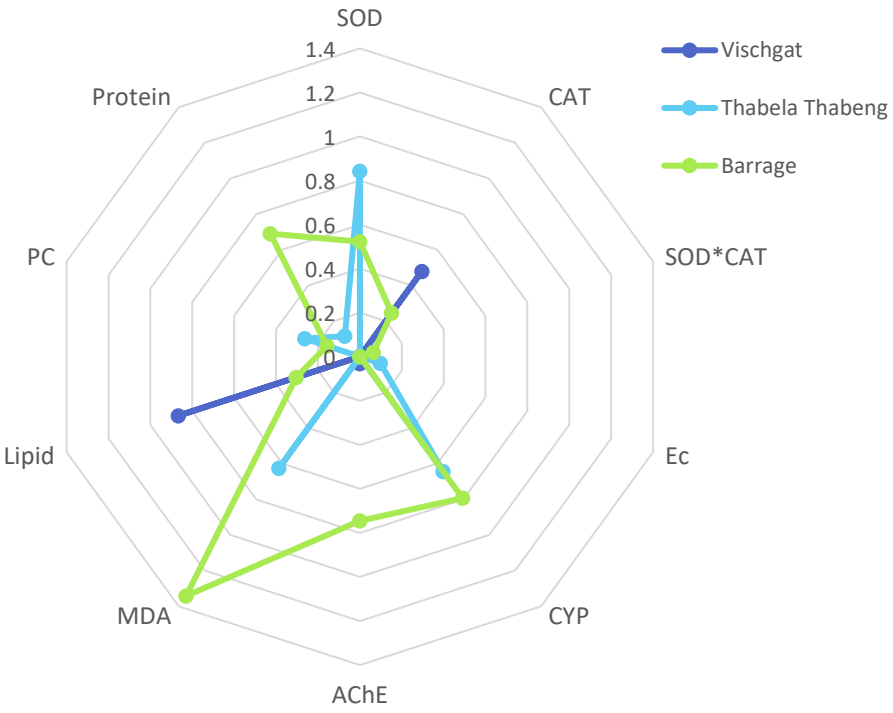


Figure 4.12 Integrated Biomarker Responses (IBR) calculated from the standardised biomarker data from the three sites in the Vaal River.

4.4 Discussion:

The various biomarker responses and causes are summarised in Table 4.4, and will aid the interpretation of the results obtained in this study.

Table 4.4. Summary of the biomarker responses and the cause for the up- or down regulation of the enzymes. Adapted from Van der Oost *et al.* (2003)

Category	Biomarker	Response (Increase or decrease)	Effect interpretation or exposure
Exposure	AChE	↓	Presence of neurotoxic compounds, e.g. pesticides
	CYP450	↑	Expressed to metabolise organic pollutants
Effect	SOD	↑	Stimulation in response to ROS
	CAT	↑	Produced in response to ROS
Oxidative stress damage	PC	↑	Indicative of protein damage in presence of ROS
	MDA	↑	Indicative of lipid peroxidation in presence of ROS
Energy allocation	CEA	↑↓	Decrease due to stress compensation, or increase due to increased energy reserves

4.4.1 Phongola

The ΣOCPs were greatest in the high flow period for all species except *H. vittatus*, which had higher ΣOCPs during the low flow (see Chapter 3 Table 3.10). The difference between flow periods in *O. mossambicus* and *C. gariepinus* was minor. More biomarker activity is expected to coincide with when the organism has higher OCP concentrations, with the exception of AChE which is expected to be inhibited. Generally, all species had the greatest biomarker activity during the high flow period.

In the current study the AChE levels were lower in the low flow compared to the high flow period (Figure 4.1 A)—indicating inhibition because AChE activity is inhibited when the organisms are exposed to various toxicants (see Chapter 1 for list). The apparent inhibition of AChE during the low flow was unexpected because ΣOCPs were generally greater in the high flow period and the greater ΣOCPs should have caused more inhibition of the AChE. There were, however, a few instances where some of the xenobiotic classes had concentrations that were greater in the low flow period. These were: HCB for *S. zambezensis*, Σchlors for *C. quadricarinatus* and *O. mossambicus*, Σdrins in *C. quadricarinatus,* and ΣDDx for *H. vittatus* (Table 3.10), these compound classes might account for the lower AChE in the low flow period. The results of the RDA indicate that the responses in AChE were caused by the presence of Σchlors, Σdrins, ΣHCHs, and ΣDDx (Figure 4.5). It also showed that *C. quadricarinatus* was highly associated with AChE. In both the high and low flow periods *C. quadricarinatus* had high AChE contributing greatly to the IBR/n scores, further indicating that this species is experiencing detrimental effects via the inhibition of this enzyme caused from exposure to the

OCPs. It is likely that there were other influencing factors at play that could explain the other species' responses because the AChE was a large influencer to the IBR scores in both seasons (Table 4.2). This might include the presence of other xenobiotics that are known to effect these biomarkers. AChE inhibitors, such as those listed previously, could be in greater concentrations during the low flow period corresponding to the lower AChE responses during this flow period. Compounds such as organophosphates, pyrethroids, carbamates, thiazine, and thiocarbamates are used widely in the northern part of KZN (Naidoo *et al.*, 2008). Some studies had shown that the herbicides atrazine and cyanozine, and the insecticide chlorpyrifosn inhibited AChE synergistically (Jin-Clark *et al.*, 2002), and synergism between compounds present in the low flow period may also explain the greater inhibition of AChE during the low flow. Wepener *et al.* (2012) sampled *H. vittatus* from the Pongolapoort Dam and found a range of PCBs and PBDEs, as well as the same OCPs that were analysed for this study. Generally, these compounds had higher concentrations in the low flow season compared to the high flow, more related to the trends seen with the biomarker results from this study. Although metal analyses were not conducted in this study at any time, a range of metals were quantified in the sediment, and tissue of *H. vittatus* sampled in Phongola in July 2011 (Tate, 2014). The presence of these metals could account for the lower AChE activity in the low flow period.

The CYP450 activity was higher during the high flow period, indicting presence of CYP450 activating compounds, which could include DLC, tryptophan derivatives, flavonoids, naphthoquinones (Tian *et al.*, 2015) (Figure 4.1 B). This is because CYP450 is activated in the presence of DLC to metabolise them (Cajaraville *et al.*, 2000; Parkinson and Ogilvie, 2001; Newman, 2010; Hecker and Giesy, 2011). The CYP450 activation could be explained by high toxicant burdens in the species during this flow period, however, the DLC compounds, which are the most potent activators of the CYP450 pathway, were not analysed. Even though DLC are the most potent activator of CYP450, numerous compounds are capable of inducing the CYP450 enzymes, including some PAHs, oxidised carotenoids, heterocyclic amines, and tryptophan-derived products, and pharmaceuticals (Hecker and Giesy, 2011; Burkina *et al.*, 2015). The results of the RDA displayed that there were no xenobiotics associated with the CYP450 biomarker; this supports the fact that the CYP450 activating compounds were not targeted in this study (Figure 4.5). But, it is probable that CYP450-inducing compounds are present within the Phongola system, particularly in the high flow, because *O. mossambicus* and *C. gariepinus* orientated closely with the CYP450 biomarker. The results of the IBR supports this—CYP450 contributed heavily to the IBR/n scores of these species, especially in the high flow period (Table 4.2).

It is possible that there might be CYP450 inhibition occurring in the organisms, which had been shown to occur when organisms were exposed to metals, carbamates, PCBs (particularly PCB 77, -126, -138, -169), DDx (Whyte *et al.*, 2000; Binelli *et al.*, 2006), and organophosphate pesticides (Stegeman and Hahn, 1994; Hahn and Chandran, 1996; Payne *et al.*, 1996; Besselink *et al.*, 1998; Camusso *et al.*, 2001). As mentioned before, many of these compound classes have been detected in the region (Wepener *et al.*, 2012).

Because AChE controls physiological and behavioural responses, changes in the functioning of this enzyme are detrimental to the organism (Kirby *et al.*, 2000). From studying the biomarkers of exposure (CYP450 and AChE) it can be deduced that organisms in Phongola had been exposed to xenobiotics, although they respectively showed greater responses in different seasons—AChE indicated that there was more exposure during the high flow season, while CYP450 was activated more during the low flow season. As already mentioned, AChE is inhibited by a range of neurotoxic compounds (Kang and Fang, 1997; Guilhermino *et al.*, 1998; Guilhermino *et al.*, 2000; Kirby *et al.*, 2000; Corsi *et al.*, 2003; Roy *et al.*, 2006; Bervoets *et al.*, 2009). Some of these compounds (example chlorpyrifos, aldicarb, and diazinon) do not directly affect the AChE or to a lesser extent, but become toxic once being biotransformed by CYP450, creating a much stronger AChE inhibitor (Reddy and Rao, 1987; Forsyth and Chambers, 1989; Perkins and Schlenk, 2000; Vittozzi *et al.*, 2001; Jin-Clark *et al.*, 2002). This difference in a compound's activating/inhibiting potential to CYP or AChE might also explain the discrepancies between responses in the AChE and CYP450 activity.

The biomarkers of oxidative stress, SOD and CAT, are expected to increase when ΣOCPs are higher—which was during the high flow period. SOD is responsible for metabolism of ROSs and the by-product of this metabolism, peroxide, is detoxified by CAT (Bannister *et al.*, 1987; Zelko *et al.*, 2002; Lobo *et al.*, 2010). It is therefore expected that both SOD and CAT would show a similar trend, and that CAT production would be high when ΣOCPs concentrations are high. However, this was seldom the case. *Oreochromis mossambicus* and *H. vittatus* had a similar trend regarding the enzyme activity: high SOD and CAT in the high flow period. However, *H. vittatus* had greater ΣOCPs in the low flow period (Table 3.10). The other species had opposite enzyme activity in the two flow periods, and no clear trend relating to the presence of the OCPs that were analysed. Although *C. quadricarinatus* had ΣOCPs in the high flow season that were on average two orders of magnitude greater than any of the other species in this flow period, the SOD activity was not significantly different to any species other than *O. mossambicus*, which had the lowest ΣOCPs. *Oreochromis mossambicus* sampled in the low flow period had slightly higher ΣOCPs than *S. zambezensis*, yet the latter had significantly greater SOD activity. It might be possible that SOD activity might be inhibited,

explaining why high SOD and/or CAT activity is not always occurring when ΣOCPs are high. It had previously been reported that an increase in ROS production may decrease the functioning of the antioxidant enzyme activities. This was reported by Kono and Fridovich (1982), where excess radicals were not detoxified by SOD and directly inhibited CAT. A similar result was observed by Faria *et al.* (2009) with freshwater muscles that were exposed to PCBs and metals. Nitrates have also been shown to inhibit the activity of CAT (Arillo and Melodia, 1991). Glutathione peroxidase could have reduced the levels of hydrogen peroxide instead of the CAT pathway being activated (Ross, 1988; Newman, 2010).

High SOD activity had been reported from fish exposed to pollutants in previous studies and it had been attributed to high exposure of superoxide anion radicals (Roberts *et al.*, 1987; Rodriguez-Ariza *et al.*, 1991; Rodriguez-Ariza *et al.*, 1992; Rodriguez-Ariza *et al.*, 1993). Superoxide radicals can be produced by various xenobiotics like drugs, pesticides, and organic solvents (Ebadi, 2001). Therefore, it is likely that the SOD responses were not only attributed to the concentrations of xenobiotics analysed. Alternatively, there could also be compounds that inhibited the up-regulation of SOD, which might account for lower SOD content when the xenobiotic loads were high. Some physiological activities can also produce radicals, such as inflammation, exercise, phagocytosis (Ebadi, 2001), and environmental fluctuations (Ross *et al.*, 2001) which can also up-regulate SOD and/or CAT. This was the case in this study based on the results of the RDA—the environmental parameters explained the up-regulation of the SOD rather than the analysed xenobiotics (Figure 4.5). The SOD responses were influenced by an increase in temperature and pH, and a decrease electrical conductivity and dissolved oxygen. The CAT activity was explained by the presence of ΣHCBs, although this was a very small influence. The activity was explained better by the increase of electrical conductivity and dissolved oxygen, and the deduction of temperature and pH—the opposite trend to that of SOD.

The biomarkers of oxidative stress damage, MDA and PC, indicate the breakdown of lipids and protein, respectively, in response to ROSs. Malondialdehyde is an indication of lipid peroxidation due to exposure to oxidative stress. This is a degenerative process whereby the entire body can experience detrimental effects due to cell membrane damage caused by oxidation of lipids, fatty acids, and cholesterol (Flohr *et al.*, 2012). High PC levels are due to oxidation of the amino acids resulting in the formation of protein carbonyls (Lobo *et al.*, 2010). Effects of protein oxidation include changes in enzyme activity, heat stability, and the signal transduction mechanisms, which in turn leads to aging (Lobo *et al.*, 2010). It is expected that these biomarkers would correlate with the ΣOCPs, and each other. Only *S. zambezensis* and *H. vittatus* had a similar trend between these two biomarkers—higher activity during high flow

period. *Cherax quadricarinatus* also had higher MDA in the high flow period, yet lower PC in the same period. With the exception of *C. quadricarinatus*, all other sampled species had higher PC activity in the high flow season, which corresponds to high ΣOCPs, and this is an expected response.

Added to the action of ROSs, PC can also be formed by increased oxidative stress as a result of increased physical activity, such as in the case of elevated swimming which is what would have happened in high flow periods (Hackenberger *et al.*, 2015). In the current study, the environmental parameters were shown to be the greater driving factor in the biomarker responses (Figure 4.5). the protective function of SOD was surpassed, and the oxidative stress caused by an increase of temperature and pH, and the decrease of electrical conductivity and dissolved oxygen, and possibly other environmental impacts, thus resulting in production of protein carbonyls and lipid peroxidation. This was evident for *H. vittatus* and *S. zambezensis* particularly in the high flow period. These species also indicated that an increase in PC and MDA, and a decrease in protein and lipid content contributed greatly to the IBR/n scores (Table 4.2). Protein carbonyls can also form via secondary mechanisms resulting from reactions of free radicals with other cellular constituents, such as lipids, carbohydrates, and nucleic acids (Grune *et al.*, 2003). The formation of carbonyl derivatives is non-reversible, causing conformational changes, decreased catalytic activity in enzymes, and ultimately resulting in breakdown of proteins by protease due to increased susceptibility (Almroth *et al.*, 2005). Oxidised lipid/amino acid reactive products were shown to be involved in feed-back inhibition where they act as antioxidant defence mechanisms, preventing or delaying damage caused by ROSs (Zamora *et al.*, 1997). Therefore, an increase in lipid peroxidation (increase of MDA) could have prevented protein carbonyl formation. Peroxides can form adducts with proteins, thereby inhibiting proteolysis which may have been the case for the difference in trend between PC and MDA for *O. mossambicus, C. gariepinus,* and *C. quadricarinatus* (Grune *et al.*, 2003). It is also possible that different oxidants will result in different by-product formation, either effecting lipids or protein (Almroth *et al.*, 2005). Presence of metals for example can cause formation of protein carbonyls, however, do not cause much damage to lipids (Stadtman and Oliver, 1991). As mentioned previously, metals have been detected in the system (Tate, 2014), and their presence may account for the lack of a trend between the two biomarkers.

Carbohydrate and lipid content had similar trends, they were lower in the low flow than the high flow period. The lipid content in *S. zambezensis* and *C. quadricarinatus* did not follow this trend because they had slightly higher lipid content in the low flow period. Organisms use carbohydrates to maintain growth rates. When the carbohydrate levels are depleted, lipids and proteins are catabolised for energy (Wilson, 1994), which may explain the common trend in

carbohydrates and lipids—when the carbohydrates are low the organism uses lipids for energy. Carbohydrates and lipids also orientated together in the RDA (Figure 4.5). The greater protein in the low flow season could be caused by synthesis of defence related proteins such as defence enzymes, antigens, and globulins (Smolders *et al.*, 2004). The fish likely have not depleted the carbohydrate and lipid reserves in the low flow completely, because the protein content was high in the low flow, while carbohydrates and lipid were low in this flow period. Lipids are critical for survival, fitness, and reproduction (Adams, 2001). The lipid stores of *O. mossambicus*, *C. gariepinus,* and *C. quadricarinatus* coincide with the results of the MDA: elevated lipid peroxidation in the low flow season which correspond to the low lipid content. *Synodontis zambezensis* and *H. vittatus* consistently had the lowest measures of carbohydrates, lipids, and protein in both seasons compared to the other species. The reduction in protein was the greatest contributor to these species' IBR/n scores in both seasons (Table 4.2). This might be due to species differences, such as the way they utilise the energy stores.

There was a similarity in the general trend for energy allocation (Ea), energy consumption (Ec), and cellular energy allocation (CEA)—higher in the low flow period. This was also similar to that of the protein content. This indicates that there were fewer energy reserves in the fish during the high flow period. This can be caused by an increase in energy demand from dealing with stressors—environmental and xenobiotic exposure, and maintaining homeostasis (Smolders *et al.*, 2004). This could also have been by a scarce food supply during the previous winter season, or the organism had used the energy reserves for growth and preparation of spawning. Additionally, it could indicate that the organism was dealing with a stressor (Smolders *et al.*, 2004). Lower available energy generally results in reduced growth, and decline of health condition. This leads to the impairment of reproductive and developmental processes (Smolders *et al.*, 2004). A lower CEA can be a result of a lower respiration rate, and in turn a lower metabolic fuel demand (Verslycke *et al.*, 2003).

Biomarker responses were not influenced heavily by the xenobiotics that were analysed in this study, but rather by the physicochemical parameters, as indicated by the results of the RDA (Figure 4.5). The only exception to this was *C. quadricarinatus*, which displayed inhibition of AChE with Σdrins, Σchlors, ΣDDx, and ΣHCHs as the causative factors. This species did not have variation between the seasons. They also had high available energy, and low energy consumption based that the CEA was high as well. This indicates that this species is dealing with the stress of the high OCP loads, and other stressors. The *H. vittatus* and *S. zambezensis* low flow samples, and to a lesser extent the high flow samples, showed an upregulation of SOD, the biomarker of oxidative stress, is not coping with its role to metabolise ROSs due to

the and an increase in MDA and PC present. This indicated that the protective role of SOD is being surpassed and is unable to prevent oxidative damage. This trend is also supported by the decrease in lipid and protein content. These species also have high energy consumption (Ec), possibly due to coping with the effects of the oxidative stress. These responses were best explained by increases of pH and temperature, and a decrease of electrical conductivity (EC) and dissolved oxygen (DO2). This is probably the effect of flow and seasonal variations. The *C. gariepinus* and *O. mossambicus* samples collected in the high flow period had high CYP450 activity, and the energy reserves: protein and carbohydrates were also high. The driving factors of this biomarker response was an increase in electrical conductivity and dissolved oxygen, and a decrease in temperature and pH. These are not expected to influence CYP450 activity, and it is more likely that other CYP450 inducing compounds are present, which have not been analysed.

The higher IBR indicated that the species had more severe biomarker responses during the low flow period compared to the high flow period. Because the ΣOCPs were generally greater in the high flow period this is likely not the cause for the poorer health status. The species also responded differently, indicating possible effects of species differences that may include trophic position, habitat selection, and responses to the contaminants or environmental conditions. Other studies successfully utilised IBR values to quantify the impact of environmental pollution. Bocquené *et al.* (2004) used IBR with the responses of *Mytilus edulis* biomarkers: AChE, CAT, MDA, and glutathione-S-transferase (GST) to determine the effects of an oil spill on the French coast. Broeg and Lehtonen (2006) found IBR values of *Zoarces viviparous* and *Mytilus* sp from the Baltic to correlate with the organochlorine concentrations in the tissues. Similarly, IBR values (based on SOD, CAT, GST, MDA, and AChE) of *Nerita chamaeleon* collected from Goa, India were positively correlated with PAH concentrations in snail tissues (Bhagat *et al.*, 2016). IBR values calculated from AChE, GST, CAT, and MDA results were correlated with concentrations of PCBs and copper found in transplanted mussels from the North-Western Mediterranean Sea. However, the IBR did not correlate well with the concentrations of PAHs (Damiens *et al.*, 2007), this is the opposite trend to what was observed by Bhagat *et al.* (2016).

4.4.2 Vaal

As mentioned already–AChE is inhibited by a variety of toxicants (Kang and Fang, 1997; Guilhermino *et al.*, 1998; Guilhermino *et al.*, 2000; Kirby *et al.*, 2000; Corsi *et al.*, 2003; Van der Oost *et al.*, 2003; Buet *et al.*, 2006; Nunes *et al.*, 2006; Roy *et al.*, 2006; Bervoets *et al.*, 2009; Oliveira *et al.*, 2013; Vidal-Liñán *et al.*, 2016), while the CYP450 activity would increase

in species that have high DLCs present. It is therefore expected that fish sampled at the Barrage site would have the lowest AChE, and the greatest CYP450 levels because highest Σxenobiotics were present in fish from this site (see Chapter 3 Results). The lowest recorded AChE was from *C. carpio* from Thabela Thabeng, which in turn had the greatest CYP450 activity, whereas *C. carpio* from Barrage had higher AChE levels, and low CYP450 content compared to those as Thabela Thabeng. This relationship was not expected considering the concentrations of Σxenobiotics in the *C. carpio* at Barrage were at least three times greater than those at Thabela Thabeng. But without having a control sample it is difficult to determine what an unexposed fish's baseline responses would be in order to determine activation/inhibition of these enzymes. The expected activity from the Barrage samples were seldom observed, and often the CYP450 activity was the lowest in fish from Barrage instead of the expected highest, while *C. carpio* and *L. aeneus* from Thabela Thabeng had the greatest CYP450 activity—indicating that these fish are exposed to compounds that activate the CYP450 pathway. The results of the RDA show that activation of the CYP450 was associated with the ΣPCBs, ΣPBDEs, and ΣDDx, however not very strongly (Figure 4.11). This is probably because these are not the most potent CYP450-activating compound. AChE was shown to be inhibited by these compounds. The IBR/n score, particularly for Barrage consisted dominantly of CYP450 activation and AChE inhibition.

The unexpected responses could be due to other compounds that are present that were not analysed for in this study (Hecker and Giesy, 2011; Burkina *et al.*, 2015): a synergistic effect could explain the low AChE responses, and high CYP450 responses for relatively lower exposed sites (Vischgat and Thabela Thabeng) (Jin-Clark *et al.*, 2002), or even inhibition of the CYP450 enzymes (Stegeman and Hahn, 1994; Hahn and Chandran, 1996; Payne *et al.*, 1996; Besselink *et al.*, 1998; Camusso *et al.*, 2001). PCB 138, amongst others, has been shown to inhibit the CYP450 pathway (Besselink *et al.*, 1998). This PCB congener was one of the targeted compounds in this study, and was highest at the Barrage site. It is therefore possible that this is the reason for the lower than expected CYP450 activity.

A study at Rietvlei Wetland, in Gauteng, that sampled *O. mossambicus* also displayed inhibition of AChE, which was attributed to exposure of organic and metal pollutants (Wepener *et al.*, 2005). These fish generally had a slight increase in the production of CYP compared to the control, however not significantly. These are similar findings to the current study. In the Vaal River or its tributaries there have been other studies conducted that have also reported presence of compounds known to affect these enzymes (Bucibo, 2011; Crafford and Avenant-Oldewage, 2011; Wepener *et al.*, 2011; Pheiffer *et al.*, 2014; Chokwe *et al.*, 2015; Gilbert *et al.*, 2017; Pheiffer *et al.*, 2018b; Pheiffer *et al.*, 2018c). Fish sampled from the Vaal River at

similar sites as those in the current study had significant inhibition of AChE at Barrage compared to the other sites, and the CYP activity was also significantly greater within the fish sampled from this location (Wepener *et al.*, 2011), which is what was expected in the current study. Another study at the Barrage determined some inhibition of AChE in *Labeobarbus umbratus* compared to fish from the control site over a two year sampling period (Barnhoorn and Van Vuren, 2004). *Clarias gariepinus* sampled from the Klip River in Gauteng, a tributary of the Vaal that enters upstream of Barrage, had inhibition of the AChE enzyme compared to the control, and there was up-regulation of the CYP enzyme in the fish (Pheiffer *et al.*, 2015; Pheiffer, 2017). Another study in the Klip River on *C. gariepinus* reported that the fish had increased levels of AChE and CYP in the lower reaches compared to the upper reaches of the river (Mahlangu, 2013), this is a similar trend that was observed in some of the fish in the current study.

It is expected that SOD activity would be greatest where xenobiotic concentrations are high because SOD is expressed to catalyse the dismutation of the superoxide anion radical—which is formed due to exposure to pollutants—into oxygen and hydrogen peroxide (Pandey *et al.*, 2003; Lobo *et al.*, 2010). Catalase is upregulated to break-down the toxic hydrogen peroxide produced via the SOD pathway into water and oxygen (Lobo *et al.*, 2010; Mihailović *et al.*, 2016). These enzymes generally had a similar trend. But many of the species have large variation in the enzyme activity, which makes interpreting trends more complicated. The SOD results indicated that *L. aeneus* sampled from Thabela Thabeng had the greatest SOD activity. This species had fairly high xenobiotic concentrations, however not as high as those from Barrage, which typically did not have very high SOD activity. Only *L. capensis* sampled from Barrage had the expected highest SOD content in comparison with the results from the other sites. *C. gariepinus* sampled at Vischgat had the greatest CAT activity, however had relatively low concentrations of xenobiotics, especially compared to the fish sampled from Barrage. It is possible that compounds present, particularly at the Barrage, could have caused inhibition of the biomarkers of oxidative stress. Presence of crude oil, which contains PAHs, had been shown to inhibit the production of SOD (Otitoloju and Olagoke, 2011). Although the presence of crude oil has not been thoroughly documented in the Vaal area, studies from the Klip River, which is a tributary that enters the Vaal upstream of the Barrage, have identified petrogenic signatures which indicate the presence of oils (Pheiffer *et al.*, 2016), there is also an oil refinery situated within the catchment area (Nieuwoudt *et al.*, 2009). It is therefore likely that the presence of these can explain the low SOD responses while high loadings of xenobiotics are present. It had been reported that high levels of the superoxide anion radical can inhibit the activity of CAT (Kono and Fridovich, 1982) and high nitrates have also been demonstrated to

inhibit CAT production (Arillo and Melodia, 1991). These inhibitory effects may be the reason for and the seemingly low responses at the Barrage, the most impacted site, based on chemical concentrations. It is also possible that there is a differing response due to these enzymes response to the different toxicant make-up at the different sites. From the RDA results SOD and CAT are explained by the presence of ΣPCBs, ΣPBDEs, and ΣDDx (Figure 4.11).

Few other studies in the area have analysed organisms for SOD and/or CAT. The Klip River flows through highly industrialised and urban areas before it flows into the Vaal River upstream of the Barrage, this transports various anthropogenic inputs into the Vaal. One study conducted in the Klip River indicated that *C. gariepinus* generally did not have much of an increase in SOD, compared to the control, and only one site had significantly higher SOD content in the fish (Pheiffer, 2017). The CAT activity was significantly higher in the fish in every instance (Pheiffer, 2017). These were similar to the responses in this study. Another study from the Klip River had indication of exposure to ROSs based on increased levels of SOD and CAT in the lower reaches of the river compared to the upper reaches (Mahlangu, 2013). Metals have been shown to induce SOD and CAT activity in fish (Okamoto and Colepicolo, 1998; Collén *et al.*, 2003), which have been measured in the Vaal River and tributaries, although not analysed in this study (Wepener *et al.*, 2011; Pheiffer *et al.*, 2014; Gilbert *et al.*, 2017). And the presence of the metals could be the cause for the activation of the enzymes.

The breakdown product produced in response to lipid peroxidation, MDA, is caused by degradation of lipid membranes by free radicals (Livingstone, 2001; Lobo *et al.*, 2010). PC, which measures the level of amino acid oxidation due to exposure of free radicals (Lobo *et al.*, 2010), follows a similar trend to the MDA responses, indicating that similar variables are causing the oxidative responses. *Labeobarbus aeneus* from Thabela Thabeng however, had the highest PC content, while the MDA content was fairly low compared with the other samples (Figure 4.9). The greatest MDA activity was measured in *C. carpio* collected from Thabela Thabeng; these samples also had the second greatest PC content, and the greatest CYP450 activity, although the measured xenobiotics were not very high compared to other species and other sites. *Labeo capensis* from Thabela Thabeng also had high MDA activity, with fairly low xenobiotic concentrations. The protective function of SOD and CAT were surpassed, and this lead to the formation of PC and MDA (Figure 4.11), again ΣPCBs, ΣPBDEs, and ΣDDx explained these responses. The IBR/n score from Barrage was heavily influenced by the up regulation of MDA. It is likely however that there are other contaminants or environmental stressors present that are causing the greater breakdown of lipids or protein at sites that are seemingly low in xenobiotics. It has been demonstrated that pesticides, metals, and industrial effluents cause lipid peroxidation (Avci *et al.*, 2005), and many of these have been determined

in the region (Bouwman *et al.*, 2008; Burger, 2008; Wepener *et al.*, 2011; Jordaan and Bezuidenhout, 2013; Teklehaimanot *et al.*, 2014; Pheiffer *et al.*, 2018c), while PC can also be produced due to higher oxidative stress caused from higher physical activity (Hackenberger *et al.*, 2015). Considering the fish from Thabela Thabeng and Vischgat generally had higher PC content than those from Barrage, which is the most polluted, it is likely these fish were exposed to higher flow rates and this required greater physical activity.

Another study in the Vaal River noted significantly greater MDA and PC content in fish also sampled from the Barrage site compared to the other sites they sampled (Wepener *et al.*, 2011), one of which corresponds to the Vischgat site. In the Klip River there was no indication of lipid peroxidation (increase in MDA content) in the fish, and fish from only one site had presence of protein damage, indicated by elevated levels of PC (Pheiffer, 2017). Mahlangu (2013) indicated that there was some activity of MDA and PC in the fish sampled from the Klip River.

The energy reserves (carbohydrate, lipid, and protein) and the available energy (Ea) had a similar pattern for *L. aeneus* and *L. capensis*—fish collected at Thabela Thabeng had high energy reserves, while those collected at Vischgat had the lowest reserves. There was a trend between protein and the PC content in *C. gariepinus* and *C. carpio*: when the PC content was high the protein content was low, this indicates the depletion of protein due to oxidative stress. However, these same species had the opposite trend for lipid and Ea. Energy reserves can become depleted due to the organism dealing with stressors, be it in the form of a toxicant, an environmental parameter, or increased energy expenditure for growth and spawning (Smolders *et al.*, 2004). Other environmental stressors like temperature and food availability have also been shown to cause decreases in carbohydrate content (Fearman *et al.*, 2009; Blicher *et al.*, 2010). It would seem that xenobiotic concentrations were not the main driver in the reduction of energy reserves, because the highly contaminated Barrage site did not have the lowest energy reserves. These samples were, however, collected towards the end of winter and fish were exposed to low temperatures, this and other physicochemical parameters are possibly of more influence. The RDA displays that the energy reserves were impacted more from the environmental parameters (TDS, TOC, temperature, pH, and dissolved oxygen) (Figure 4.11).

In the study by Wepener *et al.* (2011) the fish sampled from the four sites in the Vaal River showed no changes in the CEA values. The sampling for that study was conducted in September, therefore roughly the same time of the year as the current study. The results in the current study also show fairly similar CEA responses, with *L. aeneus* and *L. capensis* from

Thabela Thabeng being slightly greater than the other samples, indicating these fish had greater energy reserves than what they were expending. The fish sampled from the Klip River had significantly greater CEA levels in 2014 compared with the 2013 samples, this was attributed to reduced energy reserves in the fish sampled from 2013 (Pheiffer, 2017).

The RDA showed that the variables analysed in the study only accounted for a small amount of the variation observed in the species responses. This indicates that the variables analysed in this study were not the main driving factors behind the biomarker responses in the sampled species. The species collected from each of the different sites clustered together. The fish sampled from Vischgat orientated with HCB, Σchlors, and ΣHCH. These OCPs are utilised in agricultural practices, and agriculture, amongst others, is a known stressor in the region (Wepener *et al.*, 2011). These OCPs caused a response in SOD activity, indicative of responding to ROSs, however these fish are dealing with the ROSs due to the lack of MDA and PC orientating to these samples. The samples collected from Thabela Thabeng orientated with the energy biomarkers: lipids, CEA, Ea, protein, and to a lesser extent carbohydrates and Ec. The explanatory variables were the physicochemical parameters: TDS, TOC, temperature, pH, and dissolved oxygen. This indicates that the fish from Thabela Thabeng were mostly influenced by these physicochemical parameters. The samples collected from Barrage orientated with CYP450, and AChE was roughly opposite these sites. This indicates that these species have an up-regulation of CYP450 and inhibition of AChE—this trend was not very clear when biomarkers alone were examined (Figure 4.7). These fish also displayed breakdown of lipids (MDA) and to a lesser extent protein (PC), explained by SOD and CAT not coping with the toxicant loadings. The explanatory variables were the xenobiotics: ΣPCBs, ΣDDx, ΣPBDEs, and ΣHCH. These are also the xenobiotics mostly expected to cause upregulation of the CYP450 pathway. (Hecker and Giesy, 2011).

The IBR scores between Barrage and Thabela Thabeng were not very different, however the highest score was at Barrage, and this indicates that fish from this site are in the poorest condition, which was expected based on their xenobiotic loads. The IBR/n derived for fish from Vischgat indicated the fish were in a good condition. The triangular star plot for Barrage indicated that SOD had the greatest contribution to the IBR/n score, whereas lipid contributed the greatest to the score at Thabela Thabeng. MDA, SOD, and CAT also contributed similarly to the IBR/n value. The high contribution of SOD to the IBR/n score was also seen by the RDA (Figure 4.11), and it is likely the result of the xenobiotics (ΣPCBs, ΣPBDEs, ΣDDx, and ΣHCHs). As for Thabela Thabeng, lipids were also a driving variable in the RDA results, and is likely a result of the higher nutrients measured at this site.

4.5 Conclusion

Organisms are exposed to various toxic and bioaccumulative compounds in the environment that are present as mixtures. Because of the mixture effects, synergism and additivity, can potentiate the toxicity of these compounds. Using biomarkers, the health of the organisms can be assessed by monitoring for oxidative stress and the effects that the exposure to the stress cause. In doing so it is possible to infer exposure to toxic compounds, and measure effects that these compounds have on the organisms that have been exposed—in a way that chemical analysis alone would be unable to predict, although using chemical analysis may be able to pin point the likely culprits of the observed effects.

In this study the organisms from both systems had evidence of stress response based on the biomarkers. There were differences in the biomarker responses between the two systems, and this is likely due to the different anthropogenic impacts in the two regions. The levels of the expected suspect compounds supposed to be present in the Phongola area due to its rural and agricultural nature did not seem to have a real effect on the biomarker responses observed in this study, it was rather the environmental parameters that caused physiological stress that was demonstrated in the biomarker responses. In contrast to this the levels of the industrial pollutants quantified for the Vaal River seemed to have been a driving force in causing the biomarker responses.

The individual species from Phongola did not have much correlation between biomarker responses and the chemical analyses. *Cherax quadricarinatus,* however, was the only species sampled that showed that the biomarkers were influenced by the analysed compounds. This was also the only invertebrate that was sampled, it is possible that because this is a benthic species it is more exposed to the xenobiotics, which adhere to the sediment, than the fish. But the general trend was that the species were highly affected by flow—temperature was a significant explanatory variable—and there was greater stress on the species during the high flow period than the low flow period. *Hydrocynus vittatus*, *S. zambezensis*, and *C. quadricarinatus* they had the highest OCP concentrations, and this may be attributed to their trophic position: *H. vittatus*, *S. zambezensis*, are top predators, and *C. quadricarinatus* is a detritivore. The IBR results also indicated that the samples collected during the high flow period were in a poorer state than those from the low flow period, which supports the greater influence of the environmental parameters rather than OCP concentrations. It is also possible that other compounds, or factors that have not been measured and/or analysed play a greater role in the biomarker responses.

It was expected that the Barrage site would be the most heavily impacted due to the xenobiotic concentrations present in the fish collected from this site compared to the other Vaal sites. When the individual biomarkers were analysed, often the other sites had greater biomarker responses—indicating that the Barrage was less impacted than originally thought. However, when the interactions of the biomarkers were considered, by means of the IBR, Barrage had the highest score, indicating the fish sampled from this site were in the poorest condition, which matches the results of the chemical analyses. There were clear site differences, the biomarkers responses from Barrage were highly influenced by xenobiotic concentrations of PBDEs, PCBs, DDx, and HCH, Thabela Thabeng was influenced by the physicochemical parameters, and Vischgat was influenced by the pesticides: HCHs, HCB, and chlordanes.

Because the explanatory variables only accounted for a small portion of the variation observed in the biomarker responses it is suggested that other influencing factors should also be analysed in future studies, these should include metals, other pesticides specifically for the Phongola region, and more industrially related xenobiotics, such as PCDD/Fs and PAHs, for the Vaal region. A greater number of individuals should be collected from the Vaal region, this will make determining effects more clear. It would also be worthwhile to determine if seasonality plays a large role in the Vaal as it does in Phongola.

5 Stable Isotope Analysis to determine bioaccumulation

5.1 Introduction

Anthropogenic pollutants such as organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), and flame retardants like polybrominated diphenyl ethers (PBDEs) have been released into the environment during the last century. These compounds, known as persistent organic pollutants (POPs), are slow to degrade, and are lipophilic and can therefore be transferred through the food web via bioaccumulation and biomagnification (Jones and De Voogt, 1999). Additionally, POPs have a multitude of toxic effects to organisms. This results in the food web being exposed to adverse effects, and the higher trophic level organisms are most at risk. Therefore, POP burdens would be greatest in higher trophic level species.

Stable isotope analyses (SIA) had been used to determine patterns in food web structures, and to trace nutrient sources. Because of these features, SIA can also be used to characterise trophic transfer of POPs in the food web (Ikemoto *et al.*, 2008). Making use of the ratio of the stable isotopes of nitrogen ($^{15}N/^{14}N$) the trophic position of an organism can be determined because there is an increase in abundance of ^{15}N at each trophic level (Hobson and Welch, 1992). This is based on the dietary assimilation of nitrogen in organisms because typically the "heavier" isotope, in this case ^{15}N, will be incorporated into the body tissue, while the lighter isotope, ^{14}N, is eliminated (Minagawa and Wada, 1984). This takes into account many meals, rather than using stomach contents, as was done in the past (Hobson and Welch, 1992; Atwell *et al.*, 1998; Ikemoto *et al.*, 2008).

The carbon isotopes can determine the original source of carbon in food webs. This is because terrestrial plants have different $\delta^{13}C$ signatures that occur during photosynthesis when carbon atoms are incorporated into the sugar formations. The 3-carbon (C_3) plants (trees, shrubs, and grasses) have a lower $\delta^{13}C$, around -28‰, while the 4-carbon (C_4) plants (corn, sugar cane, and dryland grasses) are higher, approximately -13‰ (O'Leary, 1988; Ehleringer and Cerling, 2001). These changes of $\delta^{15}N$ and $\delta^{13}C$, fractionate at approximately 3–4 ‰, and 1‰, respectively. Using these changes, the trophic position can be calculated (Hobson and Welch, 1992).

Linking trophic position with chemical concentrations can determine bioaccumulation and biomagnification in systems (Borgå *et al.*, 2012). It was demonstrated that the $\delta^{15}N$ values are

correlated with the bioaccumulation of xenobiotics (Gray, 2002; Borgå *et al.*, 2012). Trophic magnification factors (TMFs) are a reliable and conclusive means to determine bioaccumulation of environmental pollutants (Gobas *et al.*, 2009). This assessment method uses field measures of both relative trophic level and pollutant concentration to derive an empirical TMF that gives an "average food web biomagnification factor" (Fisk *et al.*, 2001). The method corrects for possible baseline variability in $\delta^{15}N$ that can result from anthropogenic inputs from wastewaters or agriculture (Anderson and Cabana, 2005). Dietary absorption occurs faster than elimination and therefore biomagnification is indicated by a TMF>1, this implies that there is no equilibrium between the organism and the abiotic matrix that increases with increasing trophic level. A TMF=1 indicates that the xenobiotic does not biomagnify, and a TMF less than 1 indicates that the pollutant decreases with increasing trophic level. This is also referred to as trophic dilution (Gobas and Morrison, 2000).

In this chapter the aims are to determine the trophic position of the samples collected from the Vaal River and in the Phongola system. And to assess bioaccumulation of xenobiotics in these organisms, which have been analysed in previous chapters, using the TMF model. This can be used to gauge potential risk to humans who subsistence fish in these systems.

5.2 Methods

5.2.1 Sample collection

Biotic and abiotic matrices were collected from the Vaal River in October 2014 and Phongola River September 2013, and therefore only low flow samples will be dealt with. These included sediment, plant matter, algae, invertebrates, and fish. A composite sample of surface sediment was collected from each site consisting of three subsamples collected approximately 3 m apart using a Van Veen grab. This sample was transferred to amber glass bottles. Plant specimens that were in and around the aquatic system were collected and placed inside aluminium foil. Biofilm was collected from rocks using a toothbrush, and a composite sample consisting of triplicate subsamples. The composite sample was stored in a 50 mL centrifuge tube. Plankton was collected by allowing a 64 µm plankton net to sit in flowing water for 15 minutes, after which the collected sample was transferred to a 50 mL centrifuge tube.

Macro-invertebrates were collected from different habitat types in the systems—stones, vegetation, sand, and mud using a 500 µm sweep net. The organisms were identified to family level, based on the identification guide by Gerber and Gabriel (2002). Samples comprised of individuals belonging to one family were placed in centrifuge tubes. Fish were caught as

described in previous chapters by means of electro-shocking and fyke nets. A small portion of the muscle tissue, without bone and scales, was transferred to a centrifuge tube.

5.2.2 Stable isotope analyses

All samples were stored in a portable freezer while in the field and transferred to a -20°C freezer for storage. Subsamples of all samples were transferred into centrifuge tubes and frozen at -80°C overnight. The samples were freeze dried in a Labconco (cat. no: 775303) freeze dyer. Once dry the samples were ground into a fine powder with a mortar and pestle. Approximately 1 g of sample was transferred to a microcentrifuge tube. The macro-invertebrate and fish samples were treated with 1 mL 2:1 chloroform:methanol (v:v) for 12 hours to remove lipids. The samples were centrifuged at 1 500 g for 2 minutes and the supernatant removed and discarded (Bligh and Dyer, 1959). This was done because lipids are depleted in ^{13}C compared with protein and variation in lipid content can confound the interpretation of the diet (Oppel *et al.*, 2010; Elliott and Elliott, 2016). The pellet was removed, and the remaining solvent was evaporated in an oven at 60°C (Elliott and Elliott, 2016). Approximately 0.5 mg, 5 mg, and 10 mg of consumer, producer, and sediment matter respectively, was weighed on a Cubis® Micro Balance (MSE6.6S-0CE-DM) and encapsulated into Elemental Microanalysis D1008 ultrapure tin capsules, 5 mm x 8 mm, for analysis. The analyses were conducted at Hokkaido University, Japan, using an Elemental IsoPrime 100 with Vario MICRO cube Isotope Mass Spectrometer (IRMS) to measure the ration of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$). Reference standard (L-alanine, SI Science Co., Ltd., Saitama, Japan) was measured in triplicate between every six samples to monitor accuracy. An acceptable error range was ±0.02‰ for both $\delta^{13}C$ and $\delta^{15}N$. Atmospheric nitrogen and PeeDee belemnite were used as the standards for nitrogen and carbon respectively (Yohannes *et al.*, 2013).

Stable isotope ratios were expressed in the standard δ notation, as defined as:

$$\delta^{13}\mathrm{C}‰ \text{ or } \delta^{15}\mathrm{N}‰ = \left[\left(\mathrm{R}_{\mathrm{sample}}/\mathrm{R}_{\mathrm{reference}}\right) - 1\right] \times 1000$$

Where R = $^{13}C/^{12}C$ for carbon and R = $^{15}N/^{14}N$.

Relative trophic levels of the organisms were calculated by substituting the $\delta^{15}N$ values of each species into the following equation (Post, 2002):

$$TP_{consumer} = \left(\frac{\delta^{15}N_{component} - \delta^{15}N_{reference}}{2.8}\right) + 1$$

Where $TP_{consumer}$ refers to the trophic position of the organism, $\delta^{15}N_{component}$ is the $\delta^{15}N$ of the organism, $\delta^{15}N_{reference}$ is the mean $\delta^{15}N$ of the sediment, 1 is the trophic level of the primary producer, the denominator, 2.8, is the trophic enrichment factor between fish and their food source (Jepsen and Winemiller, 2002). Because the trophic positions were corrected for differences in the $\delta^{15}N$ in the primary sources from each of the sites, in this case sediment, it is possible to directly compare the TP across sites.

Trophic magnification factors (TMFs) were calculated using the lipid normalised contaminant concentration in the organisms and the relative trophic positions (TP). These were calculated from the regression-slope of the log transformed concentrations versus the trophic level calculated (Borgå *et al.*, 2012). Such that:

$$\mathrm{Log}\,[\mathrm{xenobiotic}] = \mathrm{a} + \mathrm{b}\,\mathrm{TP} \qquad \mathrm{TMF} = 10^{\mathrm{b}}$$

5.2.3 Persistent organic pollutant (POP) analyses

Analysis of xenobiotics have been discussed in previous chapters (see Chapters 2 and 3). In brief, the samples collected from the Vaal River were extracted with hexane:acetone (3:1 v:v) using an automated hot Soxhlet. The extract was subjected to acidified silica (44% H_2SO_4, w:w). The eluent was concentrated and resuspended in isooctane. The target analytes were quantified by gas chromatography mass spectrometry (GC MS). Mean internal standard (PCB 143, PBDE 77, and ε-HCH) recoveries were >86%, and analyses of National Institute of Standards and Technology standard reference material (NIST, SRM 1945) was within an acceptable range (10%) of the certified values.

The samples collected from Phongola were extracted with 3:1 hexane:acetone (v:v) in an automated hot Soxhlet. The extract was treated to 5% deactivated Florisil. The eluent was concentrated and resuspended with *n*-decane containing surrogate standard (tetrachloro-*m*-xylene). The target compounds were quantified using a GC coupled to a ^{63}Ni electron capture detector. The mean recovery of target compounds for spiked blanks was 90 ± 11%, and the mean recovery of fortified samples, *Oreochromis niloticus,* were in the acceptable range of 70–110%. Additionally SRM (NIST 1947, Lake Michigan fish tissue) was analysed with satisfactory recoveries of 75–115%, and a relative standard deviation of 12%. The xenobiotic concentrations have been calculated using a surrogate value of ½ LOD for instances of <LOD (Verhaert *et al.*, 2013).

5.3 Results

5.3.1 Phongola

Frogs: *Chiromantis xerampelina*, *Xenopus muelleri* (adults and tadpoles), *Amietophrynus garmani*, and *Ptychadaena anchietae* that were collected for another study during the same sampling period (Wolmarans, 2015), were included in the food web and trophic magnification analyses. The trophic positions of the species sampled from Phongola are listed in Table 5.1. Libellulidae, an invertebrate, had a lower trophic position, and therefore a lower $\delta^{15}N$ than the sediment which was used as the basal reference value for the calculation of the trophic levels. The primary producers: leaf litter and grasses were in trophic level 1, while biofilm was in the second trophic level, and the water hyacinth, *Eichhornia crassipes* was calculated as being in trophic level 3. Other invertebrates that were sampled, positioned in the first trophic level and these were the bivalve, Corbiculidae, insects: Baetidae and Gomphidae, and Oligochaeta. The snail from the family Lymnaeidae, was in trophic position two, and the shrimp, belonging to Atyidae, was in position three. Frogs occupied positions two and three, and *X. meulleri* adults and tadpoles were in different trophic levels. *Hydrocynus vittatus* and *A. bicolor* had the highest $\delta^{15}N$, and were positioned at the top of the food web in trophic position four. *Synodontis zambezensis* had a slightly lower $\delta^{15}N$, and was also in position four. The remaining fish species that were targeted for this study were in trophic position three.

The primary source of energy was the leaf litter and grasses (Figure 5.1). Water hyacinth, is a primary producer, although it has a high $\delta^{15}N$, and would possibly serve as a carbon source for the higher trophic level species such as *C. quadricarinatus*, *O. mossambicus*, and *X. meulleri*. Corbiculidae was an exception, and its primary carbon source was not sampled, but it correlated well with the sediment.

Table 5.1 The mean ± standard deviations of the $\delta^{15}N$ and $\delta^{13}C$ values and trophic positions of the composite samples collected from the Phongola system in September (low flow).

Sample	Guild	$\delta^{15}N$	$\delta^{13}C$	TP
Libellulidae (Lib)	Insectivore[1]	4.2 ± 1.0	-27 ± 0.13	0.4
Sediment	Organic matter	5.9 ± 1.1	-22 ± 1.2	1
Corbiculidae (Corb)	Filter feeder[2]	6.7 ± 0.22	-16 ± 1.6	1.3
Leaf litter (LL)	Primary producer	6.9 ± 0.62	-29 ± 0.79	1.4
Baetidae (Baet)	Herbivore/detritivore[3]	7.6 ± 1.1	-25 ± 1.6	1.6
Grasses	Primary producer	7.8	-26	1.7
Oligochaeta (Oligo)	Detritivore[4]	8.1 ± 0.03	-22 ± 2.1	1.8
Gomphidae (Gomp)	Invertivore[1]	8.3 ± 1.4	-25 ± 0.99	1.9
C. xerampelina (C.xera)	Insectivore[10]	8.3 ± 0.01	-23 ± 0.1	1.9
X. muelleri tadpoles *(X. meu T)*	Filter feeder/herbivore[5]	9.1 ± 2.9	-26 ± 7.0	2.2
Plankton	Primary producer	9.3 ± 0.39	-26 ± 0.48	2.2
A. garmani (A. gar)	Invertivore[10]	9.3 ± 0.5	-23 ± 1.0	2.2
P. anchietae (P.anch)	Insectivore[10]	9.6 ± 0.08	-20 ± 0.02	2.3
Lymnaeidae (Lymn)	Scraper/detritivore[3]	9.6 ± 1.1	-19 ± 3.2	2.3
Small fish (Barbs)	Invertivore[6]	9.9 ± 1.3	-22 ± 1.2	2.4
Biofilm	Primary producer	10 ± 0.33	-22 ± 0.47	2.5
Atyidae (Aty)	Omnivore/detritivore[7]	12 ± 1.0	-23 ± 1.2	3.1
Hyacinth	Primary producer	12 ± 0.71	-24 ± 0.25	3.2
X. muelleri (X.meu)	Opportunistic/nektonic[8,9]	12 ± 0.6	-22 ± 0.77	3.4
C. quadricarinatus (C. quad)	Omnivorous detritivore[11]	13 ± 1.2	-24 ± 0.78	3.4
C. rendalli (C. ren)	Omnivore (more herbivorus)[6]	13 ± 0.06	-19 ± 0.66	3.4
O. mossambicus (O. mos)	Detritivore/omnivore[6]	13 ± 0.59	-22 ± 1.7	3.5
C. gariepinus (C. gar)	Omnivore[6]	14 ± 0.44	-24 ± 0.69	3.8
S. zambezensis (S. zam)	Invertivore[6]	15 ± 0.58	-24 ± 2.1	4.1
A. bicolor (A. bic)	Invertivore/Piscivore[6]	16 ± 0.36	-20 ± 2.0	4.5
H. vittatus (H.vit)	Piscivore[6]	16 ± 0.53	-23 ± 1.6	4.5

[1]de Moor *et al*., 2003; [2]Bódis *et al.,* 2006; [3]Baptista *et al*., 2006; [4]Marco *et al*., 2001; [5]Cannatella *et al*., 1993; [6]Skelton, 2001; [7]Day *et al*., 2001; [8]Vogt *et al.,* 2017; [9]Paul and Shi., 2003; [10] Poynton and Carruthers, 2004; [11]Daniel, 2020.

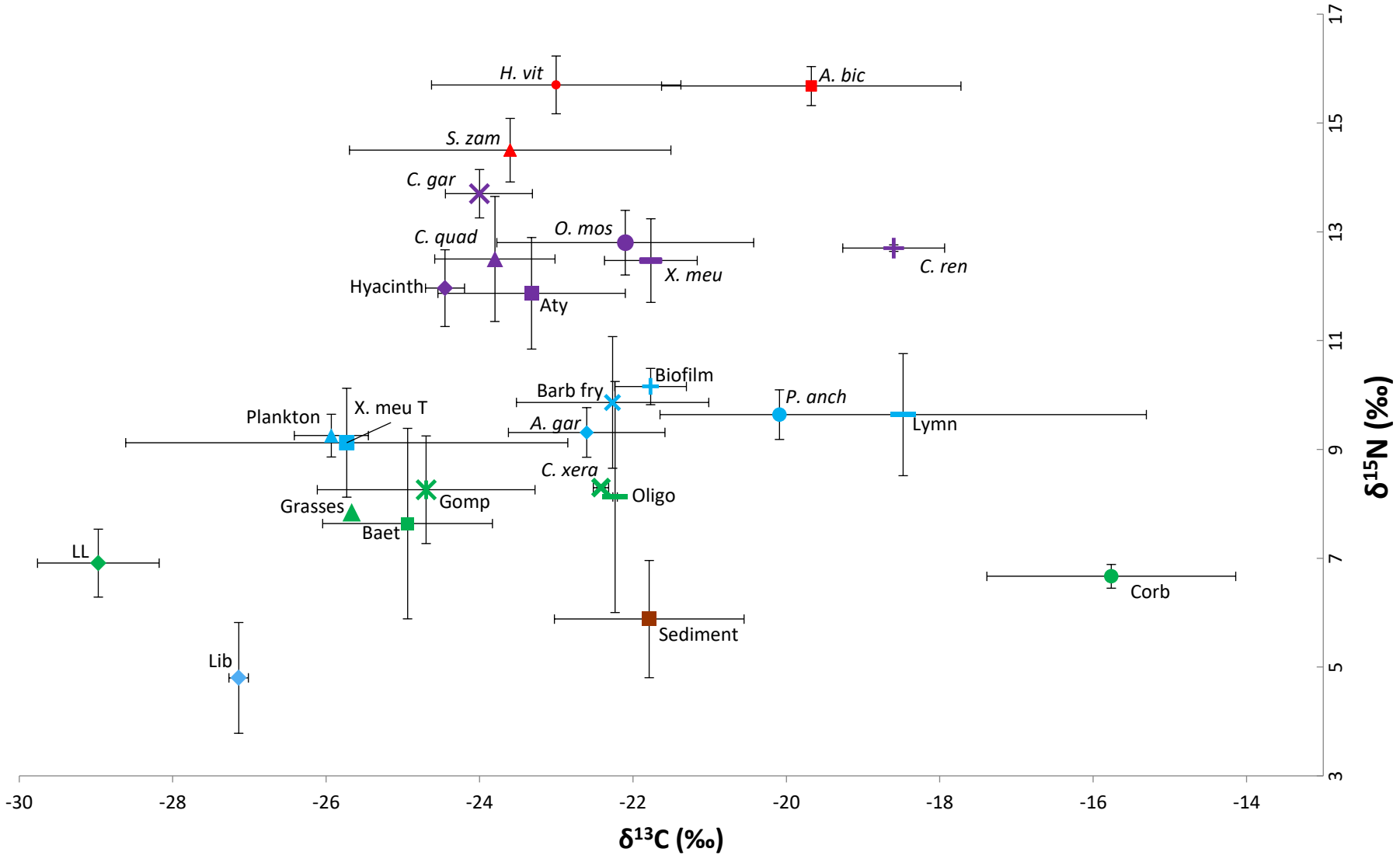


Figure 5.1. Stable isotope biplot of $\delta^{13}C$‰ (mean ± standard deviation) and $\delta^{15}N$‰ (mean ± standard deviation) ratios for the food web of the Phongola River

The trophic magnification factors of the xenobiotics are listed in Table 5.2. Typically, the TMFs were all <1, with the exception of ΣDDx and ΣOCPs, which had a TMF>1. The Σdrin was just less than one.

Table 5.2 Mean concentrations (±standard deviation) of the analysed OCPs (ng/g lm), $\delta^{15}N$‰ (±standard deviation), and the trophic position (TP) of biota samples collected from Phongola in September, low flow period

Species	$\delta^{15}N$	TP	ΣDDx	ΣHCH	ΣChlor	ΣHCB	Σdrin	ΣOCPs
			ng/g lm	ng/g lm	ng/g lm	ng/g lm	ng/g lm	ng/g lm
C. xerampelina	8.3 ± 0.01	1.86	240 ± 160	9.0 ± 4.9	2.8	0.61	1.1	260 ± 160
A. garmani	9.3 ± 0.46	2.23	69 ± 75	15 ± 12	2.8	0.61	1.05	110 ± 74
P. anchietae	9.6 ± 0.08	2.34	9.2 ± 15	50 ± 55	2.8	0.61	1.05	120 ± 110
C. quadricarinatus	13 ± 0.7	3.1	1200 ± 970	4700 ± 1900	720 ± 790	0.61 ± 0	1900 ± 890	8600 ± 3200
C. rendalli	13 ± 3.6	3.2	53 ± 110	48 ± 34	29 ± 37	1.5 ± 2.7	2.5 ± 6.2	130 ± 48
O. mossambicus	13 ± 0.6	3.2	320 ± 450	120 ± 260	110 ± 210	0.6 ± 0	1.9 ± 2.5	560 ± 220
X. muelleri	12 ± 0.6	3.35	1500 ± 2200	2300 ± 4800	240 ± 700	0.61	6.7 ± 20	6700 ± 2600
*C. gariepinus**	13 ± 0.4	3.5	270	0.11	0.06	0.05	NA	270 ± 150
S. zambezensis	15 ± 0.6	3.8	21 ± 40	4.0 ± 3.2	5.5 ± 5.6	0.7 ± 0.2	1.9 ± 3.8	33 ± 13
A. bicolor	16 ± 0.4	4.2	6600 ± 3200	300 ± 170	80 ± 64	4.4 ± 4.6	98 ± 62	7100 ± 3500
H. vittatus	16 ± 0.5	4.2	1100 ± 1800	1.6 ± 0	3.2 ± 0	1.61 ± 0	1.05 ± 0	1100 ± 580
TMF			**1.55**	**0.33**	**0.73**	**0.51**	**0.96**	**1.07**

* Chemical data from Volschenk *et al.* (2019) because *C. gariepinus* concentrations from September were not calculated in this study, the fish were sampled at the same time and location as those in the current study. Other than ΣDDx, all other OCP classes were <LOD and the means are calculated from ½ LODs

NA: not analysed.

5.3.2 Vaal

The three sample regions from the Vaal: Vischgat, Barrage, and Thabela Thabeng had very different food web structures (Figure 5.2–Figure 5.4), and the trophic positions of common species were different at each site (Table 5.3). The $\delta^{15}N$‰ of sediment was greatest at Vischgat, and lowest at Thabela Thabeng, while the $\delta^{13}C$ was greatest at Vischgat and lowest at Barrage. The Vischgat food web contained algae, aquatic vegetation, and crab (Potamonautidae) that positioned below the sediment. Plankton collected from Barrage was far more nitrogen enriched that plankton collected from Vischgat, trophic positions 20‰ and 12‰ respectively. Thabela Thabeng and Barrage were far more enriched with nitrogen than Vischgat. The food webs of Barrage and Thabela Thabeng had trophic lengths of 5.8 and 6.6, respectively, whereas the trophic length at Vischgat was only 3.2. The target species for trophic magnification (*L. capensis*, *C. gariepinus*, *C. carpio*, and *L. aeneus*) were in tropic position two as Vischgat, position five in Barrage, and between five and six at Thabela Thabeng. The shrimp

(Atyidae) collected from Vischgat occupied the top position in the food web, whereas those sampled from Thabela Thabeng were in positon 4.7, below the target species. *Clarias gariepinus* from Thabela Thabeng was the highest trophic organism sampled from this site with a trophic position of 6.6 compared with those collected from Vischgat which had a trophic position of 2.2. *Labeobarbus aeneus* was in the highest trophic position of the target species at Vischgat and Barrage, and the second highest at Thabela Thabeng.

There were different trends in the TMFs between the Vaal sampling sites; ΣDDx, ΣOCPs, and ΣPCBs had TMF >1 at every site (Table 5.4). ΣChlors, ΣHCH, and ΣPBDEs had TMF>1 at Thabela Thabeng and Barrage, and <1 at Vischgat. HCB had TMF>1 at Vischgat and Thabela Thabeng, however these TMFs were based solely on <LODs that were replaced with ½ LOD surrogate values, whereas Barrage had TMF <1, yet this was derived from actual calculated concentrations.

Table 5.3. Stable isotope ratios, $\delta^{15}N‰$ (±standard deviation) and $\delta^{13}C‰$ (±standard deviation) of composite samples, and trophic positions of organisms collected from the three sites in the Vaal River: Vischgat, Barrage, and Thabela Thabeng.

Sample	Guild	Vischgat			Barrage			Thabela Thabeng		
		$\delta^{15}N$	**$\delta^{13}C$**	**TP**	**$\delta^{15}N$**	**$\delta^{13}C$**	**TP**	**$\delta^{15}N$**	**$\delta^{13}C$**	**TP**
Algae	Primary producer	8.64	-16.1	0.26		NA			NA	
Aquatic vegetation	Primary producer	9.2 ± 1.2	-27 ± 1.1	0.46		NA			NA	
Potamonautidae (Potam)	Detritivore/opportunistic scavenger[1]	10	-21	0.75		NA			NA	
Sediment	Organic matter	11	-26	1	9.8 ± 2.7	-23 ± 1.4	1	7.2 ± 2.8	-25 ± 0.7	1
Hirudinea	Parasitise on invertebrates and vertebrates[2]		NA		19.6	-26	4.5		NA	
Beatidae (Baet)	Herbivore/detritivore[3]	12 ± 4.2	-21 ± 0.92	1.5		NA			NA	
Plankton	Primary producer	12	-23	1.6	20 ± 1.3	-22 ± 0.38	4.7		NA	
Gambusia affinis	Insectivore[4]	14	-21	2.1		NA			NA	
Gyrinidae	Herbivore/detritivore[5]		NA			NA		16 ± 0.6	22 ± 0.8	4.4
L. capensis	Detritivore[4]	14 ± 1.04	-18 ± 1.9	2.2	22 ± 0.7	-22 ± 0.85	5.4	21 ± 0.13	-22 ± 2.0	5.9
C. gariepinus	Omnivore[4]	14 ± 0.3	-20 ± 0.02	2.2		NA		23 ± 0.51	-21 ± 0.69	6.6
Biofilm	Primary producer	15	-17	2.6		NA			NA	
C. carpio	Omnivore[4]		NA		22 ± 0.15	-24 ± 0.5	5.5	22 ± 0.68	-22 ± 0.19	6.2
L. aeneus	Omnivore[4]	15 ± 0.84	-19 ± 0.8	2.6	23 ± 0.7	-24 ± 0.76	5.8	22 ± 0.28	-23 ± 0.52	6.4
Atyidae	Omnivore/detritivore[6]	17	-22	3.2		NA		18 ± 0.51	-22 ± 0.7	4.7

NA: not analysed

[1] Peer *et al.,* 2015; [2] Langer *et al.,* 2018; [3]Baptista *et al*., 2006; [4]Skelton, 2001; [5]de Moor *et al*., 2003; [6]Day *et al*., 2001;

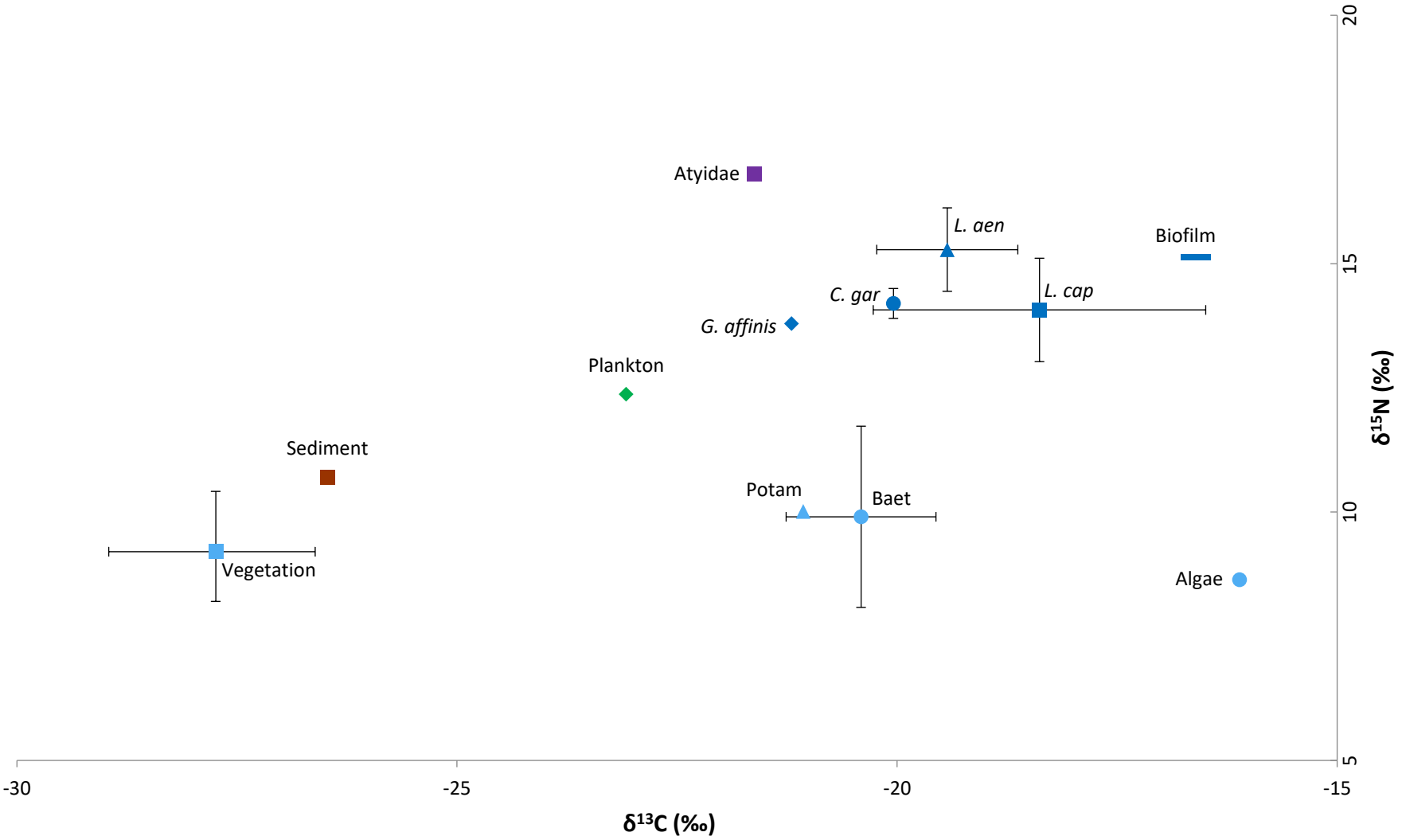


Figure 5.2 Stable isotope biplot of $\delta^{13}C$ (mean ± standard deviation) and $\delta^{15}N$ (mean ± standard deviation) ratios for the food web of Vischgat, in the Vaal River

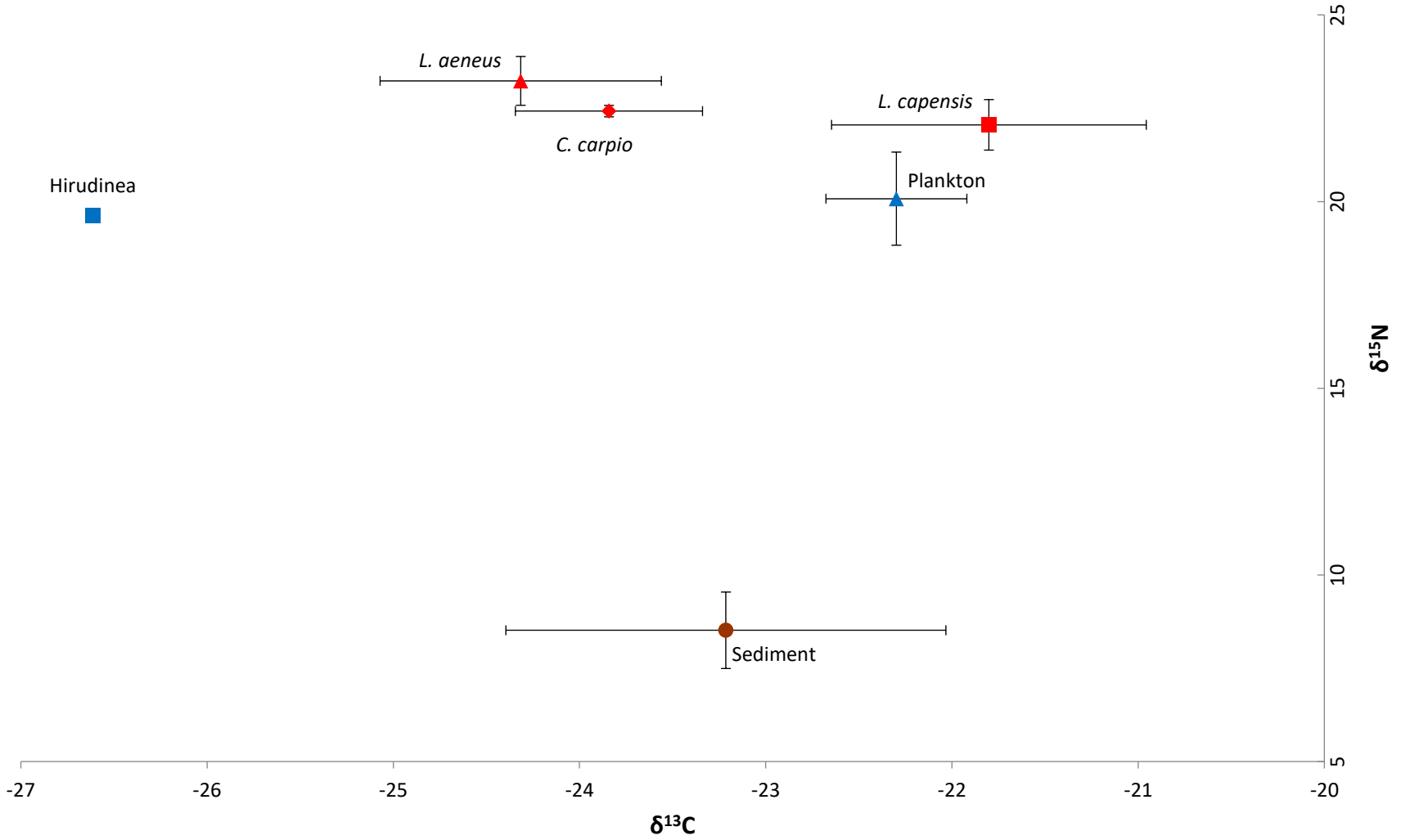


Figure 5.3 Stable isotope biplot of $\delta^{13}C$ (mean ± standard deviation) and $\delta^{15}N$ (mean ± standard deviation) ratios for the food web of Barrage, in the Vaal River

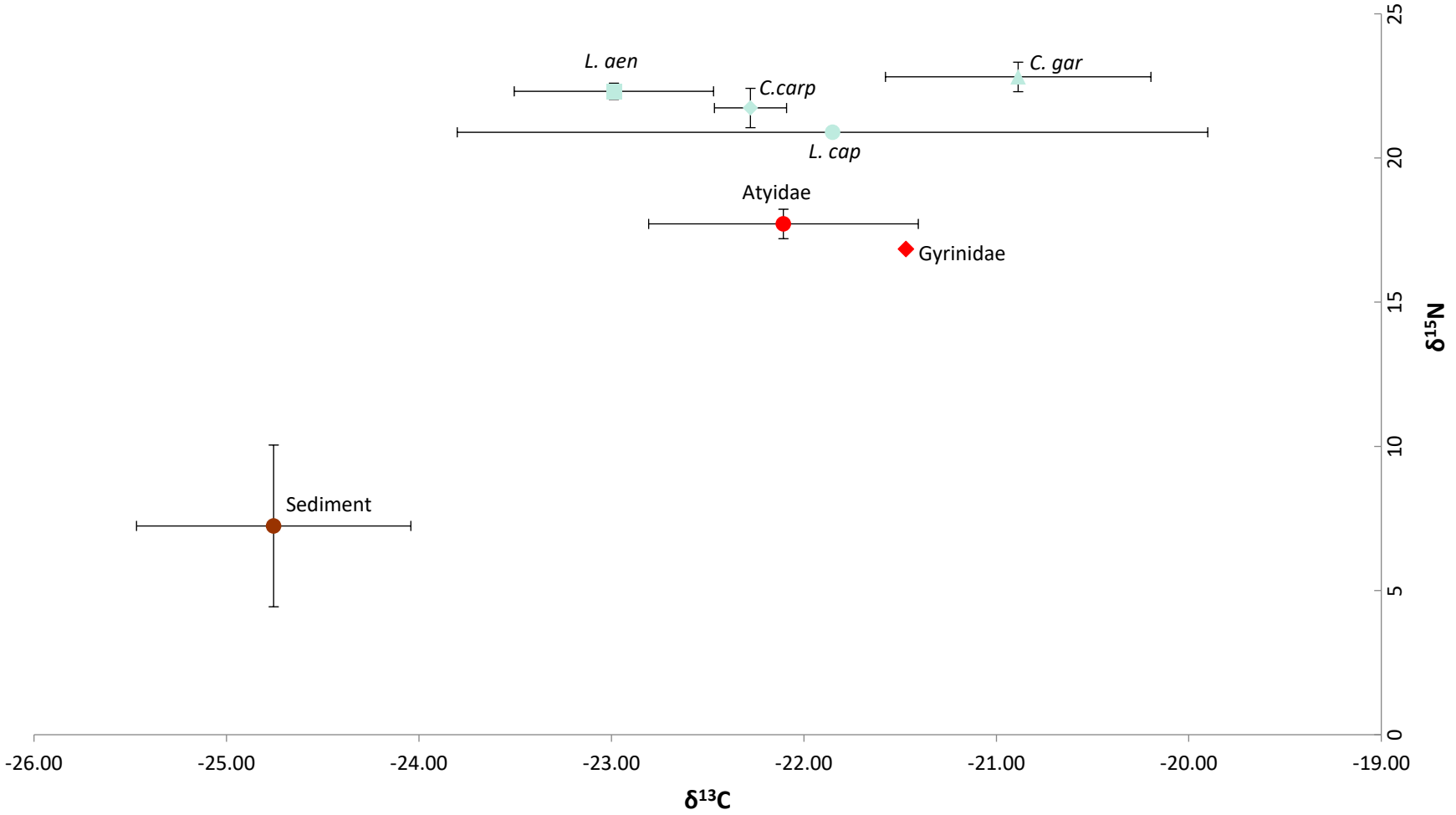


Figure 5.4 Stable isotope biplot of $\delta^{13}C‰$ (mean ± standard deviation) and $\delta^{15}N‰$ (mean ± standard deviation) ratios for the food web of Thabela Thabeng, in the Vaal River

Table 5.4. $\delta^{15}N‰$, trophic position, xenobiotic concentrations (ng/g lipid mass), and trophic magnification factors (TMFs) of the sites from the Vaal River.

Site	Species	δ15N	TP	ΣChlors	HCB	ΣDDx	ΣHCHs	ΣOCPs	ΣPCBs	ΣPBDE
Vischgat	*C. gariepinus*	14.20	2.25	3.0	1.0	61	2.0	67	135	3.5
	L. aeneus	15.28	2.64	3.0	1.0	162	2.0	169	169	3.5
	L. capensis	14.07	2.20	7.0	1.0	133	23	163	74	8.8
	TMF			**0.56**	**1.84**	**6.45**	**0.06**	**4.89**	**7.38**	**0.50**
Thabela Thabeng	*C. gariepinus*	22.81	6.56	8.2	1.0	215	2.8	227	241	10.3
	C. carpio	21.73	6.18	3.0	1.0	82	2	88	129	8.5
	L. aeneus	22.31	6.38	3.0	1.0	84	7	95	120	66.2
	L. capensis	20.89	5.88	3.0	1.0	49	3.6	57	237	2.7
	TMF			**5.93**	**1.77**	**11.95**	**2.18**	**10.73**	**1.51**	**33.41**
Barrage	*C. carpio*	22.42	5.52	3.0	1.0	173	11.6	189	536	21.3
	L. aeneus	23.23	5.81	17	2.8	314	18	352	944	46.7
	L. capensis	22.05	5.39	8.6	8.7	142	8.5	168	311	20.2
	TMF			**14.01**	**0.26**	**9.14**	**7.68**	**8.34**	**16.87**	**11.13**

5.4 Discussion

Most of the organisms in the systems seemed to have logical trophic positions based on their feeding guild (Table 5.1 and 5.3; Figures 5.1–5.4). Some of the organism have higher than expected $\delta^{15}N$, and therefore TP. This could be explained by nitrogen enrichment in the systems, and is explained in more detail later in this discussion.

There are limited studies in Africa using the $\delta^{15}N$ stable isotope to track trophic levels, and even fewer that have similar species to those in the current study (e.g. Kidd *et al.* (2001); Kidd *et al.* (2004); Verhaert *et al.* (2013)). Table 5.5 compares the $\delta^{15}N$ values obtained in the current study to those of other studies in Africa. $\delta^{15}N$ was used rather than trophic position because the TP depends on which base organism or sediment was used.

Common species between the Phongola and Vaal Rivers, typically had higher $\delta^{15}N$ values from the Vaal sites, with the exception of *C. gariepinus*, which had similar values in the Phongola River and from the Vischgat site. The $\delta^{15}N$ values were greatest in the species from the Vaal compared with the other regions in Africa, while the values were roughly similar between Phongola Floodplain and the other African sites, with the exception of Libellulidae which was much lower in Phongola Floodplain than those collected from Luvuvhu and Olifants Rivers. Lake Chad, in central Africa, did not have identical species sampled to those in this study, however had some genera overlap (Kidd et al., 2004). They determined the $\delta^{15}N$ values of *Oreochromis niloticus* (5.7 ± 1.1‰), *Synodontis schall* (7.0 ± 0.6‰), *Hydrocynus forskahlii* (8.8 ± 0.9‰), and C*. gariepinus* (10.2 ± 2.2‰). These $\delta^{15}N$ values were far lower than those from the Phongola and Vaal Rivers.

High $\delta^{15}N$ values indicate nitrogen enrichment within the system. Possibilities for the enrichment is allochthonous inputs such as sewage and agricultural practices (animal waste and animal derived fertilisers i.e. manure) which can cause $\delta^{15}N$ enrichment of up to 25‰. Synthetic fertilisers either do not affect the $\delta^{15}N$, or cause depletion of the $\delta^{15}N$ (McClelland et al., 1997; Hebert and Wassenaar, 2001). The Olifants River is regarded as one of the most polluted rivers in the country with similar activities to those in the Vaal region, such as mining, agriculture, and urbanisation (De Villiers and Mkwelo, 2009; Heath et al., 2010), and the Luvuvhu River has similar impacts to the Phongola River including agriculture and forestry (Kleynhans, 1996). Gerber (2012) demonstrated that the Olifants River was nitrogen enriched, compared to the Luvuvhu River and was attributed to the effect of anthropogenic impacts in the region. The Vaal system, however, had even greater $\delta^{15}N$ values, indicating severe nitrogen enrichment. The Vaal River is known for being polluted by industrial effluents, sewage,

acid mine drainage, and agricultural runoff (Burger, 2008; Jooste *et al.*, 2008; Quinn *et al.*, 2009; Jordaan and Bezuidenhout, 2013; Teklehaimanot *et al.*, 2014), all of which can cause eutrophication. A study in the Vaal River at similar sampling sites to the Barrage and Thabela Thabeng indicated high levels of dissolved inorganic nitrogen in the system, and Barrage had the greatest levels out of the sample sites (van Vuuren and Pieterse, 2005). This eutrophication is likely the cause for the increase in $\delta^{15}N$ at Barrage and Thabela Thabeng compared to Vischgat. This nitrogen enrichment had been demonstrated to affect growth, relative abundance, and feeding dynamics of the functional groups within systems, as well as the diversity of the community (Deegan *et al.*, 2002; Huxel *et al.*, 2004; Tewfik *et al.*, 2005).

Table 5.5 Mean $\delta^{15}N‰$ (±standard deviation) values for organisms collected in this study compared with other studies.

Organism	Phongola	Vischgat	Barrage	Thabela Thabeng	Luvuvhu, SA	Olifants, SA	Olifants (site OG), SA	Lake Koka, Ethiopia	Lake Chad, central Africa
	This study				Gerber *et al.* (2016)		Verhaert *et al.* (2017); (2019)	Deribe *et al.* (2011)	Kidd *et al.* (2004)
Libellulidae	4.2 ± 1.0				9.4 ± 1.1	12.9 ± 0.4			
Algae		8.64							
Aquatic vegetation		9.2 ± 1.2							
Potamonautidae		10							
Sediment	5.9 ± 1.1	11	9.8 ± 2.7	7.2 ± 2.8	5.9 ± 0.9	6.5 ± 0.3			
Corbiculidae	6.7 ± 0.22								
Leaf litter	6.9 ± 0.62				5.9 ± 1.4				
Baetidae	7.6 ± 1.1	12 ± 4.2							
Grasses	7.8								
Oligochaeta	8.1 ± 0.03								
Gomphidae	8.3 ± 1.4				9.3 ± 1.6	12.6 ± 0.7	11 ± 0.46		
C. xerampelina	8.3 ± 0.01								
Gambusia affinis		14							
X. muelleri tadpoles	9.1 ± 2.9								
Plankton	9.3 ± 0.39	12	20 ± 1.3		9.3 ± 0.4	7.9 ± 1.3			
L. capensis		14 ± 1.04	22 ± 0.7	21 ± 0.13					
A. garmani	9.3 ± 0.5								
P. anchietae	9.6 ± 0.08								
Lymnaeidae	9.6 ± 1.1								
Small fish (Barbs)	9.9 ± 1.3				10.4 ± 0.9	13.7 ± 0.5			
Biofilm	10 ± 0.33	15			8.5 ± 2.1	8.5 ± 1.1			
L. aeneus		15 ± 0.84	23 ± 0.7	22 ± 0.28					
Atyidae	12 ± 1.0	17		18 ± 0.51	11.7 ± 1.1				
Hyacinth	12 ± 0.71								

Organism	Phongola	Vischgat	Barrage	Thabela Thabeng	Luvuvhu, SA	Olifants, SA	Olifants (site OG), SA	Lake Koka, Ethiopia	Lake Chad, central Africa
	This study				Gerber *et al.* (2016)		Verhaert *et al.* (2017); (2019)	Deribe *et al.* (2011)	Kidd *et al.* (2004)
Xenopus muelleri	12 ± 0.6								
C. quadricarinatus	13 ± 1.2								
C. rendalli	13 ± 0.06				11.9 ± 0.2				
O. mossambicus	13 ± 0.59				11.1 ± 1.1	13.1 ± 0.9			
Oreochromis niloticus									5.7 ± 1.1
C. gariepinus	14 ± 0.44	14 ± 0.3		23 ± 0.51	13.2 ± 0.2	14.7 ± 0.5	13 ± 1.1	17.5	10.2 ± 2.2
Synodontis forskahlii									7.0 ± 0.6
S. zambezensis	15 ± 0.58						14 ± 0.41		
Gyrinidae				16 ± 0.6					
Hirudinea			19.6						
A. bicolor	16 ± 0.36								
H. vittatus	16 ± 0.53				15.2 ± 0.6	18.3 ± 0.5	16 ± 0.36		
C. carpio			22 ± 0.15	22 ± 0.68				14.5	

Biomagnification of a compound or compound class in the food web occurs when the TMF is greater than one, whereas a TMF less than one indicates dilution through the food web and a TMF equal to one indicates no biomagnification (Gobas and Morrison, 2000). In the Phongola system ΣDDx and ΣOCPs had TMFs>1 (1.55 and 1.07, respectively). The ΣOCPs TMF is highly influenced by the DDx concentrations. ΣDrins had a TMF of 0.96—indicating that there is only a very slight dilution effect, bordering on no bioaccumulation. All the other compound classes had TMFs < 1 indicating there is trophic dilution of the compounds through the food web.

Typically TMFs are linked to the octanol-water coefficient (K_{ow}); the greater the K_{ow} the greater the potential for the compound to biomagnify (Walters *et al.*, 2011). The K_{ow} is constant in different environments and acts as a main controller of biomagnification of persistent compounds in aquatic food webs (Kelly *et al.*, 2007; Walters *et al.*, 2011). Organic pollutants that have a K_{ow} <5 have a low biomagnification potential, while those with a K_{ow} between 5 and 7 have the greatest potential to biomagnify. The DDx have a K_{ow} of 6.9 (Walters *et al.*, 2016), which aids in it bioaccumulation potential. The drins, HCB, and most of the chlors, with the exception of heptachlor-epoxide, had K_{ow} > 5. Yet these compounds did not biomagnify. The "borderline" magnification of the drins may have been assisted by the high K_{ow} in this compound class, which were 5.4–6.5. The HCHs had K_{ow} < 5, and the lowest TMFs were found from this class.

Within the Vaal River system, every compound class biomagnified at Thabela Thabeng, at Barrage all but HCB, and at Vischgat all but the chlors, HCHs, and PBDEs biomagnified. As stated above, DDx and chlors have K_{ow} values >5. The PCBs, PBDEs, and HCB also have K_{ow} values >5. All of which can explain the biomagnification of these compounds. The HCHs are the only compounds analysed in this region that have K_{ow} values <5, despite the low K_{ow}, values these compounds were still able to biomagnify in the food webs at Thabela Thabeng and Barrage.

Bioaccumulation in food webs have been noted from other areas in Africa as well. In the Olifants River there was trophic transfer of PCBs, DDx, and HCHs in the food web (Verhaert *et al.*, 2017), like in the Vaal River, HCHs biomagnified although they have a lower K_{ow} value. In Lake Koka and Lake Awassa, Ethiopia, and Lake Malawi, East Africa, DDx was also found to bioaccumulate in the food web (Kidd *et al.*, 2001; Deribe *et al.*, 2011; Yohannes *et al.*, 2013). Increasing POP concentrations were found in higher trophic species from Lake Chad, central Africa—indicating bioaccumulation in this food web (Kidd *et al.*, 2004). Bioaccumulation of PCBs, DDx, PBDEs, HCB, and HCHs were found in the food web of the Congo River Basin in

the Democratic Republic of the Congo (Verhaert *et al.*, 2013). Groffen *et al.* (2018) conducted a study at the same sites and times as the Vaal study, and concluded that perfluorinated alkyl substances were bioaccumulating in the food web.

5.5 Conclusion

Trophic positions of the species collected from the Vaal and Phongola Rivers were assessed. Within the Phongola system *H. vittatus*, *A. bicolor*, and *S. zambezensis* occupied the highest trophic position of the studied food web. In the Vaal River, the target species all occupied the same trophic positions at the sites, however the positions differed between sites. When comparing the $\delta^{15}N$ values between the systems, and to other studies in Africa, it was evident that the Vaal system was highly enriched with $\delta^{15}N$, particularly at the more downstream sites—Barrage and Thabela Thabeng. It is likely that this enrichment is caused from anthropogenic impacts in the area such as industrial effluents, urbanisation, and sewage leaks, all of which have been widely studied in the Vaal River system.

Within the Phongola system ΣDDx and ΣOCPs bioaccumulated in the food web. In the Vaal River, all the compound classes bioaccumulated in the food web at the Thabela Thabeng site, while all but HCB bioaccumulated at Barrage. The food web at Vischgat showed the least amount of bioaccumulation of the analysed compounds. The TMFs typically associated with the K_{ow} of the compounds and the trophic position of the sampled organisms. The most at risk regions are Thabela Thabeng and Barrage.

6 Conclusions and recommendations

Organisms from the Vaal River were analysed for the industrially relevant persistent organic pollutants (POPs): polychlorinated biphenyls (PCBs) and polybrominated diphenylethers (PBDEs); as well as organochlorine pesticides (OCPs). The concentrations of all the pollutants were greatest at the Barrage site compared to the other sites, Thabela Thabeng and Vischgat, and often this difference was significantly so. This was the expected outcome, as stated in the hypothesis. The concentrations of ΣPCBs and ΣPBDEs in fish from the Barrage remained similar to *L. capensis* sampled from the same site in 2009 (Wepener *et al.*, 2011), five years before the current study. The concentrations of the OCPs were lower in the current study than those sampled in 2009. The OCP, dichlorodiphenyltrichloroethane (DDT) was lower in the current study, although not drastically. It is evident that the presence of PCBs, and DDTs are persisting in this river system, from old sources in the case of PCBs and DDTs because these have been banned by the Stockholm Convention for many years and the DDTs were mainly metabolites indicating historical use. In contrast to this, the PBDEs were listed on the Stockholm Convention after this study was conducted (Stockholm Convention, 2017), and the fairly similar concentrations to those in 2009 (Wepener *et al.*, 2011) were likely from active use. It was hypothesised that the industrially related xenobiotics, PCBs and PDBEs would be high, and this was observed. Although it was not expected that the concentrations of DDx would be the second greatest contributor to the ΣOCPs, second to the PCBs.

The exact same compounds were not targeted for in the Phongola Floodplain because the industrially associated pollutants (PCBs and PBDEs) were not suspected to pose a threat in this region since it is predominantly agricultural. When comparing the common compound classes between the Vaal River and the Phongola Floodplain, many of the concentrations were comparable, with the exception of *C. quadricarinatus* and *A. bicolor* that had exceedingly greater OCP concentrations than any of the other species in the Phongola Floodplain system. The ΣDDTs at Thabela Thabeng and Vischgat were similar to most of the fish from the Phongola system, while the concentrations at the Barrage site were similar to the concentrations in *S. zambezensis* that were sampled in the high flow period. This is one of the high trophic position organisms in the Phongola system and it is therefore expected to have high toxicant burdens. Additionally, there DDT is still being sprayed, as a malaria vector control method in northern KwaZulu-Natal, and its neighbouring country, Mozambique, that occurs in December and March annually (Brooke *et al.*, 2013). It is therefore unexpected that there would be such similar concentrations in the Vaal River, where DDT is not permitted for use.

The concentrations of OCPs in the Phongola Floodplain were generally greater in the April (high flow period). It was hypothesised the ΣDDx would be high in the species sampled in the Phongola Floodplain, however, the concentrations of ΣDDTs in *H. vittatus* sampled in 2009 from the Pongolapoort Dam (Wepener *et al.*, 2012) were much higher than the concentrations of ΣDDTs in any of the species sampled from Phongola Floodplain in the current study. The concentrations of ΣHCHs in *H. vittatus* between the two studies were comparable, however most of the other species in the current study had concentrations up to two orders of magnitude greater, and these were in lower trophic level organisms. This is unusual considering these compounds typically bioaccumulate. The concentration of Σchlors were typically greater in all the species in the current study than those in the *H. vittatus* sampled in 2009. HCB was generally not detected in the current study, and when it was detected it was lower than those quantified in the (Wepener *et al.*, 2012) study. The concentrations in the current study were similar to slightly lower than those reported by (Wolmarans, 2015) in frogs sampled during the same period as the current study. This might indicate that there has been a shift in pesticide use in the Phongola area.

Within the Vaal system the fish sampled from the Barrage site were in the poorest condition based on biomarker responses assessed with the integrated biomarker response (IBR), and this was hypothesised. The responses were attributed to the presence of the industrially associated POPs (PCBs, and PBDEs) and DDTs. The responses at Thabela Thabeng were predominantly explained by the environmental parameters, while the fish at Vischgat were in the best condition based on the responses from the biomarkers, the responses at this site were driven by the presence of HCHs, chlordanes, and HCBs—this indicates a more agricultural impact. Although these sites are relatively close to each other the organisms are clearly impacted by different stressors at the different sections of the river. This is likely an effect of the tributaries that enter the Vaal River, bringing with them very different stressors from the areas these rivers flow though. The results of the stable isotope analyses also indicated that the three site are impacted differently, based on the $\delta^{15}N$ values of common species between systems. The species from the Barrage and Thabela Thabeng sites had much higher $\delta^{15}N$ vales than those from the Vischgat site. This is explained by nitrogen enrichment from allochthonous inputs such as sewage, which has been reported to enter the Vaal River (Jordaan and Bezuidenhout, 2013). Although the species sampled from the various sites all were of a similar trophic position a biomagnification factor was still able to be calculated and all of the compounds analysed in the Vaal River were capable of biomagnifying in the food web, at at least one of the sites.

The biomarker responses from the Phongola Floodplain were influenced greatly by the environmental stressors rather than the presence of the POPs—indicating that the seasonal fluctuations are the stressors. The organisms were typically in a poorer state in the April (high flow) period compared to the September (low flow) period based on the IBR, although the differences between seasons were small. This was in accordance with the hypothesis that species would be in a poorer state in the April period. The stable isotope analyses revealed that the only common species between the Vaal River and the Phongola Floodplain, *C. gariepinus*, had similar concentrations only at the Viscghat site, the $\delta^{15}N$ values from the other Vaal River sites were much higher—indicating these regions have similar nitrogen sources, or fewer anthropogenic impacts. The only OCP class that bioaccumulated in the Phongola floodplain was the DDTs.

The study indicates that the Phongola Floodplain is far less impacted than the Vaal River system—based on the concentrations of pollutants, the severity of the biomarker responses, and the causative agents responsible for the responses. Because of the clear bioaccumulation of these compounds through all the food webs, higher trophic organisms are at risk of the various detrimental effects that these compounds cause. Many people subsistence fish from these systems, which puts them at risk due to the pollutants present within the fish. The most at risk regions are Thabela Thabeng and Barrage.

For future studies in the Vaal River, it is recommended that studies focus on the most impacted sites that were determined in this study—the Barrage and Thabela Thabeng. There should be a greater number of individuals sampled, and a wider range of species to include species from more diverse trophic levels. This will allow for a more complete food web to be created, and to better assess the biomagnification potential of the toxicants in the food web. It would be beneficial to include other compounds into the analyses, such as PAHs and their metabolites, and metals. These compounds are associated with industrial activities and have been detected in the Vaal River and its tributaries in previous studies (Wepener *et al.*, 2011; Pheiffer *et al.*, 2018a; Pheiffer *et al.*, 2018b). It would also be worthwhile analysing the sediment for these compounds to use as a basal source in the food web, because sediment is a sink for these compounds (Barakat *et al.*, 2002; Kilemade *et al.*, 2004). It would be interesting to determine whether seasonal fluctuations impact the Vaal River as was evident with the Phongola Floodplain.

Within the Phongola Floodplain it would be recommended that a wider range of compounds are analysed in future studies, in both sediment and organisms. This should include metals, PCBs and PBDEs because these have been found in the system in previous studies (Wepener

et al., 2012; Tate, 2014). Monitoring the species in this system is crucial considering the floodplain forms part of the Ndumo Game reserve, a Ramsar site. As mentioned above, many people subsistence fish in both the Vaal and Phongola Rivers, it would be recommended that human health risk assessments are conducted on both system's fish.

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