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The determination of dioxin-like POPs in sediments and fish of the Vaal Triangle region, Gauteng, South Africa.

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Abbreviations and Acronyms

A	
ADD	Average daily dose
AHH	Aryl hydrocarbon hydroxylase
AhR	Aryl hydrocarbon receptor
Arnt	AhR nuclear translocator
ASE	Accelerated solvent extractor
AT	Averaging time
AWER	Aquatic, Watershed and Earth Resources
B	
BC	Blank control
BM	Body mass
BPA	Bisphenol A
C	
CF	Condition factor
ClB	Chlorinated biphenyls
ClDD	Chlorinated dibenzo-*para* -dioxins
ClDF	Chlorinated dibenzofurans
C_M	Average concentration
CCMS	Committee on Challenges of Modern Society
CR	Cancer risk
CT	Cytotoxicity
CV	Coefficient of variation
CYP 1A1/1A2	Cytochrome P450 A/ 1A2
D	
DCM	Dichloromethane
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethene
DDT	Dichlorodiphenyltrichloroethane
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DRE	Dioxin responsive elements
DWAF	Department of Water Affairs and Forestry
E	
EC20, EC50, EC80	Effective concentration causing 20%, 50% or 80% response
ED	Exposure duration
EDCs	Endocrine disrupting chemicals

F	
f	Correction factor
FBS	Foetal bovine serum
FHAI	Fish Health Assessment Index
FOW	Fishing Owl's World
G	
GC	Gas chromatography
H	
HEPES	[4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer
HPLC	High performance liquid chromatography
HSP	Heat shock proteins
HpCDD/F	Heptachlorodibenzo-*para*-dioxins/furans
HxCB/DD/DF	Hexachlorobiphenyls/dibenzo-*para*-dioxins/dibenzofurans
I	
IR_M	Average intake rate
I-TEF	International toxic equivalency factor
K	
K_{OW}	Octanol/water partition coefficient
L	
LADD	Lifetime average daily dose
LC	Lipid content
LOD	Limit of detection
LT	Expected lifetime
M	
MFO	Mixed function oxidase
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MTT	3-[4,5-dimethyltiazol-2yl]-2,5-diphenyl tetrazolium bromide
N	
NADH	Nicotinamide-adenine dinucleotide
NADPH	Nicotinamide-adenine dinucleotide phosphate
NATO	North Atlantic Treaty Organization
NILU	Norwegian Institute for Air Research
NIR	Near-infrared
NRF	National Research Foundation
O	
OCDD/F	Octachlorodibenzo-*para*-dioxins/furans
P	
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PCBs	Polychlorinated biphenyls

PCDD	Polychlorinated dibenzo-*para* -dioxins
PCDF	Polychlorinated dibenzofurans
PeCB/DD/DF	Pentachlorobiphenyls/dibenzo-*para*-dioxins/dibenzofurans
POPs	Persistent organic pollutants
psi	Pound per square inch
R	
R^2	Correlation coefficient
REP	Relative potency
RLU	Relative light/luminescence units
S	
SC	Solvent control
SCPOPs	Stockholm Convention on Persistent Organic Pollutants
Sf	Slope factor
Stdev	Standard deviation
T	
TCDD/F	2,3,7,8-tetrachloro dibenzo-*para*-dioxin/furans
TeCB	Tetrachlorobenzenes
TEF	Toxic equivalency factors
TEQ	Toxic equivalent quotient
TLTC	Too low to calculate
TOC	Total organic carbon
U	
UNEP	United Nations Environment Programme
USA	United States of America
US EPA	United States Environmental Protection Agency
UV	Ultra violet
W	
WHO	World Health Organization
WRC	Water Research Commission

Abstract

The determination of dioxin-like POPs in sediments and fish of the Vaal Triangle region, Gauteng, South Africa.

Water resources in South Africa are scarce, and should therefore be protected against pollutants, also from persistent organic pollutants (POPs). This is emphasised by the Stockholm Convention on POPs, which aims at reducing and ultimately eliminating POPs. South Africa signed and ratified the treaty, and it became international law on 17 May 2004.

POPs are highly stable, toxic, hydrophobic and lipophilic compounds, with the ability to accumulate in biological tissues. Previous research had shown that dioxin-like POPs are present in the aquatic environments of South Africa, with the highest concentrations of these substances measured in industrialised areas of South Africa. The present study aimed at investigating the extent of polychlorinated dibenzo-*para*-dioxin (PCDD), polychlorinated dibenzo-furan (PCDF) and polychlorinated biphenyl (PCB) pollution in the Vaal Triangle, by targeting aquatic sediments and biota.

Sediment samples were collected from the Blesbok Spruit, Taaibos Spruit, Leeu Spruit and Suikerbosrand River, and fish tissue samples were collected from Blesbok Spruit and Suikerbosrand River, to determine bio-accumulation. The samples were extracted with organic solvents, cleaned-up and fractionated. Raw extracts and fractions were analysed with the H4IIE-*luc* reporter gene bio-assay. This bio-assay is a rapid, sensitive and relatively cost-effective method, which measures the effects of dioxin-like compounds on rat hepatoma cells, transfected with firefly luciferase gene. Selected samples were analysed with gas chromatography/mass spectrometry (GC/MS) to confirm results.

Only one site had quantifiable amounts of dioxin-like substances in the sediment, measured to be 52.35 ng/kg [Effective Concentration 50 (EC 50)]. This value exceeds many of the European and USA quality guidelines, proposed for sediments. No dioxin-like substances were found in fish tissues. The absence of PCDD/Fs and PCBs in aquatic sediments and fish tissues from the Vaal Triangle area might be due to the climatic conditions of the area, dilution effects in streams, and degradation of these compounds by UV-radiation and microbial organisms.

Key words: Vaal Triangle region, polychlorinated dibenzo-*para*-dioxins, polychlorinated dibenzo-furans, polychlorinated biphenyls, sediment, fish tissue, H4IIE-*luc* reporter gene bio-assay.

Opsomming

Die bepaling van dioksienagtige POB's in sediment en vis van die Vaaldriehoekstreek, Gauteng, Suid-Afrika.

Waterhulpbronne in Suid-Afrika is skaars, en daarom moet dit beskerm word teen besoedelstowwe, ook teen persisterende organiese besoedelstowwe (POB's). Dit word beklemtoon deur die Stockholmkonvensie vir POB's, wat dit ten doel stel om POB's te verminder, en uiteindelik te elimineer. Suid-Afrika het die verdrag onderteken en bekragtig, en op 17 Mei 2004 het die Konvensie internasionale wet geword.

POB's is baie stabiele, toksiese verbindings, wat hidrofobies en lipofilies van aard is, en in biologiese weefsel kan akkumuleer. Vorige navorsing het aangetoon dat dioksienagtige POB's in die akwatiese omgewings in Suid-Afrika teenwoordig is, en die hoogste vlakke van hierdie stowwe is in geïndustrialiseerde gebiede van Suid-Afrika gevind. Hierdie studie het die omvang van poli-gechloreerde dibenso-*para*-dioksien- (PCDD), poli-gechloreerde dibensofuraan- (PCDF) en poli-gechloreerde bifeniel- (PCB) besoedeling, in die Vaaldriehoek ondersoek, deur te fokus op akwatiese sediment en biota.

Sedimentmonsters is uit die Blesbokspruit, Taaibosspruit, Leeuspruit en Suikerbosrandrivier versamel. Visweefselmonsters is uit die Blesbokspruit en Suikerbosrandrivier versamel. Die monsters is met organiese oplosmiddels geëkstraheer, gesuiwer en gefraksioneer. Die rou ekstrakte en die fraksies is met die H4IIE-*luc* biosiftingstoets geanaliseer. Die biosiftingstoets is 'n vinnige, sensitiewe, relatief koste-effektiewe metode om die effekte van dioksienagtige stowwe op rotlewerkankerselle, wat getransfekteer is met die vuurvliegielusiferasegeen, te bepaal. Vier sedimentmonsters is met gas chromatografie/massa spektrometrie (GC/MS) geanaliseer, om die resultate van die biosiftingstoets te bevestig.

Kwantifiseerbare hoeveelhede van dioksienagtige stowwe, van 52.35 ng/kg [Effektiewe Konsentrasie 50 (EK50)], is by slegs een van die sedimentmonsters gemeet. Hierdie waarde is hoër as wat die meerderheid van die kwaliteitsriglyne van die VSA en Europa vir sediment aanbeveel. Geen dioksienagtige stowwe is in visweefsel gemeet nie. Die afwesigheid van PCDD/F's en PCB's in akwatiese sediment en visweefsel kan moontlik toegeskryf word aan die klimaatstoestande van die omgewing, verdunningseffekte van strome, en afbreek van hierdie verbindings as gevolg van UV-bestraling en mikroörganismes.

Sleutelwoorde: Vaaldriehoekstreek, poligechloreerde dibenso-*para*-dioksiene, poligechloreerde dibensofurane, poligechloreerde bifeniele, H4IIE-*luc* biosiftingstoets, sediment, visweefsel.

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Chapter 1. Introduction

1.1. The water crisis in South Africa

South Africa is an arid to semi-arid region, which receives an average rainfall of less than 500 mm annually. This includes areas with high water demands receiving insufficient precipitation (O'Keefe, Uys & Bruton, 1994).

It is expected that the water demand of South Africa will exceed the water supply by 2040 (DWAF, 1986). It is not only the quantity of water resources that is important; the quality of water is of equal significance. Anthropological practices generating pollutants have an immense impact on water quality. It is therefore necessary that these limited resources are protected against pollutants, or else it may possibly bring about a severe water crisis in South Africa.

1.2. The Stockholm Convention on Persistent Organic Pollutants (SCPOPs)

Especially one class of pollutants, persistent organic pollutants (POPs), has aroused global concern, since these pollutants are dispersed world-wide and it even affects areas where it has never been produced. These pollutants are a huge challenge to deal with, because they are persistent in the environment, bio-accumulative and they have adverse toxic effects (UNEP, 2005).

To take action against these problematic pollutants, the United Nations Environment Programme (UNEP) initiated the Stockholm Convention on Persistent Organic Pollutants (SCPOPs) in May 1995. This Convention is an international, legally-binding treaty intended to reduce and eliminate the twelve most harmful POPs (UNEP, 2001). These twelve POPs, also known as the "dirty dozen", include the chlorinated pesticides (aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, mirex and toxaphene), two groups of industrial chemicals known as hexachlorobenzene and polychlorinated biphenyls (PCB), and unintentional combustion by-products generally known as polychlorinated dibenzo-*para*-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) (UNEP, 2005).

Almost all of these pollutants have been banned in most countries of the world, although use of DDT still occurs in developing countries, among them South Africa, for the control of pests. There are, however, many sites, which are already contaminated with these chemicals that continue to release POPs into the environment (UNEP, 2005).

The SCPOPs is best understood as having five essential objectives or aims.

- The first aim of the Convention is to terminate the release and use of the twelve most toxic POPs. The Convention bans and limits the production and use of the intentionally produced POPs and it aims at reducing releases of the unintentionally produced POPs, which are formed as by-products of combustion and industrial processes (UNEP, 2005).

- Secondly, the Convention supports the replacement of harmful POPs with safer, cost-effective alternatives. This process may pose a huge challenge to developing countries, as they may lack the financial and technological resources to use and manufacture less threatening chemicals and develop new techniques. The Convention calls on developed nations to share their knowledge and lend financial support to developing countries in aiding their transition to more suitable alternatives (UNEP, 2001).

- In addition to the twelve POPs listed in Section 1.2., there might be other POPs harming human health and the environment. The third aim of the Stockholm Convention is to identify these additional POPs and to aim at the reduction and elimination of these substances (UNEP, 2005).

- Fourthly, the Convention aims at cleaning up stockpiles and equipment containing POPs. Stockpiles and waste sites should be identified and managed in an environmentally safe manner (UNEP, 2005).

- The fifth aim of the Convention is to increase public awareness and provide information regarding these pollutants through educational programmes and other national action plans. The Convention will only succeed if everyone participates and works together to a future without POPs. The Convention calls on industries, public interest groups, politicians and scientists to work together to establish a global partnership as a component of the Stockholm Convention on POPs (UNEP, 2005).

South Africa played a major role in the negotiations and implementation of the Stockholm Convention. The final text of the Convention was successfully negotiated in Johannesburg in December 2000. On 22 to 23 May 2001 the world's governments held a conference in Stockholm, Sweden and adopted the SCPOPs. South Africa signed and ratified the treaty on 4 September 2002, and the Convention entered into force, becoming international law on 17 May 2004 (Bouwman, 2004).

As a party of the Stockholm Convention, South Africa is legally obligated to abide by the objectives of the Convention. In undertaking this research, we are fulfilling a part of our responsibility to the Convention, in providing a better understanding of the unintentionally produced POPs, since very little research has been done in South Africa, regarding these pollutants.

1.3. Persistent organic pollutants

As the name implies, POPs are highly stable compounds, which persist in the environment, by resisting photolytic, biological and chemical degradation. Because these toxic pollutants are lipophilic and cannot be broken down, it leads to the accumulation of these substances in the environment and in tissues. This means that even though these pollutants are initially distributed in small amounts, it gradually builds up in every organism of a food chain, posing significant threats to organisms at the top of long food chains (Schecter, Birnbaum, Ryan & Constable, 2006).

Furthermore, adding to the problems these pollutants pose, they are capable of long-range transport allowing the pollutants to become widely distributed geographically, even to regions where they have never been used or produced, therefore endangering environments and people all over the world (Ritter, Solomon & Forget, 2005).

Although some research has been done in South Africa on the pesticide POPs, such as DDT (Bouwman, Sereda & Meinhardt, 2006), and the intentionally produced PCBs, there is still very much to learn about the unintentionally produced dioxin-like POPs, namely PCDDs, PCDFs and PCBs.

In the past, PCBs were deliberately produced for industrial purposes, but because this pollutant is dangerous to animals and humans, its production has been banned globally since the mid-1980s (Ritter *et al.*, 2005). However, the emission of PCBs to the environment is still a problem today, because co-planar PCBs are produced unintentionally along with PCDD/Fs. This study will focus on these lesser-researched, unintentionally produced POPs and the remainder of this literature review will be directed towards PCDD/Fs and PCBs.

1.4. Aims of the project

A previous study conducted by Vosloo & Bouwman (2005) indicated that PCDD, PCDF and PCBs are present in selected aquatic environments throughout South Africa. Of the 22 aquatic sites they had chosen for the study, the highest levels of PCDD/F and PCB were measured in the Vaal Triangle region, Gauteng. It is important to determine the extent of dioxin-like pollution in the Vaal Triangle area, since the rivers of this region drain into the Vaal Dam (27°00' S, 28°19' E), which provides potable water for the region. This means that a large number of people may be exposed to dioxin-contaminated water, and some even to contaminated fish. The aim of the study was to do a more comprehensive investigation of dioxin-like persistent organic pollution in the Vaal Triangle area.

The objectives of this research project were:

- To gain a better understanding of dioxin-like pollution in the aquatic environment of the Vaal Triangle region by determining the presence of these pollutants in sediment and fish tissue.
- To quantify the amount of PCDD/Fs and PCBs in sediment and fish tissue by calculating 2,3,7,8-tetrachloro dibenzo-*para*-dioxin equivalents (TCDD-equivalents) using the H4IIE-*luc* reporter gene bio-assay.
- To determine bio-accumulation of PCDD/Fs and PCBs in biota by comparing the quantities of dioxin-like pollutants in sediment and fish tissue to one another.
- To estimate the probable human cancer risk associated with the consumption of dioxin-contaminated fish, by making use of a scenario-based risk assessment.
- To compare TCDD-equivalent values, obtained with the H4IIE-*luc* bio-assay, with results obtained from chemical analysis, as an additional measure to confirm the levels of dioxin-like substances measured with bio-analysis.

Chapter 2. Literature review

2.1. The Vaal Triangle area – An overview

The aim of the study was to investigate dioxin-like pollution in the Vaal Triangle area, one of the largest industrialised areas in South Africa. The Vaal Triangle is represented by a triangular area of land formed by Vereeniging (26°39' S, 27°58' E) and Vanderbijlpark (26°41' S, 27°49' E), located in Gauteng, and Sasolburg (26°49' S, 27°50' E), located in the northern Free State. Together these three cities form an extensive urbanised complex, located in the interior high-plateau of South Africa, approximately 1580 m above sea level (Fig. 1).

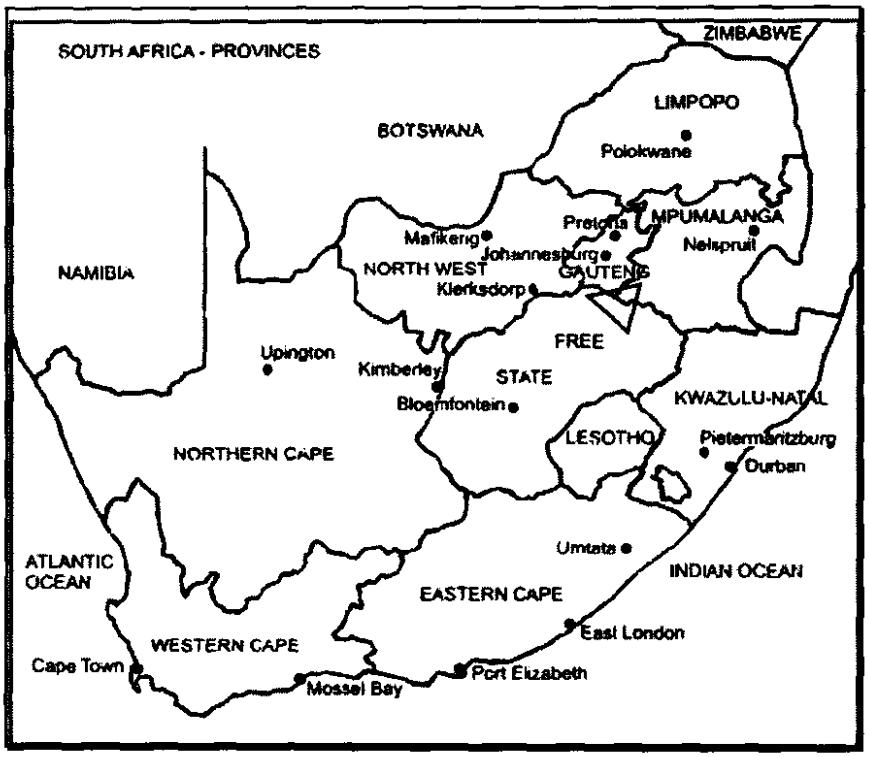

Figure 1. A map of southern Africa indicating the approximate location of the Vaal Triangle area (adapted from Southern Africa Places, 2006).

Because the occurrence and distribution of PCDD/Fs and PCBs is influenced by factors such as temperature, rainfall and dominating wind direction, it is important to take the climate of the area into consideration: The Vaal Triangle has warm summers with temperatures reaching 30 – 35 °C, and it receives summer rainfall (October to April) of roughly 683 mm, annually (South African Weather Service, 2006).

The winters are dry and cold with frost occurring in June and July, but the days are generally sunny. North-westerly and north-easterly winds frequently blow due to anti-cyclonic circulation (South African Weather Service, 2006).

This industrialised area, as we know it today, had its origin in 1878 when coal was discovered in the beds of the Vaal River. Coal and clay processing works were the first industries to arise in this area in 1882. Vereeniging had all the essentials for an industrial explosion when a power station was built in 1912: water supply from the Vaal River and electric power from coal combustion (Leigh, 1968).

As a number of steel corporations were established, the industrial growth of the area required more power and water supply. A further four power stations were built from 1936 to 1959, and the construction of the Vaal Dam was completed in 1938 (Leigh, 1968). The demand on steel production increased beyond the capacity of the steel works, during the Second World War. This required the establishment of giant steelworks together with housing and recreational facilities for workers, leading to the development of Vanderbijlpark in 1949 (Nieuwoudt, 1983). The Vaal Triangle was growing rapidly and the founding of an oil and gas corporation, which converted low grade coal into oil, further expanded the industry, giving rise to Sasolburg in 1954 (Schmitt, 1979). Thus, the Vaal Triangle area has been subjected to industrial pollution since the 1800's.

Since then, the area had become increasingly industrialised and today the Vaal Triangle is home to a number of large corporations and manufacturers, some of them possibly contributing to the production of large amounts of dioxin-like pollutants.

2.2. Dioxin-like persistent organic pollutants: PCDD/Fs and PCBs

2.2.1. Physico-chemical properties of PCDD/Fs and PCBs

PCDDs and PCDFs are two groups of planar tricyclic compounds with similar chemical structures and properties (Fig. 2.1 a and b). They consist of 12 carbon atoms, forming two aromatic phenyl rings, attached to one another by two oxygen bonds in dioxins and by one oxygen bond and one carbon-carbon bond in furans. Both PCDD and PCDF may contain between one and eight chlorine atoms in the hydrogen atom position (Schecter *et al.*, 2006).

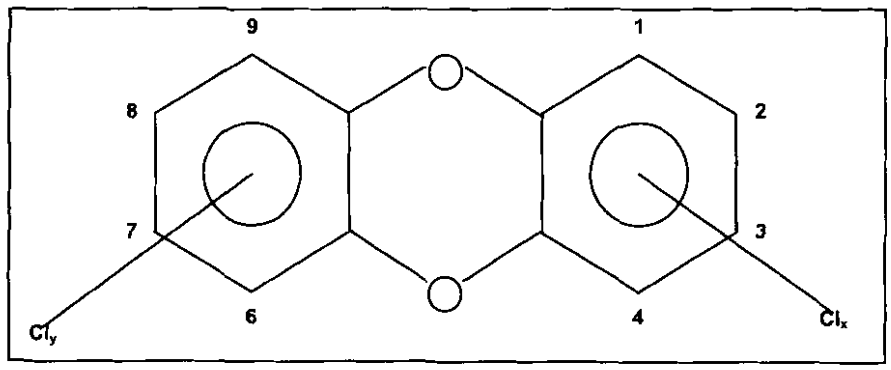

Figure 2.1.a. Structural formula of chlorinated dioxins

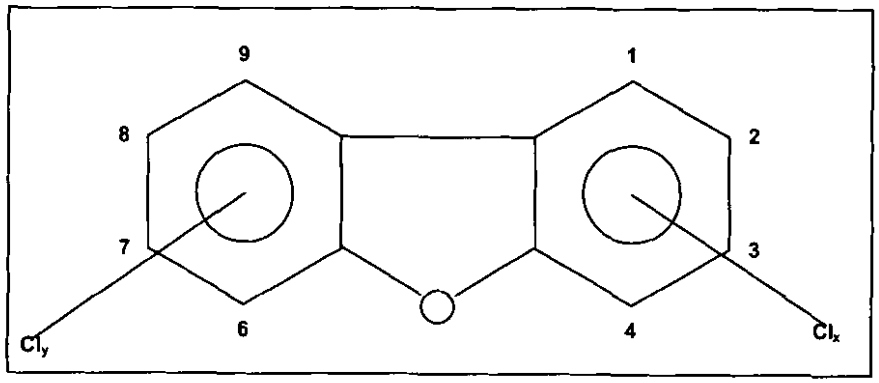

Figure 2.1.b. Structural formula of chlorinated furans

PCBs are aromatic compounds formed by two benzene rings bonded by a single carbon-carbon bond. The two benzene rings can rotate along the carbon-carbon bridge axis and they are therefore flexible in the sense that they can assume a planar conformation similar to PCDDs or a propeller-like conformation. The hydrogen atoms on the biphenyl molecule may be replaced by up to ten chlorine atoms (Fig. 2.1 c) (Schecter *et al.*, 2006).

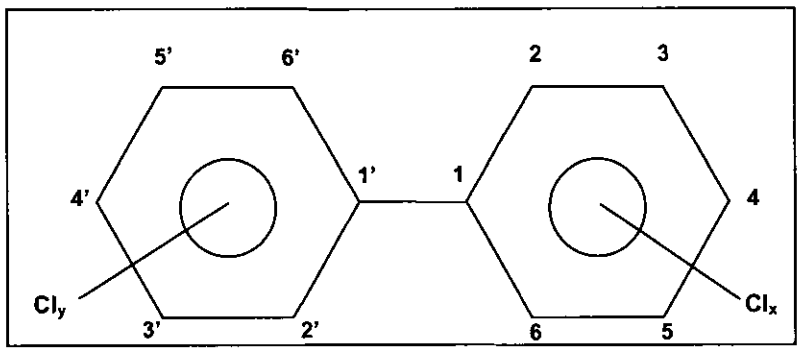

Figure 2.1.c. Structural formula of polychlorinated biphenyls

Theoretically, there are 75, 135 and 209 possible congeners for PCDD, PCDF and PCBs, respectively, but only 7 PCDD, 10 PCDF and 12 PCB congeners are of toxicological interest (Table 2.1) (WHO, 1997).

The toxicity of these compounds, as well as their physico-chemical properties (Table 2.2) are determined by the presence and structural position of chlorine atoms on the molecules. Chlorine atoms in the 2, 3, 7 and 8 positions, better known as lateral chlorines, are essential for toxicity, lipophilicity and prevention of destruction of dioxin-like chemicals, making congeners with these chlorines very persistent in the environment (Carey, Cook, Giesy, Hodson, Muir, Owens & Solomon, 1998).

Table 2.1. The IUPAC names of the most toxic PCDD/F and PCB congeners, according to the WHO (1997).

Toxic PCDD congeners	Toxic PCDF congeners	Toxic PCB congeners
2,3,7,8 –Cl_4DD	2,3,7,8-Cl_4DF	3,3',4,4'-Cl_4B
1,2,3,7,8-Cl_5DD	1,2,3,7,8-Cl_5DF	3,4,4',5-Cl_4B
1,2,3,4,7,8-Cl_6DD	2,3,4,7,8-Cl_5DF	3,3'4,4',5,5'-Cl_5B
1,2,3,4,6,7,8-Cl_6DD	1,2,3,4,7,8-Cl_6DF	3,3',4,4',5,5'-Cl_6B
1,2,3,7,8,9-Cl_6DD	1,2,3,6,7,8-Cl_6DF	2,3,3',4,4'-Cl_5B
1,2,3,4,6,7,8-Cl_7DD	1,2,3,7,8,9-Cl_6DF	2,3,4,4',5-Cl_5B
Cl_8DD	2,3,4,6,7,8-Cl_6DF	2,3',4,4',5-Cl_5B
	1,2,3,4,6,7,8-Cl_7DF	2',3,4,4',5-Cl_5B
	1,2,3,4,7,8,9-Cl_7DF	2,3,3',4,4',5-Cl_6B
	Cl_8DF	2,3,3',4,4',5'-Cl_6B
		2,3',4,4',5,5'-Cl_6B
		2,3,3',4,4',5,5'-Cl_7B

Table 2.2. The physico-chemical properties of PCDD/Fs and PCBs (adapted from Whylie, Albaiges, Barra, Bouwman, Dyke, Wania & Wong, 2003)

Physico-chemical property	PCDD/Fs	PCBs
Water solubility at 25 °C	0.43 – 0.0002 ng/L	0.01 – 0.0001 µg/L
Vapour pressure at 20 °C	2 – 0.007 x 10^{-6} mmHg	2.1 x 10^{-4} – 4 x 10^{-3} Pa
Lipophilicity (log K_{ow})	6.60 – 8.20	4.3 – 8.26
Half life in soil/sediment	10 – 12 years	> 6 years

2.2.2. Sources of PCDD/Fs and PCBs

In order to comply with the Stockholm Convention's objective to reduce the releases of unintentionally produced POPs, the sources of PCDD/F and PCB release must be identified before they can be targeted. As stated earlier, PCDD/Fs are produced unintentionally and they have never been produced deliberately for any other purpose than scientific research. This brings about difficulties in controlling the release of these substances (Schecter *et al.*, 2006).

PCBs were manufactured from the early 1930s for industrial purposes, but their production and use were banned globally in the 1980s, because of the toxicity of these chemicals. PCBs were valuable for industrial purposes, because they are chemically stable, resistant to heat, non-flammable and they have low vapour pressures and high dielectric constants (Ritter *et al.*, 2005). These characteristics made PCBs excellent as insulating materials in electrical equipment, plasticisers (softening materials) in plastic products, hydraulic fluids, adhesives, lubricants, fire retardants and dielectrics in transformers (Koppe & Keys, 2001). Although the production use of PCBs have been banned, they are still released into the environment via accidental spillages, fires and volatilisation from old stockpiles. Co-planar PCBs are also formed unintentionally, in the same way PCDDs and PCDFs are produced (Ritter *et al.*, 2005).

PCDD/Fs and co-planar PCBs are produced as by-products of industrial and thermal processes under the optimal conditions of carbon, oxygen and chlorine availability in the presence of metal catalysts at temperatures ranging from 400 to 700 °C. These optimal conditions commonly occur during incineration processes (Fiedler, Lau, Kjeller & Rappe, 1996).

In developing countries, where regulations have not been established against all of the potential sources of PCDD/Fs and co-planar PCBs, mixed waste incineration is the largest contributor of PCDD/F and PCB production. Major sources of PCDD/F and PCB releases in these countries include the combustion processes of municipal, hazardous and medical waste (Ritter *et al.*, 2005).

In developed countries, such as the United States, regulations have been established to minimise incineration processes and other processes producing POPs. The United States Environmental Protection Agency (US EPA) determined the main sources of PCDD/F and PCB emissions in the United States from 2002 to 2004. Their results indicated that the emissions of PCDD/Fs and PCBs as a result of backyard trash burning alone were estimated at 56%; greater than that from all the other sources combined. The other 44% included incineration processes, the paper industry, residential and industrial wood burning, vehicle emissions and cigarette smoke. The possible motivation for the high contribution via backyard trash burning might be the absence of control regulations regarding this uncontrolled combustion process (New York State Department of Environmental Conservation, 2004).

Many other activities can lead to the releases of dioxins, furans and co-planar PCBs. The most common of these activities include cement production, ferrous and non-ferrous metal smelting operations, pulp and paper production and fuel combustion. Smaller non-point sources include burning wood in stoves and fireplaces, landfill fires and open burning on the ground. PCDD/Fs and PCBs can also be produced by natural processes such as forest fires and volcanoes (Ritter *et al.*, 2005).

To facilitate and assist countries to create release inventories of PCDD/Fs, UNEP created a publication, Standardized Toolkit for Identification and Quantification of Dioxin and Furan Releases (UNEP, 2003). This Toolkit divides the possible sources of dioxins and furans into ten main categories according to their formation and sources of release, making it an excellent reference for the identification of the PCDD/F sources (Table 2.3). It has been introduced to and is being field-tested by a number of countries receiving assistance from UNEP (UNEP, 2003). The aim of this study was to quantify dioxin-like pollution in the Vaal Triangle area. For this purpose, sampling sites had to be selected near to, or down-stream of, potential emission sources of PCBs and PCDD/Fs. The Standardized Toolkit for Identification and Quantification of Dioxin and Furan Releases (UNEP, 2003) proved to be extremely useful in pin-pointing the possible emission sources of dioxin-like pollutants.

Table 2.3. The main categories of possible PCDD/F sources according to the Standardized Toolkit for the Identification and Quantification of Dioxin and Furan releases (UNEP, 2003).

1. Waste incineration - Municipal solid waste - Hazardous waste incineration - Medical waste incineration - Light-fraction shredder waste incineration - Sewage sludge incineration - Waste wood and waste biomass incineration - Destruction of animal carcasses	**2. Ferrous and non-ferrous metal production** - Iron ore sintering - Coke production - Iron and steel production plants - Aluminium production - Lead production - Zinc production - Brass and bronze production - Magnesium production - Other non-ferrous metal production - Shredders - Thermal wire reclamation
3. Power generation and heating - Fossil fuel power plants - Biomass power plants - Landfill/biogas combustion - Household heating and cooking with biomass - Domestic heating and cooking with fossil fuels	**4. Mineral products** - Cement production - Lime production - Brick production - Glass production - Ceramic production - Asphalt mixing
5. Transport - 4-Stroke engines - 2-Stroke engines - Diesel engines - Heavy oil fired engines	**6. Uncontrolled combustion processes** - Biomass burning - Waste burning and accidental fires
7. Production/use of chemicals and consumer goods - Pulp and paper production - Chemical industry - Petroleum industry - Textile production - Leather refining	**8. Miscellaneous** - Drying of biomass - Crematoria - Smoke houses - Dry cleaning - Tobacco smoking
9. Disposal/landfill - Landfills and waste dumps - Sewage and sewage treatment - Open water dumping - Composting - Waste oil treatment (non-thermal)	**10. Hot spots** - Production sites of chlorinated organics - Production sites of chlorine - Formulation sites of chlorinated phenols - Application sites of chlorinated phenols - Timber manufacture and treatment sites - PCB-filled transformers and capacitors - Dumps of wastes/residues from 1 – 9 - Sites of relevant accidents - Dredging of sediments - Kaolinitic or ball clay sites

By making use of a website (Vaal Triangle Info, 2005) where 44 major industries of the Vaal Triangle are listed, the most important dioxin-producing industries in this area could be identified (Fig 2.2). Of the 44 industries listed, 33 of them had the ability to produce PCDD/Fs and co-planar PCBs, strengthening the motivation for studying this area (UNEP, 2003).

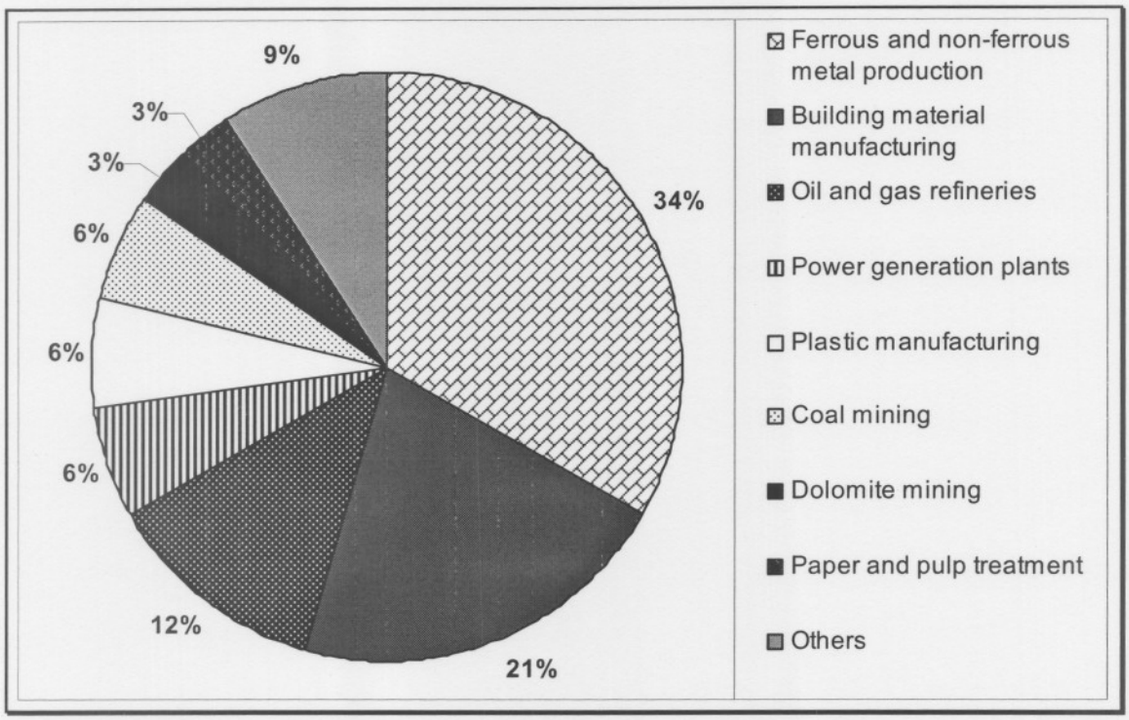

Figure 2.2. The contribution of the major industries in the Vaal Triangle to dioxin-like pollution, classified according to the number of these industries in this area.

Ferrous and non-ferrous metal production appeared to be a large possible contributor of dioxin-like pollution, since there were 11 (34%) industries producing metal products, especially steel goods (Fig 2.2). Seven (21%) of the 33 industries specialise in the production of building materials, such as bricks, cement and ceramic goods: also possibly contributing to dioxin production. Other dioxin-producing industries (listed on the website) include power generation plants, oil- and gas refineries, paper and pulp treatment plants, plastic manufacturers, coal- and dolomite mining (Vaal Triangle Info, 2005).

One should not underestimate the Vaal Triangle's contribution to dioxin-like pollution in South Africa: this area is one of the largest industrial areas in the country and there are many other potential dioxin producing sources that are not listed on the website. These sources include incinerators, crematoria, sewage works, and sources that are more difficult to quantify, such as vehicle emissions and landfills (listed in Table 2.3).

2.2.3. PCDD/F and PCB transport

Once PCDD/Fs and PCBs are formed, they may be released into atmospheric, aquatic or terrestrial compartments, depending on their emission sources. When dioxin-like pollutants are present in one of these compartments, they can cycle between the others, because their physico-chemical characteristics allow them to partition between gaseous, liquid and particle phases (Carey *et al.*, 1998) (Fig 2.3). For example, if dioxin-like pollutants are released into the atmosphere, they may condensate in the clouds and descend to the earth as precipitation. As precipitation falls to the earth, dioxins may end up in water bodies, or it may infiltrate land where it adsorbs onto soil particles. Because PCDD/Fs and co-planar PCBs are semi-volatile, they can evaporate from water bodies or from the earth's surface, allowing the cycle to be repeated once again (UNEP, 2003).

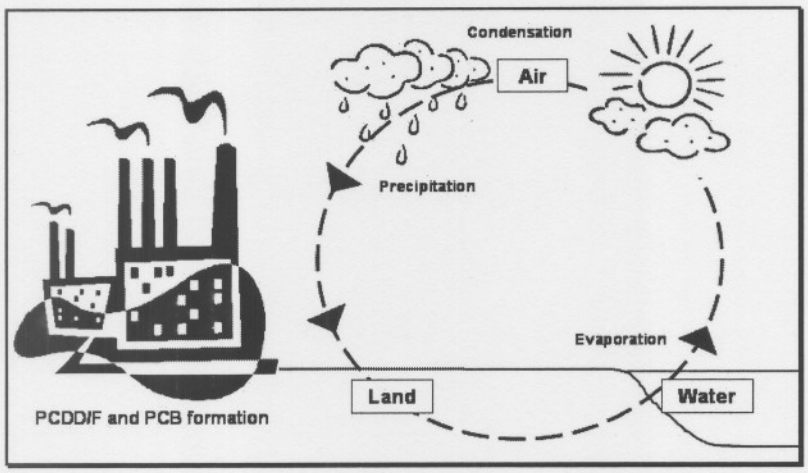

Figure 2.3. A schematic representation of the cycling of PCDD/Fs and PCBs in various environmental compartments.

Because these pollutants are present in many environmental compartments, they can be transported through various routes. These transportation routes may include ocean currents, rivers, strong winds and migratory animals such as birds, whales, dolphins and salmon, as well as anthropogenic practices such as trade (Krümmel, Macdonald, Kimpe, Gregory-Eaves, Demers, Smol, Finney & Blais, 2003).

In aquatic components, oceanic- and riverine transports play important roles in PCDD/F and PCB transportation. Due to the great volumes involved and the massive movements of these systems, they contribute significantly in transferring these pollutants all over the world. Although PCDD/Fs and PCBs are insoluble in water, these substances adsorb onto sediment particles or are taken up by biota, which carry them over long distances (Whylie *et al.*, 2003).

Since PCDD/Fs and co-planar PCBs are semi-volatile, they are capable of long-range transport, leading to the occurrence PCDD/Fs and PCBs in places where they have never been produced (Corsolini, Kannan, Imagawa, Focardi & Giesy, 2002). In Polar regions, for instance, where the production and emission sources of PCDD/Fs and PCBs are limited, significant levels of these substances are found in breast milk of women and in fatty tissues of dolphins and whales. This phenomenon can be explained by the "cold condensation"- or "grasshopper-effects" (Fig. 2.4) (Corsolini *et al.*, 2002).

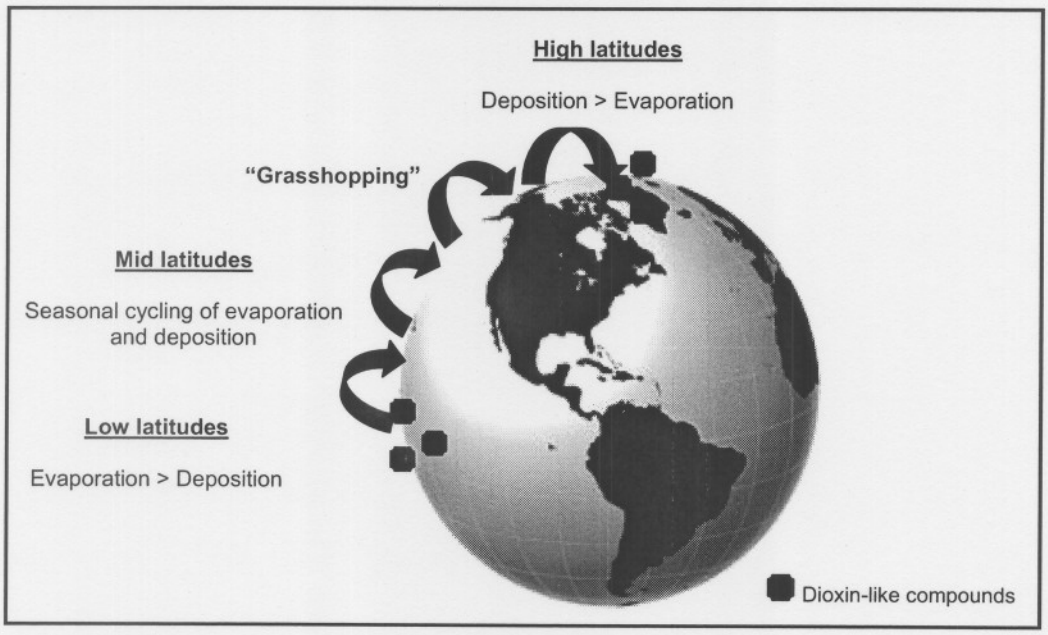

Figure 2.4. A diagrammatic representation illustrating long-range transport of dioxin-like substances, known as the "grasshopper-effect".

PCDD/Fs and PCBs evaporate into the atmosphere with ease at regions with higher temperatures, because of their volatility. Once in the atmosphere, these substances are carried by wind and air currents. When there are alterations in atmospheric conditions, POPs descend to the earth as precipitation and impaction. In this process POPs are transferred further north (Fig 2.4) (Corsolini *et al.*, 2002).

The cycle replicates itself at this point, and in colder Polar regions, POPs fall to the earth as rain or snow. The low temperatures at these locations prevent evaporation, trapping POPs at these regions. This sequence of events may be repeated several times (Corsolini *et al.*, 2002). During the "grasshopper-effect" a part of the pollutant load may be lost through deposition in sediment and absorption into vegetation or algae taking up pollutants (Tysklind, Fängmark, Marklund, Lindskog, Thaning & Rappe, 1993).

2.2.4. Environmental fate of *PCDD/Fs and PCBs*

PCDD/Fs and PCBs are distributed ubiquitously in many environmental compartments (Carey *et al.*, 1998). In terrestrial and aquatic compartments, dioxin-like substances are generally bound to particulate matter, such as soils and aquatic sediments. PCDD/Fs and PCBs can be redistributed from these abiotic compartments to bio-accumulate in lipid-rich tissues of biota and circulate through food chains (Carey *et al.*, 1998).

Dioxin-like substances can be introduced into terrestrial food chains by wet and dry deposition onto soil or plant surfaces, or by diffusion (Gao, Jiang, Wang, Wang & Bian, 2005). The uptake of PCDD/Fs and PCBs in plants via root systems has proved to be insignificant. When grazing animals consume contaminated plants, they are exposed to dioxin-like substances. Because PCDD/Fs and PCBs are attracted to lipids, these pollutants accumulate in the fatty tissues and milk of grazing animals (Fiedler, 2003).

Aquatic food chains are also impacted on by the presence of PCDD/Fs and PCBs. When aquatic biota, such as phytoplankton, collect nutrients, they may be exposed to dioxin-like chemicals present in sediments. Zooplankton and small fish feed on phytoplankton, and in this way any toxic chemicals accumulated by the phytoplankton are further concentrated in the bodies of the animals that eat them (Okumura, Yamashita & Isagawa, 2003). Although contaminants may initially be present in low concentrations, they bio-accumulate in each organism of a food chain and become concentrated. This process of increasing concentration in a food chain is known as bio-magnification (Fiedler, 2003).

The top predators of long food chains, such as lake trout, large salmon and fish-eating gulls, may accumulate concentrations of toxic chemicals high enough to cause serious deformities or even death. By eating fish or other animals exposed to high concentrations of toxic substances, humans may be seriously affected. Humans are mainly exposed to PCDD/Fs and PCBs through their diet. Once food is digested, these substances bind to lipoproteins in blood and are transported to different parts of the body. Dioxin-like compounds have a tendency to bio-accumulate in fatty tissues as well as the liver, bone marrow and cerebral tissue of mammals (Carey *et al.*, 1998).

Although PCDD/Fs and PCBs are persistent in the environment, these substances can be broken down over time by high temperatures, UV-rays and microbial organisms (Brouwer, Longnecker, Birnbaum, Cogliano, Kostyniak, Moore, Schantz & Winneke, 1999). These mechanisms of degradation will be discussed in Chapter 5.

2.2.5. Toxicity of PCDD/Fs and PCBs

2.2.5.1. Toxic effects of PCDD/Fs and PCBs

The Stockholm Convention's objective to minimise and reduce the releases of PCDD/Fs and PCBs is motivated by the adverse toxic effects these pollutants exert on animal and human health. These substances can be lethal in high concentrations but their greatest detrimental influences are in their chronic toxicity, leading to effects such as cancer and a number of non-cancerous effects, including effects on the immune system, reproductive system, nervous system and endocrine system, as well as other health effects (US EPA, 2002a).

A visible dioxin-related health effect, perceived as the hallmark of dioxin exposure, is chloracne (Fig 2.5). Chloracne can be described as the acne-like eruption of blackheads, cysts and pustules as a result of the over-exposure to dioxin-like substances. These lesions are most frequently found on the cheeks, behind the ears (Fig 2.5), in the armpits and in the groin region. This condition is commonly seen in industrial workers, who are chronically exposed to dioxin-like substances (Schecter *et al.*, 2006). The effects of dioxin exposure were recently reported in the media when Viktor Yushchenko, a presidential candidate of Ukraine, was poisoned with 2,3,7,8-tetrachloro dibenzo-*para*-dioxin (TCDD) in September 2004. His appearance changed drastically within weeks, and by November 2004 the effects of TCDD poisoning were clearly visible (Fig 2.5) (Schecter *et al.*, 2006, Ross, 2004).

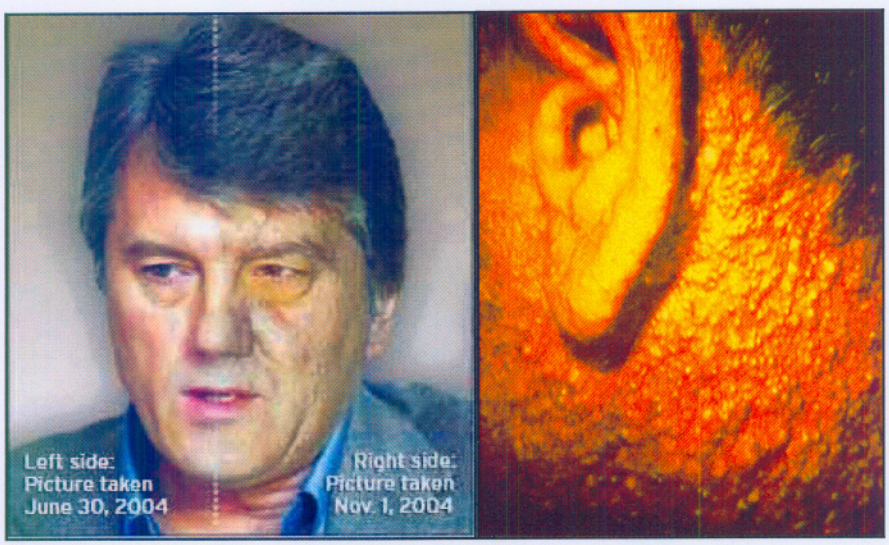

Figure 2.5 Images of chloracne: Viktor Yushchenko's appearance (left) dramatically changed within weeks, after he was poisoned with the most toxic dioxin, 2,3,7,8- TCDD (MSNBC, 2004).

In addition to chloracne, other dermal effects, such as hyperpigmentation and hypertrichosis can also be associated with dioxin exposure. Both hyperpigmentation and hypertrichosis (excessive hair growth) seem to resolve more quickly than chloracne, making it more of an acute than a chronic response (US EPA, 1994a).

A very distressing fact regarding PCDD/F and PCB exposure is that these chemicals may cause cancer (Silbergeld, 1991). The US EPA characterises mixtures of dioxin-like substances as "probable human carcinogens" based on the fact that individual components of these mixtures might have the ability to cause cancer. Especially TCDD, the most toxic dioxin congener, can be identified as a "human carcinogen". According to the US EPA, PCBs have been shown to cause cancer in animals, and it is possible that PCB exposure can be linked to rare liver cancers in humans (US EPA, 2001). Although a number of cancerous effects, such as liver, lung and bladder tumours, have been reported in animals, it is more difficult to prove that dioxin-like substances cause cancer in humans, because cancer can be caused by many factors other than dioxin-exposure (Birnbaum, 1995).

The most prominent effects of dioxin exposure can be seen on the endocrine system, nervous system and immune system. Chemicals with the potential of impeding the function of endocrine systems are called endocrine disrupting chemicals (EDCs). EDCs can be defined as exogenous agents interfering with the production, release, transport, metabolism, binding, action or elimination of natural hormones (Kavlock, Daston, DeRosa, Fenner-Crisp, Gray, Kaattari, Lucier, Luster, Mac, Maczka, Miller, Moore, Rolland, Scott, Sheenan, Sinks & Tilson, 1996). Because dioxin-like chemicals are structurally similar to natural hormones, they can mimic the action of these hormones, affecting a number of natural reactions in various systems. PCDD/Fs and dioxin-like PCBs largely affect the reproduction and growth of animals and humans by exerting their effects on the endocrine system (US EPA, 2002a).

The hormone-like activity of dioxins may alter the levels and activity of reproductive hormones directly, by altering functions of neurotransmitters involved in gonadotropin regulation, or indirectly by altering hormones not immediately acted on by these chemicals. For example, by decreasing the number of oestrogen receptors in cells, the effects of oestrogen on an organism can be reduced (Moore, Potter, Theobald, Robinson & Peterson, 1985).

A study on rats has shown that relatively high doses of TCDD and related chemicals can cause testicular and ovarian degeneration. PCDD/F and PCB exposure has also been linked to other reproductive effects such as the inability to maintain pregnancy, decreased fertility, reduced sperm counts, increased endometriosis, and lowered testosterone levels (Birnbaum, 1995).

Thyroid hormones, which are critical for normal growth and differentiation of cells, also appear to respond to dioxin exposure. By decreasing thyroid hormone levels, dioxin-like substances interfere with normal growth regulation, leading to several developmental deficits in animals and humans. The developmental effects of PCDD/Fs and PCBs are especially evident on the unborn embryo or foetus (US EPA, 2002a).

Many studies have been done on children from Japan and Taiwan, were poisoned with dioxin-contaminated rice oil consumed by their mothers before they were born (Koppe, Pluim, Olie & Van Wijnen, 1991). These children were born 6 – 12 days early on average and had shown decreases in birth weight, discolouration of the skin and nails, and abnormal teeth and gums. Along with these obvious physical differences, many of the children showed neurological and behavioural changes, such as unresponsiveness and lowered short-term memory, when compared to normal, unaffected children (Koppe *et al.*, 1991; Tanabe, 1988).

Proper development of the nervous system is critical for early learning and can have potentially significant implications for the health of individuals throughout their lifetimes. Other effects of PCDD/Fs and PCBs on the nervous system include decreases in visual recognition, learning deficits and changes in brain activity, which may lead to depression and personality changes (Corsolini *et al.*, 2002).

In addition to the neurological effects of PCDD/Fs and PCBs, these substances can have serious effects on the immune system. Studies on Rhesus monkeys, which have immune systems similar to humans and other animals, have revealed that dioxin-like exposure can cause a decrease in the thymus gland, reducing the ability to produce cell-killing T-lymphocytes, which is important for immunity. Since the immune system is critical for fighting infections, diseases of the immune system have very serious potential implications for the health of humans and animals. Individuals with diseases of the immune system may be more susceptible to other infections such as pneumonia and viral infections (Brouwer *et al.*, 1999).

2.2.5.2. Mechanism of toxicity mediated by the Aryl hydrocarbon receptor (AhR)

To induce their effects on various systems, PCDD/Fs and dioxin-like PCBs act through the AhR. The mechanism of dioxin toxicity is mediated by the AhR. The AhR, also known as the dioxin receptor, is a transcription factor, which is a member of the basic helix-loop-helix family of transcription regulators. This receptor is complexed with heat shock proteins and is located in the cytosol of cells (Carey *et al.*, 1998).

The binding of the ligand, in this case the PCDD/F or PCB molecules, to the AhR is the primary step in the action of dioxin-like compounds on cells (Elferink, 2003). Upon ligand binding, conformational changes take place in the AhR, which result in the translocation of the AhR-ligand complex to the nucleus and dissociation of heat shock proteins from the receptor. In the nucleus, the AhR-ligand complex heterodimerises with the AhR nuclear translocator (Arnt) protein, and probably other factors too (Fig 2.6) (Pocar, Fisher, Klonish & Haubach-Klonish, 2005). The binding of the ligand-AhR-Arnt transcriptionally active complex to a specific DNA recognition sequence, the dioxin-responsive element (DRE), results in increased transcription of genes in the AhR gene series such as cytochrome P450A (CYP1A1) and cytochrome P450 1A2 (CYP1A2) (Fig. 2.6) (Fiedler, 2003; Eisen, Hannah, Legraverend, Okey & Nebert, 1983).

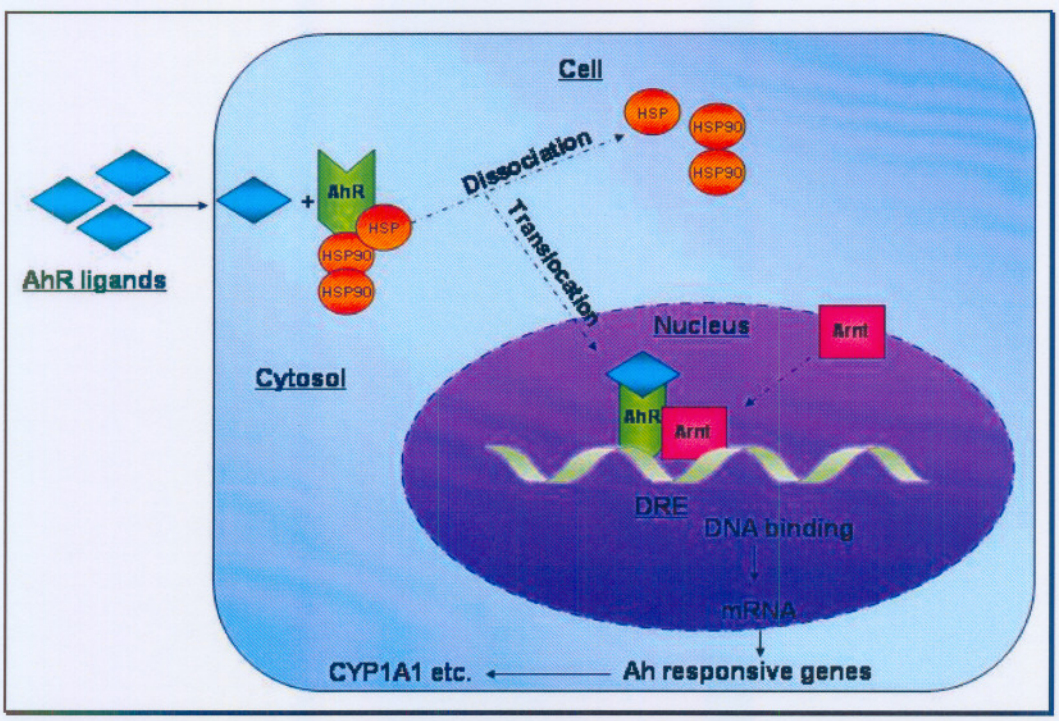

Figure 2.6. A diagrammatic representation of the AhR-mediated response (adapted from Hilscherova, Machala, Kannan, Blankenship & Giesy, 2000). [AhR = aryl hydrocarbon receptor; HSP = heat shock protein; DRE = dioxin response element; Arnt = AhR nuclear translocator].

One of the enzymes in the cytochrome P450 family, known as aryl hydrocarbon hydroxylase (AHH), catalyses a chemical reaction to initiate the breakdown of benzene-rings and similar molecules. Enzyme production is switched off once these molecules are degraded. Thus, the AhR plays an important role in the detoxification of the body. Much of this degrading activity takes place in the liver and degraded substances are eventually converted to water-soluble compounds, which are excreted by the kidneys (Silbergeld & Gasiewicz, 1989).

Thus, if dioxin-like compounds bind to the AhR, the resulting cytochrome P450 enzyme activity attempts to degrade the DNA-attached dioxin. If the dioxin cannot be broken down, the receptor remains bound to the DNA, and P450 is produced continuously. These enzymes do not break down dioxins, but they do degrade hormones and other degradable chemicals to a certain extent, making these intermediate products toxic or carcinogenic. This can explain why TCDD is a potential cancer promoter. Not all dioxin congeners have the same affinity for binding to the AhR. Thus, dioxin-like substances with lower affinities for the AhR fail to elicit the entire spectrum of dioxin-like toxic effects (Silbergeld & Gasiewicz, 1989). Most fish species, birds and mammals possess functional AhR, but it has not been detected in some plants and primitive invertebrate species (Carey *et al.*, 1998).

2.2.5.3. Methods for determining toxic concentrations of PCB and PCDD/F

The earliest method used to detect the presence of TCDD, was a rabbit skin test where test samples were applied to the inner surface of the ears and shaven bellies of albino rabbits. This experiment tested for inflammatory responses produced by TCDD, but it was not sufficiently sensitive to detect low levels of contamination (Adams, Irish, Spencer & Rowe, 1941). Improving on this method, gas chromatography (GC) methods were introduced in the 1970s to quantify dioxins, mainly TCDD. The GC method posed a problem, because the method was not sensitive enough to detect low levels of compounds, and analysis was not isomer specific. This difficulty was overcome by combining gas chromatography with a mass spectrometer. Gas chromatography/mass spectrometry (GC/MS) combinations were used for the development of isomer-specific analysis of PCDD and PCDF in the 1980s (Slonecker, Pyle & Cantrell, 1983).

Presently, high-resolution GC/MS analysis is the most generally applied chemical method used for the detection and quantification of dioxin-like chemicals (Safe, 1995). The accuracy at which chemical methods, such as GC/MS, measure the concentration of each congener, make them excellent methods to detect trace amounts of chemicals.

Unfortunately, this method is very costly and time-consuming, and it does not account for interactions among chemicals, which can alter the toxic potential of mixtures of chemicals (Hilscherova *et al.*, 2000).

Chemical methods merely determine the concentrations of congeners in complex mixtures. In order to predict the potential toxicity of a mixture of compounds on organisms, the toxic equivalency quotient (TEQ) of the mixture should be established. To calculate the TEQ values of each isomer, the concentration of the isomer is multiplied with its toxic equivalency factor (TEF). The TEQ-value of each isomer is then added together to give the total TEQ for the mixture of dioxin-like compounds (Safe, 1995). TEF-values are assigned to PCDD/Fs and PCBs to indicate their toxic potencies relative to the most toxic dioxin congener, 2,3,7,8-tetrachloro dibenzo-*para*-dioxin (2,3,7,8-TCDD), which has a TEF-value of 1 (Table 2.4) (Schecter *et al.*, 2006).

International toxic equivalency factors (I-TEFs) were established for PCDD and PCDF by the Working Group on Dioxins and Related Compounds of NATO/CCMS in the 1980s. I-TEFs are the most generally used TEFs, but these TEFs do not include values for PCB congeners (NATO/CCMS, 1988). However, the European Centre of Environmental Health of the World Health Organization (WHO) and the International Programme on Chemical Safety have initiated a programme to obtain TEFs for PCDD/Fs and PCBs. These values are used to assess the impacts of dioxin-like compounds on humans, fish and birds.

Several different schemes have been developed to determine TEF-values for dioxin-like compounds, including short-term toxicity tests and *in vitro* tests. These tests were used to calculate TCDD equivalent values for each of the dioxin-like isomers for various mammalian, bird and fish species. The average of these TCDD equivalent values was determined for each isomer, and was ultimately expressed as TEF-values. As more data becomes available, TEFs should be revised as they are order of magnitude consensus estimates, which may change over time (Van den Berg, Birnbaum, Bosveld, Brunström, Cook, Feeley, Giesy, Hanberg, Hasegawa, Kennedy, Kubiak, Larsen, Van Leeuwen, Liem, Nolt, Peterson, Poellinger, Safe, Schrenk, Tillitt, Tysklind, Younes, Waern & Zacharewski, 1998).

Since the chemical approach for measuring the toxicity of dioxin-like substances does not take interactions among molecules into account, chemical analysis may under or over estimate the potential toxicity caused by a mixture of chemicals (Slonecker *et al.*, 1983).

Due to the shortcomings in standard chemical methods, biological methods, such as bio-markers, cell- or organ-based bio-assays and protein binding assays, have been introduced during the last decade (Behnisch, Hosoe & Sakai, 2001).

Table 2.4. The TEF-values for PCDD, PCDF and PCB congeners, including I-TEFs and TEFs for mammals, fish and birds (WHO, 1997; Van den Berg *et al.*, 1998)

Congener	I-TEF	TEF for mammals	TEF for fish	TEF for birds
Dioxin congeners				
2,3,7,8 –Cl_4DD	1	1	1	1
1,2,3,7,8-Cl_5DD	0.5	1	1	1
1,2,3,4,7,8-Cl_6DD	0.1	0.1	0.5	0.05
1,2,3,4,6,7,8-Cl_6DD	0.1	0.1	0.01	0.1
1,2,3,7,8,9-Cl_6DD	0.1	0.1	0.01	0.01
1,2,3,4,6,7,8-Cl_7DD	0.01	0.01	0.001	<0.001
Cl_8DD	0.001	0.001	Unknown	Unknown
Furan congeners				
2,3,7,8-Cl_4DF	0.1	0.1	0.05	1
1,2,3,7,8-Cl_5DF	0.05	0.05	0.05	0.1
2,3,4,7,8-Cl_5DF	0.5	0.5	0.5	1
1,2,3,4,7,8-Cl_6DF	0.1	0.1	0.1	0.1
1,2,3,6,7,8-Cl_6DF	0.1	0.1	0.1	0.1
1,2,3,7,8,9-Cl_6DF	0.1	0.1	0.1	0.1
2,3,4,6,7,8-Cl_6DF	0.1	0.1	0.1	0.1
1,2,3,4,6,7,8-Cl_7DF	0.01	0.01	0.01	0.01
1,2,3,4,7,8,9-Cl_7DF	0.01	0.01	0.01	0.01
Cl_8DF	0.001	0.001	0.0001	0.0001
PCB congeners				
3,3',4,4'-Cl_4B	No values calculated	0.001	0.0001	0.05
3,4,4',5-Cl_4B		0.001	0.0005	0.1
3,3'4,4',5,5'-Cl_5B		0.1	0.005	0.1
3,3',4,4',5,5'-Cl_6B		0.01	0.00005	0.001
2,3,3',4,4'-Cl_5B		0.0001	<0.000005	0.0001
2,3,4,4',5-Cl_5B;		0.0005	<0.000005	0.0001
2,3',4,4',5-Cl_5B		0.0001	<0.000005	0.00001
2',3,4,4',5-Cl_5B		0.0001	<0.000005	0.00001
2,3,3',4,4',5-Cl_6B		0.0005	<0.000005	0.0001
2,3,3',4,4',5'-Cl_6B		0.0005	<0.000005	0.0001
2,3',4,4',5,5'-Cl_6B		0.00001	<0.000005	0.00001
2,3,3',4,4',5,5'-Cl_7B		0.0001	<0.000005	0.00001

Biological monitoring is sensitive, relatively cost-effective and results are usually obtained rapidly. Biological methods, such as bio-assays, represent the toxic effects of specific pollutants on organism level and also integrate possible interactions among chemicals. Biological monitoring therefore allows the detection of disturbances on organisms and the environment that might have been missed by performing chemical analyses alone (Chutter, 1998).

When bio-assays are used to measure the amount of dioxin-like substances in environmental extracts, the toxicity is reported as *TCDD-equivalents*. TCDD-equivalents are used as a means of normalising the toxicity of a mixture of compounds relative to a single compound, the most toxic congener, 2,3,7,8-TCDD (Schecter *et al.*, 2006). TCDD-equivalents calculated with bio-assays are the equivalent to TEQ values calculated with chemical methods such as GC/MS analysis. Both of these values are generally reported in ng TEQ/kg sample, thus these values can be compared to one another (WHO, 1997).

Since both of these methods have limitations and advantages, it is best to use these methods in combination with one another, if possible. Because high-resolution GC/MSs are very costly, it is advised that samples are analysed with bio-assays to screen them for the presence of dioxin-like substances, prior to chemical analysis (Kannan, Hilscherova, Imagawa, Yamashita, Williams & Giesy, 2001).

2.2.5.4. The H4IIE-*luc* tissue culture bio-assay

Bio-assays measure different endpoints in AhR-mediated responses (explained in Section 2.2.5.2.). The AhR response results in cytochrome P450 induction. Reactions catalysed by cytochrome P450 are known as mixed function oxidase (MFO) reactions. When an organism is exposed to toxic substances it can lead to cytochrome P450 induction, which is an indicator of toxic exposure (Stegeman, Brouwer, Di Guilio, Förlin, Fowler, Sanders & Van Veld, 1992).

During this study the H4IIE-*luc* tissue culture bio-assay was implemented to detect and quantify dioxin-like chemicals present in environmental and biological extracts. This bio-assay is a newly developed process which measures the effect of dioxins on rat hepatoma cells. These cells were stably transfected with a luciferase reporter gene under control of dioxin-responsive elements (DREs) (Hilscherova *et al.*, 2000).

When the AhR-ligand complex binds to the DREs, it results in an up-regulation of luciferase transcription and once luciferin is added to the cells, a light-producing reaction is catalysed. A luminometer measures the amount of light emitted from the cells, which is equivalent to their toxicant exposure (Fig 2.7) (Denison, Rogers, Fair, Ziccardi, Clark, Murk & Brouwer, 1996). The method of the H4IIE-*luc* bio-assay is described in Section 3.5.2.

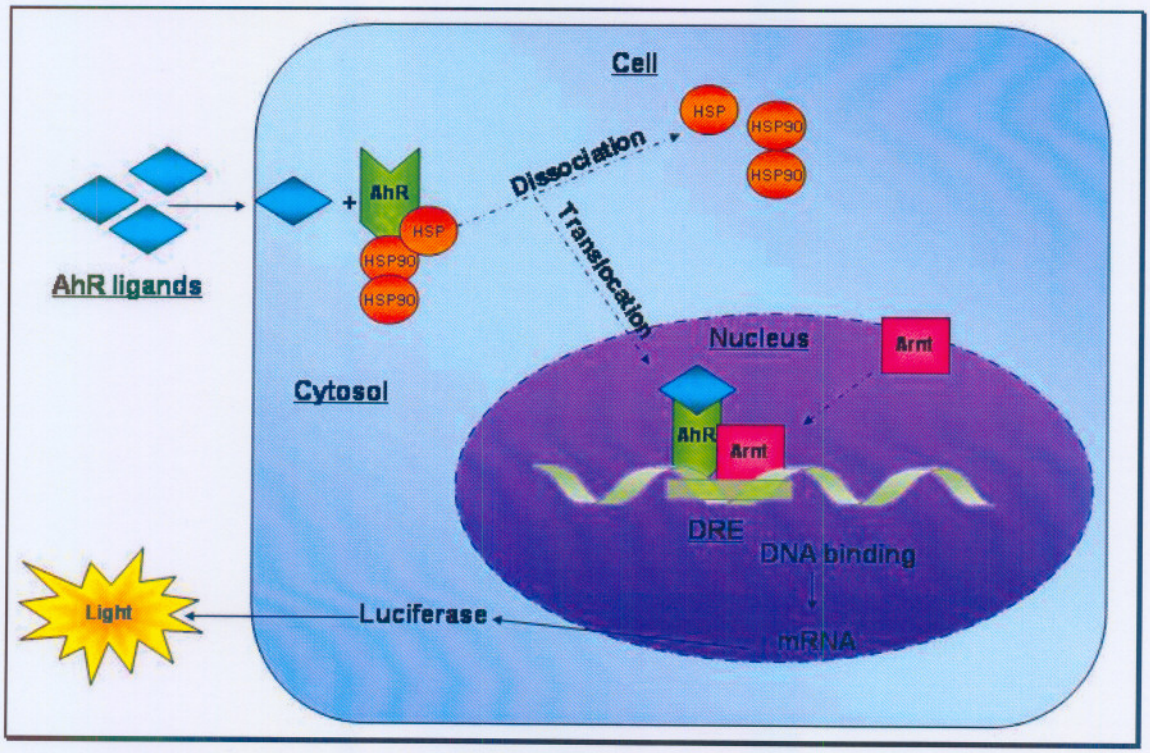

Figure 2.7. A diagrammatic representation of the light-producing response used to quantify dioxin-like substances with the H4IIE-*luc* bio-assay (adapted from Hilscherova *et al.*, 2000).

Chapter 3. Materials and Methods

The aim of the study was to determine the degree and nature of POPs pollution in various rivers of the Vaal Triangle, focusing on dioxin-like POPs. For this purpose sediment and fish samples were collected and analysed. The approach that was followed during this investigation is illustrated below in Figure 3.1.

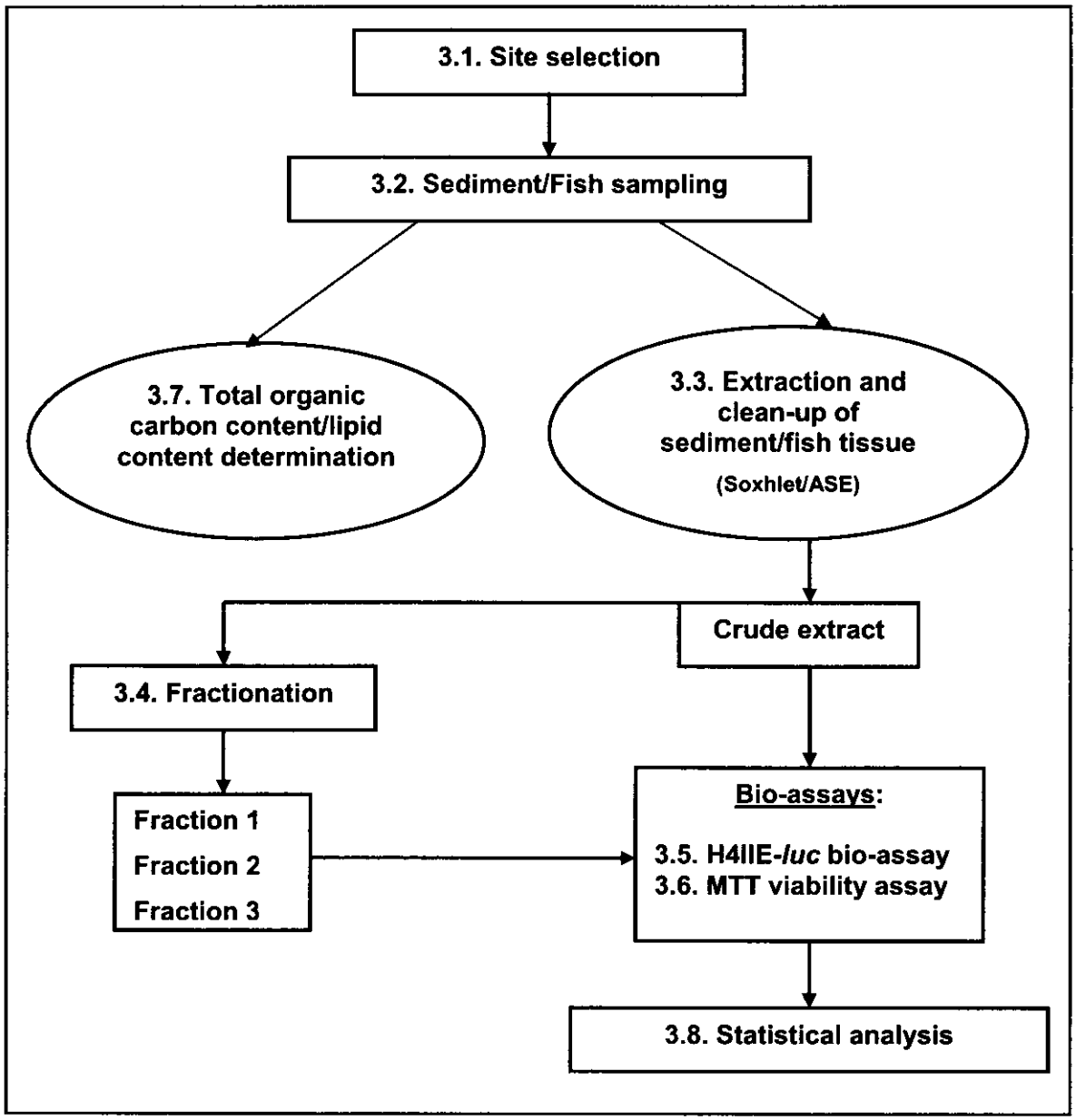

Figure 3.1. A schematic view of the POPs pollution assessment approach followed during the study.

3.1 Site selection

The motivation for choosing the Vaal Triangle as a sampling area for dioxin-like POPs pollution was based on previous work done by Vosloo & Bouwman (2005). During their study, 22 aquatic sites were selected throughout South Africa to establish the presence and levels of PCDD/F and PCB. Their sites were chosen down-stream from possible dioxin, furan and PCB sources. Results indicated that dioxin-like substances were present in all 22 of the sites, with the highest TCDD equivalent values calculated for the Vaal Triangle site. This project aimed to investigate and evaluate dioxin-like pollution in the Vaal Triangle more extensively by targeting water bodies in this area for analysis.

3.1.1. Sediment sampling sites

To cover a large part of the Vaal Triangle river system, several rivers in this region were selected as sediment sampling sites. These rivers included the Klip River, Natal Spruit, Riet Spruit (draining into the Vaal River at the Barrage), Blesbok Spruit, Taaibos Spruit, Leeu Spruit and Suikerbosrand River. This thesis reports only on the Blesbok Spruit, Taaibos Spruit and Leeu Spruit, draining into the Vaal Dam. Sites in the other rivers were investigated by a fellow student. The accessibility of the rivers played an important role in site selection, and sites were chosen that were easily accessible. The sites were selected near potential emission sources of PCBs and PCDD/F. Suikerbosrand River was chosen as a reference river, because of its expected low potential for POPs pollution due to its location.

Blesbok Spruit was represented by Sites 1, 8, 9, 11, 12, 13 and 15 (Fig 3.2). Whereas the smaller water bodies, Taaibos Spruit and Leeu Spruit, were represented by only one site each, Site 20 and Site 22 (Fig 3.3), respectively. Sites 16, 17 and 18 were the Suikerbosrand reference sites (Fig 3.4) (Table 3.1). The site numbers not included in this thesis: 2, 3, 4, 5, 6, 7, 10, 14, 19 and 21, were the sites in the Klip River, Natal Spruit and Riet Spruit.

<u>Blesbok Spruit sites</u>:

Site 1 was located near to many mines and tailings dams of previous mining activity, and down-stream of a paper and pulp producer, a paper treatment plant and a non-ferrous metal refinery, which are known sources of PCDD/F or PCBs (UNEP, 2003). The setting of Site 8 was the Putfontein residential area. Putfontein is located up-stream from the more industrialised areas; therefore less POPs pollution was expected for this site.

Site 9 was inside the Grootvaly Blesbok Spruit Wetland Nature Reserve. The nature reserve is situated down-stream of tailings dams of previous mining activity, a residential area and sewage works. Several informal settlements were also located near to Site 9.

Sampling Site 11 was chosen near Nigel. A residential area, as well as municipal dumping sites and landfills were noticed near this area. Sites 12 and 13 were selected in the surrounding area of smallholdings, beyond the point where Blesbok Spruit merged with the Suikerbosrand River. Site 15 (also in the Suikerbosrand River after confluence with the Blesbok Spruit) was situated in a residential area of Vereeniging, which was being developed at the time.

Other rivers' sites:
The Taaibos Spruit sampling area (Site 20) was near Sasolburg and was located down-stream of the afore-mentioned oil and gas refinery. Leeu Spruit flows through the suburban area of Sasolburg, and Site 22 was chosen down-stream of the oil and gas refinery, a ferrous metal refinery and a power plant.

Reference sites:
Reference sites (16, 17 and 18) of the Suikerbosrand River were selected to represent expected minimal POPs pollution, and these sites were located on farms and smallholdings, with no factories or industries in close proximity.

Sediment samples were collected from Sites 15, 16, 17 and 20, during June 2006 to be analysed with GC/MS analysis.

3.1.2. Fish sampling sites

Of the main study, only three rivers were selected for collecting fish: the Blesbok Spruit, Klip River and Suikerbosrand River. This included one major river for each of the sub-studies and Suikerbosrand River as the reference river (Table 3.2). In addition to fish samples, composite sediment samples were collected at these sites (using the method described in Section 3.2.1). The samples would be used to compare the amount of POPs in the sediment to those in the fish to determine bio-accumulation. The sites were chosen where the conditions for fishing were favourable.

The rivers had to be accessible for fishing by means of gill nets, line fishing and/or electro-fishing. This implied that the rivers had to be deep enough and sustain sufficient water flow. Site selection was assisted by previous work done by Rand Water, where the same sites were used to sample fish (Du Preez, 2005). Suikerbosrand River and Blesbok Spruit were represented by one site each, Site 1F and Site 2F (Fig 3.2), respectively.

Site 1F (Suikerbosrand River reference site) was selected in an area where POPs pollution was expected to be minimal. The site was situated 13 km South of Heidelberg on a cattle farm. No industries or factories were seen in this area (Fig 3.5). The Blesbok Spruit fish-sampling site (Site 2F) was situated in a recreational park in Heidelberg. This sampling site was located down-stream of the industrial areas of Heidelberg, Brakpan, Springs and Nigel, which were expected to produce industrial waste that might contain POPs (Fig 3.6).

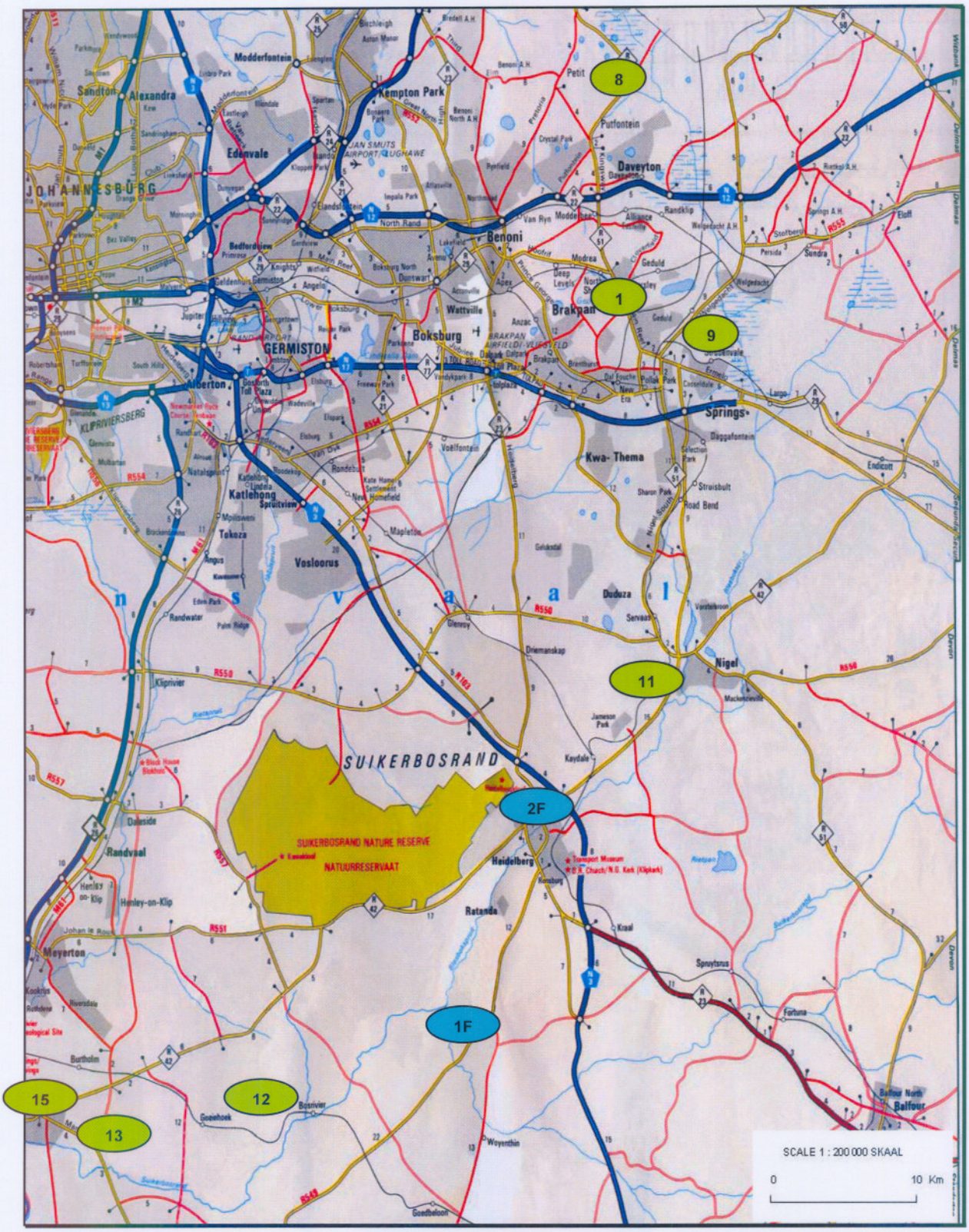

Figure 3.2. A map of the Vaal Triangle indicating Blesbok Spruit sediment sampling sites (Green) as well as the Blesbok Spruit and Suikerbosrand River fishing sites (Blue) (Map Studio, 1990).

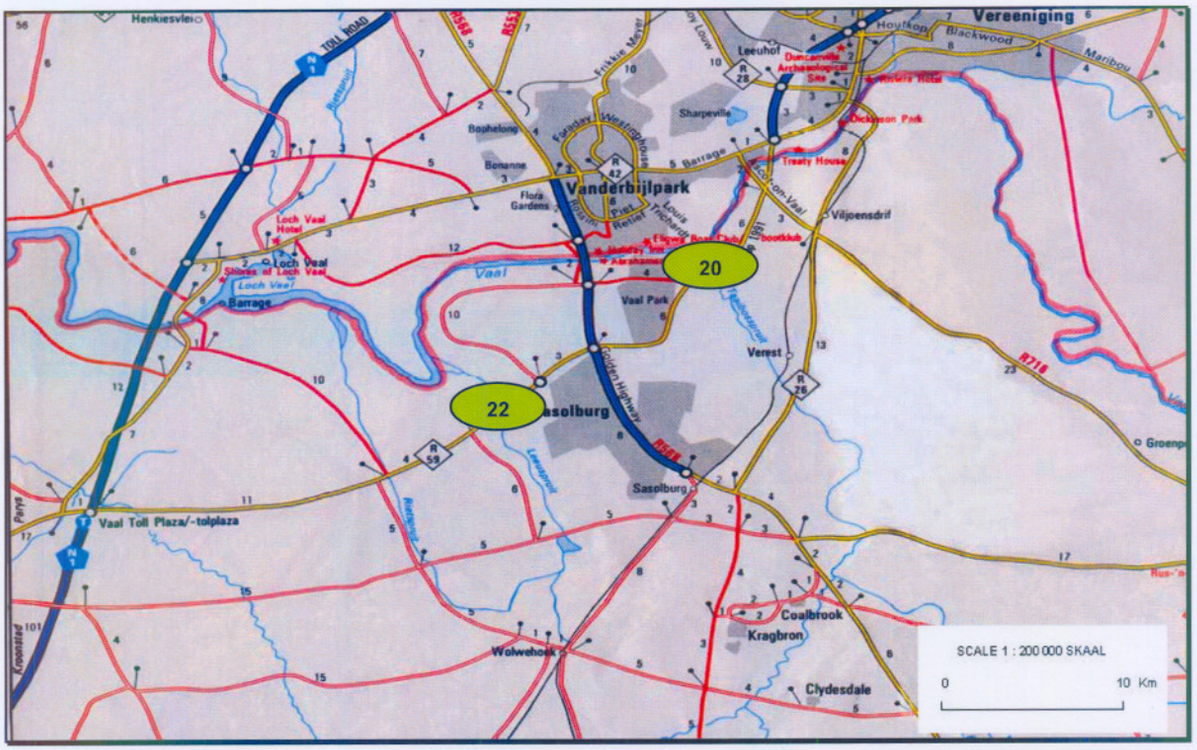

Figure 3.3. A sectional map of the Vaal Triangle indicating Site 20 (Taaibos Spruit) and Site 22 (Leeu Spruit) (Map Studio, 1990).

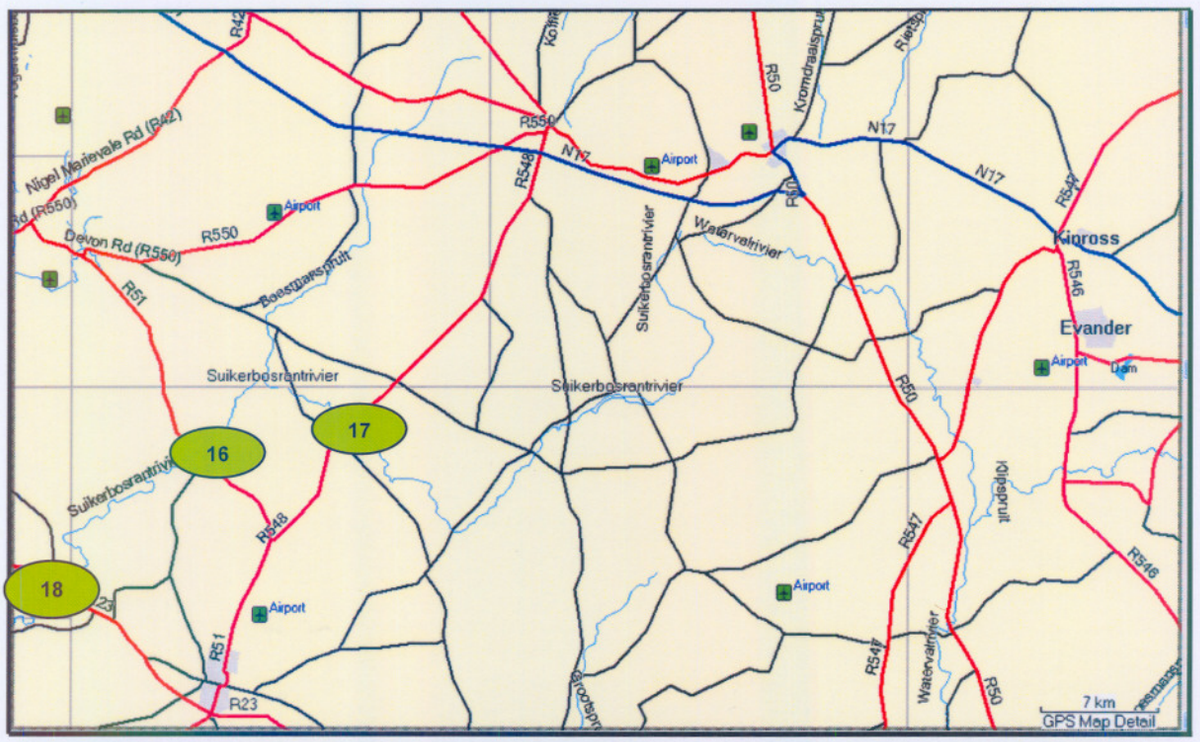

Figure 3.4. A map of the Suikerbosrand River reference area (Sites 16 – 18) (Garmap, 2002).

Figure 3.5. The Suikerbosrand fishing site was South of Heidelberg, just off the R549 on a farm.

Figure 3.6. The Blesbok Spruit fishing site was located in a residential area of Heidelberg. The site was chosen where the river was deep and wide enough for gill-net fishing.

Table 3.1. Site description of the sediment samples collected in the Vaal Triangle.

Site	Coordinates	Location	Date	River	Nearby industries	Main Category* (Table 2.3)
1	S 26°12.992' E 28°26.501'	Geduld (Brakpan) ± 200 m West of the R51-M2 intersection	21/04/2005	Blesbok Spruit	Mines and tailings dams	
					Pulp and paper producer	Category 7
					Non-ferrous metal producer	Category 2
					Paper treatment plant	Category 7
8	S 26°05.224' E 28°25.772'	Putfontein	21/04/2005	Blesbok Spruit	Residential area	
9	S 26°13.167' E 28°28.981'	Grootvaly Blesbok Spruit Wetland Nature Reserve	19/04/2005	Blesbok Spruit	Tailings dams	
					Sewage works	Category 9
					Residential area	
11	S 26°26.505' E 28°28.903'	Nigel	21/04/2005	Blesbok Spruit	Residential area	
					Waste dumps	Category 9
12	S 26°41.648' E 28°06.433'	Goeiehoek	20/04/2005	Suikerbosrand River after confluence with Blesbok Spruit	Power station	Category 3
13	S 26°38.422' E 28°13.818'	Platkoppies smallholdings	20/04/2005	Suikerbosrand River after confluence with Blesbok Spruit	None in close proximity	-
15°	S 26°40.259' E 28°00.998'	Vereeniging – Drie Riviere residential area: Fish eagle drive	20/04/2005	Suikerbosrand River after confluence with Blesbok Spruit	Residential area: being developed at the time.	-
					Near a ferrous metal producer.	Category 2
16°	S 26°32.042' E 28°34.626'	On the R51, 8 km North West of the R51-R548 intersection	04/05/2005	Suikerbosrand River reference site	In the vicinity of farms	-

* Main category - according to the Standardized Toolkit for Identification and Quantification of Dioxin and Furan Releases (UNEP, 2003).

° Sediment samples were collected again, during June 2006, at Sites 15, 16, 17 and 20 to be analysed with GC/MS.

Table 3.1. *Continued.*

Site	Coordinates	Location	Date	River	Nearby industries	Main Category* (Table 2.3)
17°	S 26°31.302' E 28°39.960'	On the R548, 9 km North East of the R51-R548 intersection	04/05/2005	Suikerbosrand River reference site	In the vicinity of farms	-
18	S 26°36.063' E 28°29.586'	On the R23, 100 m West of the Poortjie intersection	04/05/2005	Suikerbosrand River reference site	In the vicinity of farms	-
20°	S 26°45.190' E 27°52.495'	On the R59, North East of Sasolburg	29/06/2005	Taaibos Spruit	Oil and gas refinery	Category 3
22	S 26°48.128' E 27°47.907'	On the R59, North East of Sasolburg	29/06/2005	Leeu Spruit	Oil and gas refinery Power plant	Category 3
					Ferrous metal producer	Category 2

Table 3.2. Site description of fish samples collected in the Vaal Triangle.

Site	Coordinates	Location	Date	River
1F	S 26°37'47.8" E 28°17'48.2"	± 13 km South of Heidelberg on the R549 on a cattle farm.	05/12/2005	Suikerbosrand River
2	S 26°30'16.1" E 28°21'44.4"	In Heidelberg residential area. In a park across from a fuelling station (Total).	07/12/2005	Blesbok Spruit

3.2 Methods of sediment and fish sampling

3.2.1. Sediment sampling

Samples were collected from the upper sediment layer by means of a brass grab sampler or a metal spade, and deposited in stainless steel containers. These sediment samples were taken at five different locations within each site area to create a composite sample. Equal volumes (250 mL) of sediment from each of the five locations were mixed together briskly for approximately one minute to obtain a homogenous sample. The sub-samples were then transferred to labelled glass containers. The lids of the containers were lined with foil to prevent contact between the sample and the plastic lining of the lid, protecting the sample from possible contamination. To prevent ultra violet breakdown of the compounds of interest, the containers were covered in brown paper bags. Samples were transported and stored at -4°C (preventing bio-degradation) until extracted (Hilscherova, Kannan, Nakata, Hanari, Yamashita, Bradley, McCabe, Taylor & Giesy, 2003).

Each of the containers and utensils, which came into contact with the sample, was washed with phosphate-free soap (Merck) and rinsed with tap water and ultra pure water (18 MΩ) prior to sampling. The utensils were also rinsed three times with high-pressure liquid chromatography-grade (HPLC-grade) acetone (Burdick & Jackson) to remove polar particles, and then rinsed three times with HPLC-grade hexane (Burdick & Jackson) to dissolve non-polar particles. Between sampling, the utensils and containers were rinsed with distilled water followed by acetone and hexane. Glass or stainless steel containers and utensils were used at all times, avoiding plastic equipment, to prevent sample contamination (Koh, Khim, Villeneuve, Kannan, Johnson & Giesy, 2005).

3.2.2. Fish sampling

To determine if POPs were present, and if bio-accumulation and bio-magnification occurred in fish tissue, two fish species were selected for sampling namely *Labeo umbratus* and *Labeo capensis.* These fish species belong to the family Cyprinidae, which includes minnows and carps (Skelton, 2001). *L. umbratus* and *L. capensis* were chosen because they are bottom feeders and it is known that POPs associate with the organic carbon particles of sediment (Schumacher, 2002). Bottom-feeding species are in direct physical contact with sediment and they bio-accumulate high concentrations of chemical contaminants (Heath, Du Preez, Genthe & Avenant-Oldewage, 2004).

L. umbratus and *L. capensis* are abundant in the Vaal Triangle area and relatively easy to capture (South African Institute for Aquatic Biodiversity, 2005). According to Heath *et al.* (2004) these two fish species are among the test species recommended for freshwater contaminant investigations in South Africa. These species are caught by recreational and subsistence fisherman and would give an indication of human exposure to PCDD/Fs and PCBs through fish consumption.

L. capensis, better known as the Orange River mud fish, is dispersed throughout the Orange-Vaal system (Fig 3.7). They can be identified by a depressed head and a mouth with papillate outer lips and two pairs of thin barbells. Adults are a dark greyish colour and they prefer to graze on surfaces of rocks and plants (Skelton, 2001).

Figure 3.7. A map of southern Africa showing the distribution of *L. capensis* (adapted from Skelton, 2001) (Fish image: FOW, 2006; Map: adapted from DWAF, 2005).

Moggels (*L. umbratus*) are often mistaken for *L. capensis*, because of their similar appearances. They are both greyish in colour and are similar in shape and size. Moggels can be distinguished from the Orange River mud fish by their rounded heads, mouths with sub-terminal lips and their two small barbells (South African Institute for Aquatic Biodiversity, 2005). *L. umbratus* (Fig 3.8) can be found in the Orange-Vaal system, South- and South-east Cape coastal regions as well as some parts of the Eastern Cape and Mpumalanga (Nussey, Van Vuren & Du Preez, 2000).

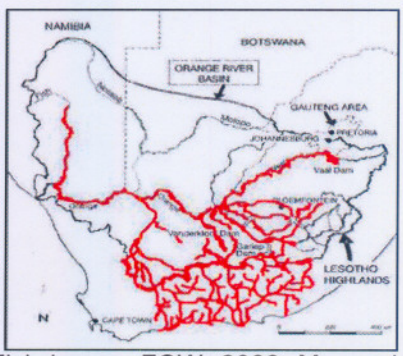

Figure 3.8. A map of southern Africa showing the distribution of *L. umbratus* (adapted from Skelton, 2001) (Fish image: FOW, 2006; Map: adapted from DWAF, 2005).

Hook and line, electro fishing and gill nets were attempted as sampling methods, with gill nets being the most effective. A single fish was caught by hook and line and only young fish were captured by electro fishing (Fig 3.9). Gill nets are easy to operate and different mesh sizes make selective catches possible (Fig 3.10). Disadvantages of gill nets include injuring or killing captured fish, which might result in physiological changes (Heath *et al.*, 2004).

Fig 3.9. Fish sampling by electro-fishing (AWER, 2005).

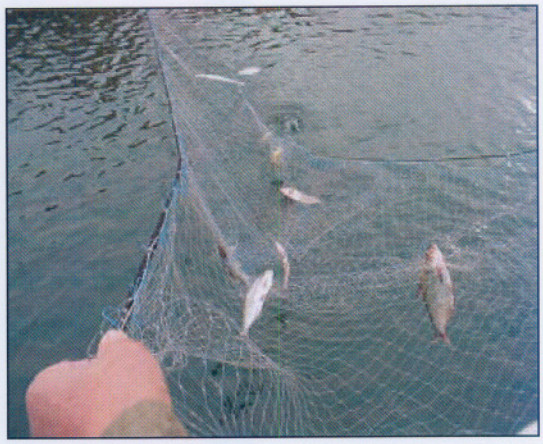

Fig 3.10. Gill nets used for sampling (AWER, 2005).

Contaminant concentrations are higher in fish of increasing size/age (Honkanen, 2004). Gill nets with mesh sizes of 75, 90 and 110 mm were used for sampling, concentrating on larger/older fish. Both *L. umbratus* and *L. capensis* were caught at Blesbok Spruit (Site 2F) and Suikerbosrand River (Site 1F). Fish of both sexes were sampled. After the fish were removed from the nets, they were identified, measured (total length), weighed and a rapid fish health assessment was done (Adams, Brown & Goede, 1993).

The fish health assessment index (FHAI), created by Goede & Barton (1990) and refined and adapted for South African conditions by Avenant-Oldewage (2001), was used during the study. This is a rapid, cost effective method used to evaluate the condition of water bodies according to the health of fish. The FHAI includes a detailed assessment of the external and internal features as well as blood analysis of fish. The examination of external features includes the skin, fins, eyes, opercula, gills and ectoparasites. The thymus, mesenteric fat, liver, spleen, hindgut, kidneys and bile should be scored as part of the internal assessment. An in-depth FHAI requires blood analysis for haematocrit, blood plasma protein, leukocrit and white blood cell count.

During the study, only selected variables were measured to determine the general health of fish. A numerical value was assigned to the condition of the eyes, skin, fins, opercula and gills, according to the criteria in Table 3.3. The colour and appearance of the liver, bile and spleen were examined and scored too.

Along with the FHAI-values, the condition factors (CF) of fish were calculated with the following formula:

$$CF = \frac{\text{Mass (g)} \times 10^5}{[\text{Length (mm)}]^3}$$

The CF is a mass-to-length ratio value indicating the condition of individual fish. Higher CF values indicate a higher body mass to length ratio, being a sign of good fish condition. This method provided a quantitative basis in order to compare the overall health and condition of fish to one another (Heath *et al.*, 2004). It was not one of the aims of this study to evaluate the water bodies, but by scoring the morphology of fish structures and organs, a quantitative impression of the fish's health can be determined. This would be valuable in linking possible POPs exposure to the general health of fish.

Fish were stunned by a forceful blow to the head, before severing the spinal cord, and dissected on pre-cleaned stainless steel trays. Each of the utensils that came into contact with the fish tissue was washed with distilled water and rinsed with acetone and hexane (Horst, Ruoff & Bluthgen, 2002).

The liver, gonads and fillets of the fish were removed on site by dissection. The fish tissue was weighed, wrapped in pre-cleaned aluminium foil, placed in waterproof plastic bags and labelled (Heath *et al.*, 2004). The samples were cooled immediately and transported and stored at -4°C (Hilscherova *et al.*, 2003). Livers and gonads, which are lipid-rich tissues, were selected for analysis, because it is known that PCDD/Fs and PCBs are lipophilic (Ritter *et al.*, 2005). The section of fish most commonly consumed by humans, is fillet tissue. Fillets were extracted and analysed to establish the potential effects of contaminated fish consumption on humans (Heath *et al.*, 2004).

Table 3.3. Score values assigned to variables according to the Fish Health Assessment Index (FHAI) (adapted from Heath *et al.*, 2004).

Variable	Field code	FHAI value
Eyes:		
Normal – No aberrations	N	0
Exophthalmia - Protrusion of one (E1) or both (E2) eyes	E1/E2	30
Haemorrhagic – Blood in eye	H1/H2	30
Blind – Eyes are dull, opaque coloured	B1/B2	30
Missing – Eye is missing	M1/M2	30
Other – Any other eye conditions	OT	30
Skin:		
Normal – No aberrations	0	0
Mild – Mild skin aberrations	1	10
Moderate – Moderate skin aberrations	2	20
Severe – Severe skin aberrations	3	30
Fins:		
No active erosion – Normal or healed lesions	0	0
Mild active erosion – Active erosion, no haemorrhage or infection	1	10
Severe active erosion – Active erosion, haemorrhage and/or secondary infection	2	20
Opercula:		
Normal – Gills are covered, no shortening	0	0
Mild shortening – Small portion of the gill is uncovered	1	10
Severe shortening – Large portion of the gill is uncovered	2	20
Gills:		
Normal – No apparent aberration	N	0
Frayed – Tips of gill lamellae are eroded	F	30
Clubbed – Tips of lamellae are swollen	C	30
Marginate – Gill has a discoloured margin	M	30
Pale – Gills are light in colour	P	30
Other – Other observations	OT	30
Liver:		
Normal – Good, solid red colour	A	0
Light red	B	0
Fatty liver – light tan colour ("coffee with cream")	C	30
Nodular – White mycobacterial cysts and incipient nodules	D	30
Focal discolouration	E	30
Colour change in whole liver	F	30
Other – Aberrations not mentioned above	OT	30
Bile:		
Yellow/Straw colour – Bladder empty/partially full	0	-
Yellow/Straw colour – Bladder full, distended	1	-
Light green/Grass green	2	-
Dark green/Dark blue green	3	-
Spleen:		
Black – Very dark red colour	B	0
Red – Red colouration	R	0
Granular – Rough appearance	G	0
Nodular – Contains nodules and cysts of varying sizes	NO	30
Enlarged – Significantly larger than normal	E	30

3.3 Sample extraction and clean up

3.3.1. Soxhlet versus Accelerated Solvent Extractor (ASE) extraction

Both Soxhlet and ASE methods are accepted by the US EPA for the extraction of PCDD/Fs and dioxin-like PCBs (Grochowalski & Maślanka, 2003). The ASE and Soxhlet apparatus have shown to be comparable in their extraction efficiency (Table 3.4) (Dionex, 2002). Sediment samples (excluding the fishing sites' sediment samples) were extracted with the ASE apparatus, since this apparatus is more time- and cost-effective. The Soxhlet apparatus was used to extract fish tissue, because the volumes of fish tissue, especially fillet tissues, were too large to be extracted with the ASE. To determine bio-accumulation, sediment- and fish tissue samples, collected at the same site, had to be compared to one another. Therefore, sediment samples collected at fish sampling sites, were also extracted with the Soxhlet apparatus, to eliminate any possible variables in extraction method.

Table 3.4. A comparison of the Soxhlet and ASE extraction methods used during this study (adapted from McCant, Inouye & McFarland, 1999).

Variable	Soxhlet extraction	ASE extraction
Extraction efficiency (recovery values)	68 %	72 %
Extraction time per sample	16 to 24 hours	30 minutes
Solvent volume required per sample	± 400 mL	± 70 - 90 mL (for a 66 mL cell)

3.3.2. Sediment extraction

To remove water from the sediment samples, they were freeze-dried for three days. Small stones, leaves and twigs were removed from the dried sediment sample by hand. A pre-cleaned mortar and pestle were used to grind the sample, increasing the reactivity surface of the sediment for extraction purposes. A copper sieve with mesh size 0.5 mm was used to obtain a homogenous sample. Samples should be homogenous to compare them to one another.

Forty grams (40 g) of sediment was mixed with an equal volume of anhydrous sodium sulphate (Merck, univAR) to remove any possible moisture still present in the sample. The sediment was placed on top of pre-cleaned glass wool. Glass wool was prepared by extracting it for 24 hours, using a methylene chloride (DCM) (Burdick & Jackson) and hexane mixture of 3:1.

Using the Soxhlet apparatus (Fig 3.11), sediment was extracted with a 3:1 HPLC-grade DCM and hexane mixture (400 mL) for 16 to 24 hours (Hilscherova, Kannan, Kong, Holoubek, Machala, Masunaga, Nakanishi & Giesy, 2001).

The ASE (Dionex, ASE 100) was used for sediment extraction. This instrument operates on the principle of pressurised fluid extraction by using elevated temperatures and pressure to achieve rapid, effective extraction (Bernsmann & Fürst, 2004).

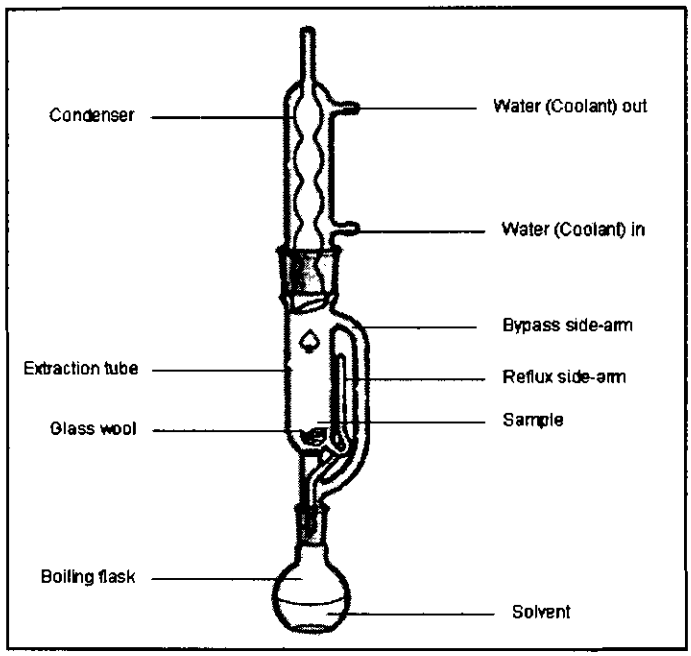

Figure 3.11. A schematic representation of the Soxhlet apparatus used for sample extraction (adapted from VELP Scientifica, 2005).

Sediment samples were freeze dried, ground and sieved as described earlier. Forty grams (40 g) of sediment was mixed with an equal volume of anhydrous sodium sulphate to remove possible moisture still present in the sample. The 100 mL extraction cell of the ASE was washed with phosphate-free soap, rinsed with 18 MΩ water and then rinsed thrice with acetone and hexane. The cell was allowed to dry completely before the cellulose filter was placed in the outlet end of the cell (Petrovic, Lacorte, Viana & Barceló, 2002). After the dried sample was added to the cell, a cellulose filter was placed on top of the sediment and the lids were closed tightly. A 3:1 mixture of DCM and hexane was used as the extraction solvent (US EPA, 1998; Hölscher, Maulshagen, Shirkhan, Lieck & Behnisch, 2004).

Sediments were extracted under the following parameters: 1500 psi, 100 °C, five minute static and heat time, a flush volume of 60%, 100 seconds nitrogen purge time and 2 cycles (McCant *et al.*, 1999). The extract was collected in a pre-cleaned collection bottle and allowed to cool.

After extraction, the extract was rotary evaporated to almost dryness. The principle of rotary evaporation is to intensify the factors that promote evaporation under controlled conditions (Hilscherova *et al.*, 2001). During rotary evaporation the extract is suspended in a heated water bath (30 – 35 °C) to increase the temperature and in this way increasing evaporation. Adding to a rapid evaporation rate, a vacuum pump lowers the vapour pressure above the solvent. The remaining extract was transferred to a pre-cleaned test tube and adjusted to 10 mL with hexane (Hilscherova *et al.*, 2001).

Some sediment extracts may contain high levels of elemental sulphur, which might be cytotoxic to the rat hepatoma cells used in the bio-assay. To remove this sulphur, a copper treatment is recommended (US EPA, 1986). Approximately 0.5 g of freshly activated copper shavings was added to the 10 mL sample extract in the test tube. [Copper shavings were activated by covering it in hydrochloric acid (32%, Merck). The shavings were rinsed with deionised water and with acetone and hexane]. The copper shavings were swirled and mixed with the extract and allowed to stand for approximately ten minutes. Copper was added until it did not turn black any longer. The sample was quantitatively transferred to separation funnels to perform an acid treatment (US EPA, 2000).

During the acid clean-up, the extract was treated with concentrated sulphuric acid (98%, Merck) to remove all traces of polycyclic aromatic hydrocarbons which are not dioxins, furans or PCBs (Vondráček, Machala, Minskova, Blacha, Murk, Kozubik, Hovmanova, Hilscherova, Ulrich, Ciganek, Neca, Svrckova & Holoubek, 2001). Fifteen millilitres (15 mL) of sulphuric acid was added to the extract, carefully mixed, ventilated often, and left to stand for at least an hour for the two phases to separate. The acid was then tapped off and fresh sulphuric acid was added. This step was repeated until the acid phase was clear (3 to 5 times).

To remove all traces of acid from the extract, the sample was washed with a 5% sodium chloride (NaCl) solution and left for an hour for the phases to separate. This step was followed by a 20% potassium hydroxide (KOH) solution treatment.

KOH is a strong base with the ability to break down the compounds of interest. Therefore, the KOH-solution was tapped off at the instant the two phases separated. This step was followed by a second 5% NaCl-solution treatment. The remaining extract was filtered through glass wool covered with anhydrous sodium sulphate to remove all traces of water (US EPA, 1994b). The crude extract was further concentrated to approximately 0.5 mL by a gentle stream of nitrogen gas and made up to 1 mL with hexane. The extract was stored in amber coloured gas chromatography (g.c.) vials at –4 °C until used in the bio-assay. Each of the utensils and apparatus used during sample extraction was washed beforehand with phosphate-free soap, rinsed with ultra-pure water (double deionised; 18MΩ) and rinsed with acetone and hexane (Vondráček *et al.*, 2001).

The sediments, collected during June 2006 for the purpose of comparing biological and chemical methods to one another, were air dried for a week to remove the majority of water from the sediments. The samples were then stored in glass containers, which were washed with acetone and hexane, and shipped to the Norwegian Institute for Air Research. The Institute was responsible for the extraction and analysis of samples (Method ISO/IEC-17025). High-resolution gas chromatography/mass spectroscopy (GC/MS) was used to measure the concentrations of dioxin-like compounds in the sediment samples.

3.3.3. Fish tissue extraction

On the day after sample collection, fish tissues were divided into composite groups, of two or three individuals per group. Three individuals per group were the ideal, but because the availability of fish was a limiting factor, some groups consisted of only two individuals. Individuals in a composite group had to belong to the same species, gender and age/size class (Heath *et al.*, 2004, US EPA, 2000). Composite samples were prepared from gonads, liver and fillet tissue, respectively.

Equal masses of tissue were weighed and combined to form a composite tissue sample. The tissue was handled with stainless steel dissection kits, which were pre-cleaned and rinsed with acetone and hexane. The combined tissue sample was wrapped in pre-cleaned aluminium foil and labelled clearly. The tissue samples were freeze-dried for three days and the dry weight of samples were determined afterwards (Adams *et al.*, 1993). Because of the nature of the tissues, fillets and ovaries were homogenised with a blender, and a mortar and pestle were used for livers and male gonads.

All of the livers and gonads, but only 10 g of fillet tissue, were extracted. The tissues were mixed with anhydrous sodium sulphate to remove any moisture remnants (Corsolini *et al.*, 2002).

The Soxhlet extraction method was used to extract fish tissue for 24 hours, with DCM and hexane (3:1, 400 mL) as solvent (Hilscherova *et al.*, 2001). Glass wool was used as a filter to extract fillets, and cellulose extraction thimbles (single thickness, 43 mm x 123 mm, Merck Whatmann) were used for livers and gonads. Each of the utensils and apparatus that came into contact with the fish samples was washed with phosphate-free soap, rinsed with ultra pure water and then rinsed thrice with acetone and hexane (Vondráček *et al.*, 2001). Extraction thimbles as well as glass wool were pre-extracted with 3:1 DCM and hexane for 24 hours, before being used in the tissue extraction.

The extract was rotary evaporated to a small volume and then transferred to clean test tubes. Hexane was added to the 11 mL mark on the calibrated test tubes. One millilitre (1 mL) of extract was used for lipid content determination, which will be discussed in detail later in this chapter (Section 3.7.2). The other 10 mL of extract was treated with concentrated sulphuric acid to remove fat and polycyclic aromatic hydrocarbons, which are not dioxin-like (Vondráček *et al.*, 2001). The acid treatment was repeated until the hexane phase was clear (3 to 5 times). Each acid phase was left to separate from the hexane phase for an hour before the acid was tapped off. The acid wash was followed by a 5% sodium chloride wash. The sample was transferred to a test tube and concentrated to 0.5 mL by a nitrogen gas purge. The concentrated extract was made up with hexane to a volume of 1 mL, and stored in g.c. vials at -4 °C until analysis using the H4IIE-*luc* bio-assay (Koh *et al.*, 2005).

3.4 Fractionation

In addition to assaying the crude extract, the extract was fractionated into separate parts that were analysed individually. Groups of compounds may have synergistic or antagonistic reactions with one another, leading to false responses in the assay. By dividing these groups of compounds into separate fractions, prior to sample analysis, the actual contribution of each fraction can be determined (Van Hoof & Hsieh, 1996). Several methods can be applied to fractionate samples containing PCDD/Fs and PCBs, for instance alumina columns, silica columns and Florisil® columns (US EPA, 1996a). Florisil® fractionation was employed during this study (US EPA, 1996b).

Twice the amount as usual (80 g) of sediment was extracted and subjected to the usual clean-up procedures. The extract was evaporated to 2 mL of which 1 mL was used in the bio-assay and the other millilitre was fractionated. A glass column (length 50 cm, diameter 10 mm) was prepared by adding 10 g of activated Florisil® PR (60 – 100 mesh, Fluka) on top of a small piece of glass wool, followed by 1.5 cm anhydrous sodium sulphate (Na_2SO_4). Florisil® was activated by drying it overnight at 130 °C. The column was activated by 10 mL HPLC-grade hexane. When the hexane was drained to just covering the Na_2SO_4, the flow was stopped. The 1 mL of sample extract was transferred to the top of the column, using a pre-cleaned Pasteur pipette (Koh *et al.*, 2005).

Analytes were separated from one another on the basis of polarity, with fraction 1 containing compounds with low polarity, and fraction 3 containing compounds with high polarity. The column was firstly eluted with a non-polar solvent, followed by solvents with increasing polarity. Hexane (100 mL) was used to elute compounds through the Florisil® column; this was the first fraction collected. A mixture of 20% DCM in hexane (100 mL) was then applied to the column and collected as fraction 2. Compounds remaining in the Florisil® column were eluted with 100 mL DCM-methanol (1:1), yielding fraction 3 (Koh *et al.*, 2005). The fractions were concentrated to 1 mL, from which a three-times dilutional series was prepared for use in the H4IIE-*luc* bio-assay (Khim, Kannan, Villeneuve, Koh & Giesy, 1999).

3.5. H4IIE-luc bio-assay

3.5.1. Maintenance of rat hepatoma H4IIE cell lines

The cells were a gift from Prof. John Giesy, then from the Michigan State University, USA. As part of the transportation requirements, cells were shipped in cryogenic vials, stabilised with a cryoprotective agent [dimethylsulphoxide (DMSO)] and frozen on dry ice (McFarland, McCant & Inouye, 1998). To avoid loss in cell viability, cells were stored in liquid nitrogen (at -180 °C), or cultured shortly upon receipt.

Sterile techniques were employed at all times, working in a bio-safety hood. All of the equipment and media used during cell maintenance were cleaned with 70% ethanol, to prevent possible contamination.

3.5.1.1. Starting the H4IIE rat hepatoma cell culture

Rat hepatoma cells were grown in sterile tissue culture dishes (100/20 mm, Greiner Bio-one). Culture dishes were prepared with 12 mL Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS). DMEM (Sigma cat no. D2906) containing L-glutamine, 5 mM HEPES and sodium bicarbonate, but not containing phenol red, was used, and will be referred to as supplemented stock media (Giesy, Jude, Tillit, Gale, Meadows, Zajieck, Peterman, Verbrugge, Sanderson, Schwartz & Tuchman, 1997).

Frozen cells in cryogenic vials were thawed, and cells were added to the tissue culture dish (already containing supplemented stock media) and aspirated well. The supplemented stock media diluted the vial contents and lowered the DMSO concentration so that the effect of DMSO on cells was insignificant. The culture dish was incubated for 24 hours in a water-jacketed incubator (ThermoForma series II, Labotec) at 37 °C and 95%/5% air/CO_2. After the 24-hour incubation period, the dish was microscopically inspected for confluency as well as contamination, and the cell media were changed. Visible signs of contamination include turbidity of the media, the presence of floating objects in media and the presence of microscopic objects with different morphology than cells (McFarland *et al.*, 1998). Cells were supplemented with fresh media every third day.

3.5.1.2. Passage of the cell line

Once the cells were 80% to 100% confluent, they were passaged to dilute the cell density. This provided more room for cell division and cell growth. Cells were perceived as confluent when their walls were wedged against one another, with little or no gaps between the cells (McFarland *et al.*, 1998).

Passaging of cells was performed under sterile conditions in the bio-safety hood. Culture dishes were prepared by adding 9 mL of supplemented stock media to each plate. Depleted media were removed from cells with a serological pipette and discarded. To restore the osmotic balance of cells, the cells were rinsed three times with Dulbecco's phosphate buffered saline (PBS, Sigma) without calcium- and magnesium-ions. After the PBS had been removed from cells, 1.5 mL trypsin was added to the dish. The role of trypsin was to hydrolyse the protein that caused cells to adhere to the tissue culture dish (Villeneuve, Richter, Blankenship & Giesy, 1999). The dish was incubated for three minutes. The trypsinisation process should not last longer than ten minutes, because the cells may be damaged by this enzyme (McFarland *et al.*, 1998).

After the 3 minutes had elapsed, the dish was tapped gently to loosen cells from the surface. To stop the action of the trypsin, 10.5 mL supplemented stock solution was added. Cells were aspirated well, to ensure that they were mixed properly into the media. Three millilitres of the cell suspension were then transferred to each of the prepared culture dishes. Tissue culture dishes were re-placed into the incubator (Hilscherova *et al.*, 2000).

3.5.2. Method of the H4IIE bio-assay

The bio-assay was performed over a period of five days. Each of the samples was assayed two to three times to confirm the consistency of results. During the bio-assay, cells were grown in DMEM, supplemented with 10% hormone-free foetal bovine serum.

On the first day of the bio-assay, 96-micro well plates (400 µL, Nunc) were seeded with a 0.25 mL suspension of cells (50 000 cells.mL^{-1}). The micro well plates were opaque white, had optical clear bottoms and lids, and were tissue culture treated. The outer wells of each plate acted as a buffer area to prevent a hydrostatic pressure from having an effect on cells. These wells were not seeded with cells, but 0.25 mL PBS without Ca^{2+} and Mg^{2+} was added to each well.

The plates were incubated for 24 hours at a temperature of 37 °C, and an air/carbon dioxide concentration of 95%/5% (Hilscherova *et al.*, 2000).

On the second day of the bio-assay, cells were dosed with 2.5 µL of the sample extract or standard. The standard used was 2,3,7,8-TCDD. The cells were exposed in triplicate to six different sample concentrations (1:1, 1:3, 1:9, 1:27, 1:81 and 1:243). The first concentration was pure extract and the following concentrations were 3 x dilutions (Giesy *et al.*, 1997). The TCDD standard, diluted in HPLC-grade hexane, was also dosed at six different concentrations (120.0, 30.0, 7.50, 1.88, 0.47 and 0.12 pg TCDD/well) (Fig 3.12). TCDD standards had to be separated from the samples by a solvent control and blank row to prevent cross-communication of cells. "Solvent control" wells were dosed with 2.5 µL of solvent (hexane) and "blank" wells contained only PBS. The plates were incubated for a 72-h time period under the same incubation conditions as stated earlier (Giesy *et al.*, 1997).

		TCDD standards			SC/ Blank	Sample A			Sample B			
A	1	2	3	4	5	6	7	8	9	10	11	12
B		TCDD (1:1)			Blank	Sample A (1:1)			Sample B (1:1)			
C		TCDD (1:4)			Blank	Sample A (1:3)			Sample B (1:3)			
D		TCDD (1:16)			Blank	Sample A (1:9)			Sample B (1:9)			
E		TCDD (1:64)			SC	Sample A (1:27)			Sample B (1:27)			
F		TCDD (1:256)			SC	Sample A (1:81)			Sample B (1:81)			
G		TCDD (1:1024)			SC	Sample A (1:243)			Sample B (1:243)			
H												

Outer wells were filled with PBS.

Figure 3.12. A schematic representation of a 96-micro well plate layout used in the bio-assay. Every second plate had to contain a TCDD standard.

The plates were inspected microscopically for viability and confluency on day 5, the final day of the bio-assay. The culture medium was removed and the cells were washed thrice with PBS with Ca^{2+} and Mg^{2+}. After adding 75 µL of Luclite™ reagent (Perkin Elmer) to each well, the cells were incubated for 10 minutes at the same incubating conditions as stated above. The plate was placed in a luminometer (Microplate fluorescence reader FLX 800, Bio-Tek Instruments Inc.) to measure the amount of light rays emitted by the cells. The sensitivity of the instrument was adjusted each time a plate was read to create optimum deviation from the background (Hilscherova *et al.*, 2001).

3.6. MTT viability bio-assay

3.6.1. Method for the MTT bio-assay:

Cell viability and proliferation greatly affect the reliability of results produced by *in vitro* assays. The MTT bio-assay is a reliable approach to examine cell proliferation, therefore determining if the samples were toxic to any extent, influencing the response of cells. This bio-assay functions on the principle of tetrazolium salt reduction (Oh, Livingston, Smith & Abrishamian-Garcia, 2004). Yellow tetrazolium salt (3-[4,5-dimethyltiazol-2yl]-2,5-diphenyl tetrazolium bromide) or MTT in short, is metabolically reduced by active cells resulting in the formation of intracellular purple formazan. The conversion of MTT tetrazolium to formazan is achieved by nicotinamide-adenine dinucleotide phosphate (NADPH) and nicotinamide-adenine dinucleotide (NADH), produced by active cells. The formazan can be dissolved by a tissue culture medium and quantified spectrophotometrically (Oh *et al.*, 2004).

Cell culturing, seeding, and dosing were performed with the same method used in the H4IIE-*luc* bio-assay, also using H4IIE-*luc* cells. On day five, the last day of the bio-assay, MTT solution was prepared. MTT solution should be prepared fresh on the day of the assay (Kangarloo, Gangopadhyay, Glück & Wolff, 2004). Plates were inspected microscopically. The cells were washed with PBS and 100 µL of the MTT solution was added to each well. The 96-well plates were incubated for 30 minutes at 37 °C and 5% CO_2 and 95% air.

After the 30-minute incubation period had elapsed, the plates were inspected microscopically for the development of formazan crystals. The yellow MTT solution was removed, and 200 µL DMSO (Saarchem, uniLAB) was added to each well. Plates were left to stand for 30 minutes to allow the crystals to dissolve in the DMSO. The plates were placed in a spectrophotometer (PowerWave X, Bio-Tek Instruments Inc.) to measure the absorbance at 492 nm and 645 nm (background) (Kangarloo *et al.*, 2004).

3.7. Determining the organic carbon content and lipid content of samples

Due to their hydrophobic characteristics, PCDD/Fs and PCBs tend to preferentially accumulate in lipid-rich tissues. For the same reason, these compounds generally associate more strongly with the organic fraction of the sediment than with the inorganic fraction (Nelson & Sommers, 1982). A higher organic carbon content of sediment, or lipid content of fish tissues, suggests that the matrix would have the potential to hold higher concentrations of dioxin-like substances than matrices with lower organic carbon or lipid contents. Therefore, the total organic carbon content (TOC) of sediment and lipid content (LC) of fish tissue were established to calculate normalised TCDD equivalent values, in order to compare samples with one another (Schmidt, 1995).

3.7.1. Organic carbon content determination of sediment samples

There are three basic forms of carbon that may be present in sediment, namely elemental carbon, inorganic carbon and organic carbon. Charcoal, graphite and coal are forms of elemental carbon and inorganic carbon is present in sediments as carbonates. Dioxins associate with the organic form of carbon (Schumacher, 2002).

The TOC content can be determined by a number of methods including analytical methods, qualitative methods, semi-quantitative methods and quantitative techniques. Quantitative techniques for TOC determination include destructive and non-destructive procedures. Currently, the most common methods used are destructive techniques, based on one of three basic principles:

(a) Wet oxidation followed by ferrous ammonium sulphate titration or photometric determination of Cr^{3+}.

(b) Wet oxidation followed by the accumulation and quantification of CO_2.

(c) High-temperature dry incineration, gathering and detecting CO_2 that developed during the process (Schumacher, 2002).

The Walkley-Black wet oxidation method was used to determine the TOC during the study. Advantages of this method include simplicity, minimal equipment needed and the fact that results can be obtained rapidly (Chan, Bowman & Oates, 2001). On the other hand, the procedure leads to the incomplete oxidation of organic carbon and is an inappropriate digester for elemental carbon. Therefore a correction factor of 1.33 is commonly applied. A correction factor of 1.4 were used during this study, because it yielded comparable TOC contents to other methods tested (Schumacher, 2002).

During execution of the Walkley-Black method, potassium dichromate (0.167 $mol.dm^{-3}$, Merck Pro analys) and 98% concentrated sulphuric acid (Merck) were added to 0.5 g – 1.0 g sediment sample. These ingredients were mixed gently and allowed to cool for 30 minutes. The sample heated up as a result of an exothermic reaction when $K_2Cr_2O_7$ and H_2SO_4 were mixed. After the solution had cooled down, distilled water was added to stop the reaction. Concentrated orthophosphoric acid (H_3PO_4) (Merck, univLAB) was added to the mixture to eliminate interferences from the ferric ion (Walkley, 1947). After 20 to 25 drops of barium diphenylamine indicator (BDH, Indicators^{TM}) were added to the solution, it was titrated with 0.5 $mol.dm^{-3}$ ferrous ammonium sulphate solution, until the colour changed from dull green to a turbid blue. The titrating solution was added drop by drop until the end point was reached when the colour shifted to a brilliant green. A blank sample (without sediment) was prepared and titrated in the same way (Walkley, 1947).

In this reaction carbon was oxidised by the dichromate ion. Excess dichromate ions were then back titrated with ferrous ions.
Dichromate ions react with carbon as follows:

$$K_2Cr_2O_7 + 3C + 16H^+ \rightarrow 4\ Cr^{3+} + 3CO_2 + 2H_2O$$

And ferrous ions react with dichromate:

$$6Fe^{2+} + Cr_2O_7^{2-} + 14H^+ \rightarrow 2Cr^{3+} + 6Fe^{3+} + 7H_2O$$

To calculate the percentage organic carbon in sediment the following equation was used:

$$\text{Org C\%} = \frac{[\text{mL } Fe(NH_4)_2(SO_4)_2 \text{ Blank} - \text{mL } Fe(NH_4)_2(SO_4)_2 \text{ Sample}] \times M \times 0{,}3 \times f}{\text{Mass of sediment sample (g)}}$$

mL $Fe(NH_4)_2(SO_4)_2$ Blank = amount of $Fe(NH_4)_2(SO_4)_2$ used to titrate the blank

mL $Fe(NH_4)_2(SO_4)_2$ Sample = amount of $Fe(NH_4)_2(SO_4)_2$ used to titrate the sample

M = the concentration of $Fe(NH_4)_2(SO_4)_2$ in mol/L

f = the correction factor of 1.4

Mass of sediment sample = between 0.5 to 1.0 grams of sediment used.

3.7.2. Lipid content (LC) determination of fish tissue samples

The most common methods for determining the lipid content of biological tissues include:

(a) Spectroscopic methods

(b) Enzymatic methods and

(c) Gravimetric methods

Spectroscopic method:

Spectroscopic methods are less frequently used for lipid content determination than gravimetric and enzymatic methods. Near-infrared (NIR) spectroscopy is the most commonly employed spectroscopic method to determine the lipid composition (Gangidi, Proctor & Meullenet, 2005). Since spectroscopic methods are not generally used for lipid content determination in fish, this method will not be discussed in detail.

Enzymatic method:

Enzymatic lipid content determination is performed by the summation of measured triglycerides, cholesterols and phospholipids in a sample. Triglyceride and cholesterol analyses are relatively rapid procedures that are performed routinely by hospitals. Phospholipid determinations are less accessible, since they are not commonly performed (Jönsson, Rylander, Rignell-Hydbom, Giwercman, Toft, Pedersen, Ludwick, Zvezday, Spanò, Bizzaro, Bonefeld-Jörgensen, Manicardi, Lindh, Bonde & Hagmar, 2003).

Enzymatic hydrolysis is employed for cholesterol and triglyceride determination, resulting in the production of a dye, which is measurable at 540 nm. The amount of dye produced is directly proportional to the amount of triglyceride and cholesterol in the sample (Grimvall, Rylander, Nilsson-Ehle, Nilsson, Strömberg, Hagmar & Östman, 1997).

Because phospholipids correlate to triglycerides and cholesterol in biological tissues, triglycerides and cholesterol can be used to determine the lipid content with the following equation (Weiss, Päpke, Bignert, Jensen, Greyerz, Agostoni, Besana, Riva, Giovannini & Zetterström, 2003):

Total lipid content = 0.9 + 1.3 x (C triglycerides + C cholesterol),

where C = concentration of triglycerides or cholesterol.

Gravimetric method:

Gravimetric methods are rapid, cost-effective methods for determining lipid content. This method has shown good correlations for LC calculation (Grimvall *et al.*, 1997).

The gravimetric method was used to determine the lipid content of the extract: Precisely 1 mL of extract was transferred to and weighed in a labelled foil measuring container. The foil container was placed in a desiccator for 24 hours and weighed after the hexane had evaporated, yielding the lipid mass of 1 mL sample extract (Honeycutt, McFarland & McCant, 1995).

The following equation was used to calculate the percentage of lipid in the fish tissue extract:

$$\% \text{ Lipids in extract (A)} = \frac{\text{Mass of measuring boat after desiccation} \times 100}{\text{Mass of measuring boat with extract before desiccation}}$$

The percentage of lipid in the fish tissue extract was converted to represent the amount of lipids per gram of extracted tissue (Schmidt, 1995).

3.8. Statistical analysis

3.8.1. H4IIE-*luc* bio-assay

Results obtained from the H4IIE-*luc* bio-assay were statistically analysed with Microsoft Excel XP, and used to calculate relative potencies, expressed as TCDD equivalent values. The statistical method used to calculate TCDD-equivalents will now be explained, with an example of a plate dosed with a TCDD standard and a sample spiked with 10 µL 2,3,7,8-TCDD at a concentration of 10 µg/mL (Sample A). After the micro well plate was inserted into the luminometer, a print-out of the amount of luciferase activity, measured in relative light units (RLUs), was obtained (Fig 3.13).

		TCDD standards			SC*/ BC	Sample A			Sample B			
A	1	2	3	4	5	6	7	8	9	10	11	12
B		1422.1	1624.2	1654.2	1176.3*	1858.1	1658.1	1923.5	1858.1	1658.1	1923.5	
C		1584.6	2276.7	1720.4	1145.2*	1574	2052.1	1433	1574	2052.1	1433	
D		1641.2	2285.6	1546.6	834.4*	1620.3	1325.3	1556.8	1620.3	1325.3	1556.8	
E		1129.3	1285.9	947.2	396.1	1035.3	1023.5	993.3	1035.3	1023.5	993.2	
F		800.3	453.0	462.7	222.3	786.3	540.6	307.0	786.3	540.6	307.0	
G		213.5	141.8	480.2	164.7	120.8	174.4	203.7	120.8	174.4	203.7	
H												

Figure 3.13. An example of a luminometer print-out containing RLU-values. The plate layout is specified on the figure indicating which wells were dosed with TCDD, solvent controls (SC*), blank controls (BC) or sample.

This data was applied to compile tables for each of the TCDD standards. The tables contained information regarding the amount of standard per well, average RLUs, standard deviation, coefficient of variation (CV), and percentage TCDD maximum (Table 3.5). The % TCDD max expressed the amount of luciferase activity induced by each sample concentration, relative to the maximal luciferase activity caused by the TCDD standard (Schramm, Klimm, Hofmaier & Kettrup, 2001).

Solvents can induce luciferase activity in cells. To bring this effect of the solvent into consideration, the average RLU of the solvent control should be deducted from each RLU. If this was done, the RLU responses of samples had negative values and were inadequate. Therefore the effect of the SC was not taken into account (Besselink, Schipper, Klamer, Leonards, Verhaar, Felzel, Murk, Thain, Hosoe, Schoeters, Legler & Brouwer, 2004).

Negative RLU values would be the first indication of no or very little PCDD/Fs and PCBs in the extract. But by not deducting the SC, it is still possible to obtain a useful dose-response curve from which one may at least learn tendencies.

Table 3.5. An example of a table showing the conversion of the measured RLUs to % TCDD maximum for wells dosed with the TCDD standard.

Amount of TCDD/well (pg/well)	log pg TCDD/ well	RLU	RLU	RLU	Mean RLU	Standard deviation	Coefficient of variation	%TCDD max
120	2.08	1422.11	1624.23	1654.21	1566.83	126.24	8.06	84.21
30	1.48	1584.62	2276.76	1720.41	1860.57	366.72	19.71	100.00
7.5	0.88	1641.21	2285.62[¢]	1546.61	1593.90	66.89	4.20	85.67
1.88	0.27	1129.35	1285.91	947.25	1120.83	169.47	15.12	60.24
0.47	-0.33	800.30[¢]	453.06	462.73	457.87	6.84	1.49	24.61
0.12	-0.93	213.51	141.81	480.22[¢]	177.70	50.69	28.53	9.55

[¢] Values were dropped from calculations to improve the CV-values.

Each variable was calculated with Microsoft Excel XP. The CV had to be less than, or as close as possible to, 20, to have acceptable variation as higher values indicate unreliable data. When CV-values were greater than 20, one of the RLU-values was dropped from the calculations to improve the CV-value (Whyte, Schmitt & Tillit, 2004). Dose-response curves were plotted for the standard with the log amount of TCDD plotted on the x-axis, and the response of cells relative to the highest TCDD RLU (% TCDD max) on the y-axis (Fig 3.14).

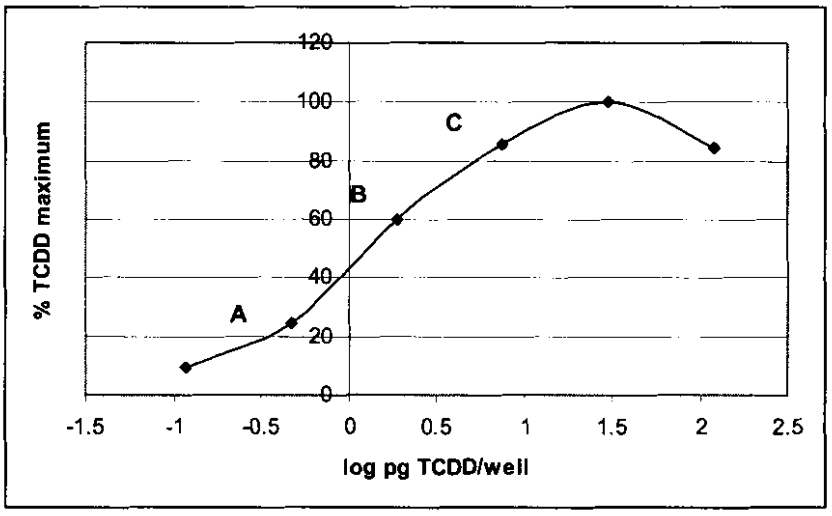

Figure 3.14. TCDD standard graph: The percentage TCDD maximum at different amounts of TCDD added to each well. A, B and C together represent a linear line on the graph.

These curves were used to calculate the amount of TCDD that was responsible for the 20%, 50% and 80% response of cells. The linear part of the curve was used to calculate the slope, intercept and correlation coefficient using the equation y = mx + c. For this purpose, at least three data points, lying on the linear line, were chosen. The data points had to be selected beyond the 20% TCDD max and below the point where the line flattened (data points A, B and C in Fig 3.14). The y-intercept had to be calculated before the corresponding x-intercepts for the 20%, 50% and 80% values could be calculated, yielding the effective concentrations (ECs) – EC20, EC50 and EC80.

The data obtained from different sediment or fish tissue samples were processed in the same way as the TCDD standards, and a table was drawn for each sample (Table 3.6). Dose-response curves were drawn for each sample, by plotting the log amount of sample (log µL sample per well) against the response of cells relative to the highest TCDD RLU (% TCDD maximum) (Fig 3.15). The EC20, EC50 and EC80 were calculated for the samples in the same way as described for calculating TCDD EC20, EC50 and EC80 (Table 3.7).

Table 3.6 An example of a table showing the conversion of the measured RLUs to % TCDD maximum for wells dosed with a "spiked" sample (Sample A).

Amount of sample/well (µL/well)	log pg sample/ well	RLU	RLU	RLU	Mean RLU	Standard deviation	Coefficient of variation	%TCDD max
2.5	0.40	1858.10	1658.13	1923.51	1813.23	138.27	7.63	98.13
0.83	-0.08	1574.00	2052.12	1433.00	1686.36	324.49	19.24	91.26
0.28	-0.56	1620.31	1325.33	1556.83	1500.81	155.27	10.34	81.22
0.099	-1.03	1035.30	1023.51	993.28	1017.37	21.65	2.13	55.06
0.03	-1.51	786.32	540.58	307.02	663.50	173.70	26.18	35.91
0.01	-1.99	120.86	174.42	203.74	189.11	20.72	10.96	10.23

The EC of the samples (µL per well) had to be converted to TCDD-equivalents to express the amount of TCDD represented by a certain amount of sample. This is termed relative potency values (REP). A REP20, REP50 and REP80 were calculated by dividing the TCDD EC-values by sample EC-values (Table 3.7).

For example: To calculate the REP20 of a sample the following equation was used:
REP20 = TCDD EC20 (pg TCDD/well) / Sample EC20 (µL sample/well)
= pg TCDD-equivalents/µL **or** pg TEQ/µL.

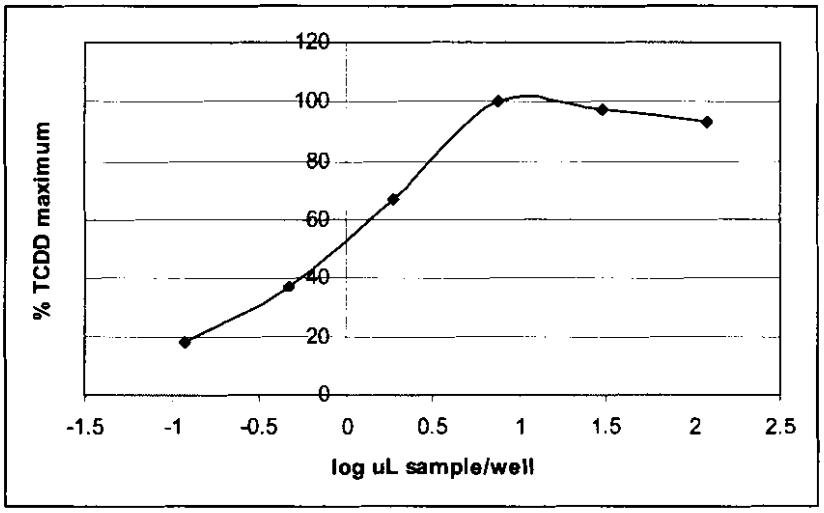

Figure 3.15. Statistical analysis example of the spiked sample (Sample A): The percentage TCDD max at different sample concentrations.

By taking the amount of sediment or fish tissue extracted into account, the REP-values were converted from pg TEQ/µL to pg TEQ/mg sample. Many authors report TCDD-equivalents in ng TEQ/kg sample (WHO, 1997), therefore that is the unit we standardised on. Thus, the relative potencies (REPs) calculated with the H4IIE bio-assay, had to be adapted to ng TEQ/kg sample (Table 3.7).

Table 3.7. The effective concentrations (ECs) and relative potencies (REPs) calculated for the TCDD standard and the spiked sample (Sample A).

	EC 20	EC 50	EC 80	REP 20	REP 50	REP 80
	µL sample/well			ng TEQ/kg sediment		
TCDD standard	0.27	1.42	7.41			
Sample A	0.015	0.06	0.28	899	1097	1338

3.8.2 MTT viability bio-assay

Microsoft Excel XP was used for the statistical analysis of MTT data. The absorbance of cells was measured spectrophotometrically at 492 and 645 nm (Fig 3.16 and 3.17) and the data were imported into Excel. The method used to calculate the viability of cells will be described by using an example.

		Sample A			SC/ Blank	Sample B			Sample C			
A	1	2	3	4	5	6	7	8	9	10	11	12
B		0.987	0.986	1.014	1.175	1.087	1.049	1.059	1.003	0.975	0.989	
C		1.034	1.003	0.972	0.958	0.974	0.924	0.967	0.968	1.019	1.024	
D		1.048	0.944	0.852	0.847	0.922	0.877	0.907	0.888	0.925	1.026	
E		1.029	1.005	0.934	0.935	0.935	0.891	0.969	0.945	0.993	0.901	
F		1.047	1.052	0.993	0.952	0.962	0.921	0.952	0.963	0.978	0.987	
G		1.057	0.962	1.035	0.949	1.084	0.962	1.045	1.105	0.936	1.071	
H												

Figure 3.16. An example of a spectrophotometer print-out containing data of the absorbance of cells measured at **492 nm** in each well. The plate layout is indicated on the figure and wells that were dosed with solvent are bordered.

		Sample A			SC/ Blank	Sample B			Sample C			
A	1	2	3	4	5	6	7	8	9	10	11	12
B		0.552	0.55	0.557	0.58	0.596	0.552	0.546	0.539	0.56	0.571	
C		0.558	0.557	0.552	0.575	0.576	0.545	0.548	0.54	0.54	0.553	
D		0.566	0.559	0.551	0.565	0.578	0.546	0.545	0.54	0.537	0.551	
E		0.56	0.566	0.557	0.573	0.573	0.54	0.553	0.542	0.541	0.541	
F		0.56	0.561	0.557	0.576	0.575	0.536	0.55	0.541	0.535	0.548	
G		0.554	0.553	0.561	0.569	0.581	0.549	0.551	0.55	0.527	0.552	
H												

Figure 3.17. An example of a spectrophotometer print-out containing data of the "background absorbances" of cells measured at **645 nm** in each well. The plate layout is indicated on the figure and wells that were dosed with solvent are bordered

The absorbances measured at 645 nm indicate the "background absorbances". To eliminate the effects of background readings on the data, the absorbances measured at 645 nm had to be subtracted for the absorbances measured at 492 nm, for each well.

The percentage viability of the cells was calculated by dividing the absorbances of each well by the average solvent control absorbances, and multiplying it by 100 to convert it to a percentage value. This data was used to draw up a table, which contained information regarding the amount of sample per well, the average percentage viability for each well, the standard deviation, and the coefficient of variation (Table 3.8). Values in the table were calculated using Microsoft Excel XP. As with the H4IIE-*luc* bio-assay analysis, the coefficient of variation had to be below 20.

The tables were used to draw a graph (Fig 3.18) of the effect of different sample concentrations on the cells. The logarithms of the amount of sample/well (log µL sample/well) were plotted on the x-axis of the graph, and the percentage viability was plotted on the y-axis of the graph. The standard deviation was incorporated into the graph, and if the viability of cells was below 80%, it was assumed that the cell viability was affected by the sample.

Table 3.8. An example of a table containing data regarding Sample A (Fig 3.16 and 3.17):

Amount of sample/well (µL/well)	log µL sample/well	% Viability	% Viability	% Viability	Mean % viability	Standard deviation	Coefficient of variation
2.5	0.40	103.57	103.81	108.81	105.40	2.96	2.80
0.83	-0.08	113.33	106.19	100.00	106.51	6.67	6.26
0.28	-0.56	114.76	91.67	71.67	92.70	21.57	23.26
0.093	-1.03	111.67	104.52	89.77	101.98	11.17	10.95
0.03	-1.51	115.95	116.90	103.81	112.22	7.30	6.51
0.01	-1.99	119.76	97.38	112.86	110.00	11.46	10.42

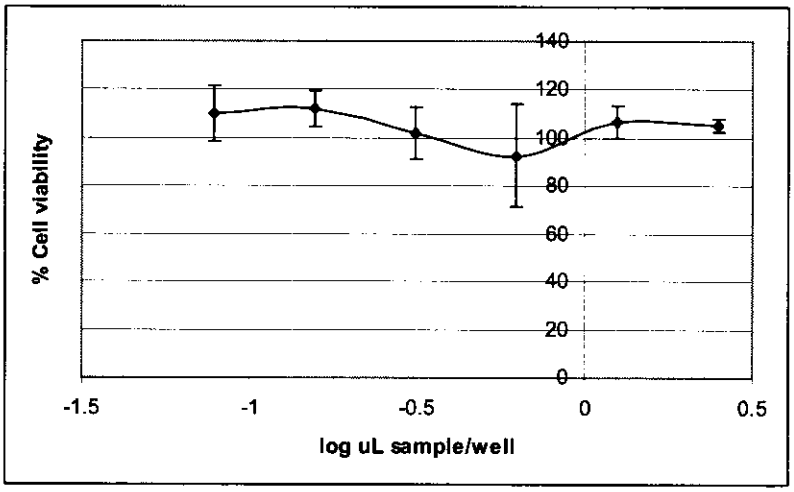

Figure 3.18. MTT statistical analysis example of Sample A: The percentage viability of cells at different sample concentrations.

3.8.3. Calculating the limit of detection (LOD)

The limit of detection can be defined as the smallest concentration of a substance that can be accurately detected, with a specific technique (in this case the H4IIE-*luc* bio-assay), but not necessarily quantified as an exact value. "Detected", in this context, suggests that a sample contains an amount of substance that is statistically different (with 95% certainty) from a blank (Van der Voet, 2002). The detection limit is matrix, method and analyte specific (Thomsen, Schatzlein & Mercuro, 2003).

The LOD was calculated by firstly determining the average TCDD concentration at which no response was elicited from the cell. Thus, the average EC0 for the entire study's standard response curves was calculated. The 95% confidence interval was subsequently determined and added to the average. This latter value was converted to ng TEQ/kg sample in the same way already explained in Section 3.8.1.

Chapter 4. Results

4.1. Bio-analysis results

The H4IIE-*luc* bio-assay was used to quantify the amount of dioxin-like substances in sediment and fish tissue samples, by measuring the amount of light emitted by cells (Section 3.5.2). The relative light units (RLUs) were converted to TCDD equivalent values (Section 3.8).

4.1.1. Sediment samples

The sediment sample extracts produced the following results when analysed with the H4IIE bio-assay (Table 4.1). The detection limit for sediment extracts was 1 – 2 ng TEQ/kg.

Table 4.1. Information regarding the TOC, cell viabilities, correlation coefficient and amount of dioxin-like substances in sediment sample extracts.

Site nr.	Total organic carbon content (%)	Cell viability	Correlation coefficient (R^2)	EC values (µL/well)	REP values (pg TEQ/µL)	TCDD-equivalents (ng TEQ/kg sample)
1	5.06	CT (43%)	-	-	-	TLTC^{+}
8	4.90	N	-	-	-	TLTC^{+}
9	14.58	N	0.95	EC20 = 0.31 EC50 = 1.11 EC80 = 3.97	REP20 = 0.52 REP50 = 1.05 REP80 = 2.12	TCDDeq20 = 25.81 TCDDeq50 = 52.35 TCDDeq80 = 106.19
11	4.92	CT (46%)	-	-	-	TLTC^{+}
12	3.22	N	-	-	-	TLTC^{++}
13	1.54	N	-	-	-	TLTC^{+}
15	2.71	N	-	-	-	TLTC^{+}
16	2.92	CT (72%)	-	-	-	TLTC^{+}
17	4.06	N	-	-	-	TLTC^{++}
18	1.77	N	-	-	-	TLTC^{+++}
20	0.75	N	-	-	-	TLTC^{+++}
22	1.02	N	-	-	-	TLTC^{+}

TLTC: Too low to calculate: The amount of dioxin-like substances in the samples was too low to quantify.

The sample elicited a maximum cell response of: $^{+}$ below 20%, $^{++}$ 20% ≥ 25% or $^{+++}$ 25% ≥ 35% (Table 4.2).

Cell viability: N = normal (above 80%), CT = cytotoxic - % viability indicated in brackets.

- No values calculated.

In order to quantify the amount of dioxins in samples, EC-values and REP-values had to be calculated. For this purpose, at least three data points beyond the 20% TCDD maximum mark, on the linear line of the dose-response graph, were needed. If the amount of the dioxin-like substances in samples were too low to comply with the above-mentioned requirements, REP-values could not be calculated. Although some of the samples produced one data point beyond 20% TCDD maximum (Sites 12, 17, 18 and 20) (Table 4.2), this was not sufficient for calculating TCDD-equivalents. This is what is referred to as "too low to calculate" (TLTC) in Table 4.1. Since the same amount of sediment (40.00 g) was extracted from each site, the % TCDD maximum values produced by each site's sediment extract are comparable.

The only sample that triggered a response in the cells, which was high enough to quantify, was sediment collected from Site 9. This sample's dose-response graph will be discussed in greater detail in Section 4.1.1. (Fig 4.1). The other sediment samples produced insignificant cell responses, with the samples' highest TCDD maximum values ranging from 35% to undetectable (Table 4.1 and 4.2).

Since PCDD/Fs and PCBs bind to the organic components of sediments, the organic carbon content of each site's sediment had to be taken into consideration (Schumacher, 2002). A high TOC suggests that sediments would have the potential to hold higher concentrations of dioxin-like substances, than sediments containing less organic carbon. The Walkley-Black method was used to calculate the percentage TOC of sediments (Table 4.1). Site 9 had the highest TOC and also produced a quantifiable amount of response from the cells. However, since the other sites produced little response, it was impossible to determine the correlation of the carbon content of a sample to its TCDD equivalent value.

Another factor which was taken into account during the analysis of samples was the viability of cells, which was determined via microscopic inspection and an MTT-viability bio-assay. Cell viabilities below 80%, suggested that the cells were affected by cytotoxic samples. It seemed that most of the samples had no toxic effects on cells, since the cells exhibited normal viabilities. However, Samples 1, 11 and 16 appeared to be toxic to cells, since cells subjected to the highest dosing concentrations (2.5 µL sample/well) of these samples had viabilities below 80% (Table 4.1). It was necessary to determine the viability of the cells after exposure to possible contaminants.

The amount of light emitted by cells with viabilities less than 80% could not be considered to produce meaningful RLU values, since these RLUs are not accurate indications of the amount of dioxin-like substances in the sample. Fewer living cells would produce lower emissions of light compared to a 100% viable well. In this instance the low response would not be due to low dioxin-like compound concentrations, but rather fewer cells.

4.1.1.1. Site 9 - Blesbok Spruit

Compared to the other sites, Site 9 had the highest TOC value of 14.58%. This high value suggests that sediment from this site would have the potential to hold larger amounts of PCDD/Fs and PCBs than the rest of the sites, since the amount of organic carbon of sediments correlates with the amount of dioxin-like substances that sediment can bind with (Schumacher, 2002).

The sediment extract collected from Site 9 produced three points beyond the 20% TCDD maximum mark on the linear section of the dose-response graph and could therefore be quantified (Fig 4.1).

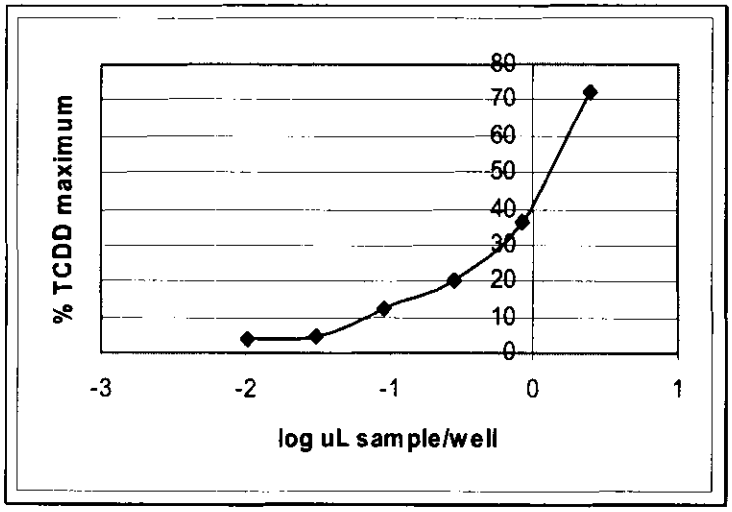

Figure 4.1. Dose-response graph calculated for Site 9 with the cell response, expressed as % TCDD maximum values, at different sample concentrations.

The correlation coefficient (R^2) of the straight line through the coordinates of the highest three concentrations is 0.95, which suggests a good correlation with the linear line. The upward slope of the dose-response graph indicates a tendency of increasing cell response at higher concentrations of sediment extract. The highest dosing concentration (2.5 µL sample/well) triggered a response in cells equal to 72.43% of the TCDD maximum. The REP20 and REP50 were calculated to be 25.81 ngTEQ/kg and 52.35 ngTEQ/kg, respectively and the extrapolated REP80 was 106.19 ngTEQ/kg (Table 4.1).

All of the cells appeared to be viable when visually inspected, and this was confirmed by the MTT-viability test. In this regard, the results produced by the bio-assay were accepted as reliable.

The sediment sample extracts (Table 4.2) were fractionated. This was done to investigate the possibility that some compounds' effects on the cells might have been masked because of synergistic or antagonistic interactions among compounds in the raw extract. Fractionation would also help to characterise the type of compounds responsible for the reaction by the cells. That is, are the compounds responsible for the response non-polar, slightly polar or polar?

4.1.1.2. Fractionation results

Each of the raw samples was fractionated into three separate parts. The fractions were separated on the basis of polarity, with fraction 1 containing compounds with no polarity and fraction 3 containing compounds with high polarity. The method of this process is described in Section 3.4. Sample 13's extract was not fractionated, because of the very low response elicited by its raw extract (Table 4.2).

Table 4.2. A summary of the response of the cells when exposed to the sediment fractions, expressed as % TCDD maximum.

Site nr.	Fraction 1 (F1)		Fraction 2 (F2)		Fraction 3 (F3)		Σ (F1 – F3)	Raw sample
	% TCDD max	Cell viability	% TCDD max	Cell viability	% TCDD max	Cell viability	% TCDD max	% TCDD max
1	6.89	CT (51%)	26.92	N	15.87	N	49.68	14.12
8	7.23	N	13.36	N	10.33	N	30.92	14.71
9	4.13	N	17.28	N	19.11	N	40.52	72.43
11	3.22	CT (59%)	7.78	N	10.55	N	21.55	12.67
12	2.34	N	3.46	N	4.23	N	10.03	20.64
13	NF	-	NF	-	NF	-	-	6.93
15	4.15	N	24.84	N	8.96	N	37.98	11.59
16	4.26	N	3.88	CT (76%)	4.55	N	12.69	11.82
17	5.46	N	10.54	N	6.89	N	22.89	22.84
18	5.21	N	16.97	N	7.82	N	30.00	29.97
20	3.56	N	16.55	N	6.33	N	26.44	26.28
22	3.89	N	5.67	N	5.68	N	15.24	15.35
Average	**4.58**		**13.39**		**9.12**			

Cell viability: N = normal, CT = cytotoxic - % viability indicated in brackets.

NF = Not fractionated. - No values calculated.

The contribution of each fraction towards the AhR binding capability of the raw sample was determined with the H4IIE bio-assay. The bio-assay results indicated that none of the fractions contained quantifiable amounts of dioxin-like substances (Table 4.2), not even the fractions of Sample 9 of which the raw extract produced high cell responses (Table 4.1).

The %TCDD maximum values produced by fractions 1, 2 and 3 were totalled and compared to the %TCDD maximum values produced by the raw samples (Table 4.2). The totalled response of Sample 9 and Sample 12 was lower than the % TCDD maximum of their corresponding raw extract. For Samples 1, 8, 11 and 15, the totalled responses were higher than the response elicited by the corresponding raw extract. In some samples the two values corresponded well, and the sum of the fractions was a good reflection of the % TCDD maximum produced by the raw sample (Samples 16, 17, 18, 20 and 22) (Table 4.2). The MTT-viability assay indicted that the fractions of Samples 1, 11 and 16 were less than 80% viable (Table 4.2). This corresponded to the cytotoxicity measured in the raw extracts (Table 4.1).

By examining the average % TCDD maximum values of each fraction (Table 4.2), it seems that the more polar fractions, fraction 2 and fraction 3, were responsible for the highest contributions of dioxin-like substances. The average TCDD maximum for fraction 2 was 13.39% and the average for fraction 3 was 9.12% (Fig 4.2). The data set was tested for normality, and since the data was non-parametric the Kruskal-Wallis ANOVA test (Statistica 7.1) was used to determine if fractions differed statistically significantly from one another (Table 4.3). Each fraction was compared to the two other fractions, and p-values smaller than 0.05 indicated that samples differed statistically significantly from one another. Fraction 1 was significantly different from fraction 2 and 3. However, fraction 2 and 3 did not differ significantly from one another. The average values, standard deviations and standard errors of these values are indicated in Figure 4.2.

Table 4.3. Multiple comparison p-values of the %TCDD max values of fraction 1, 2 and 3.

p-values	Fraction 1	Fraction 2	Fraction 3
Fraction 1	-	**0.004**	**0.027**
Fraction 2	**0.004**	-	1.000
Fraction 3	**0.027**	1.000	-

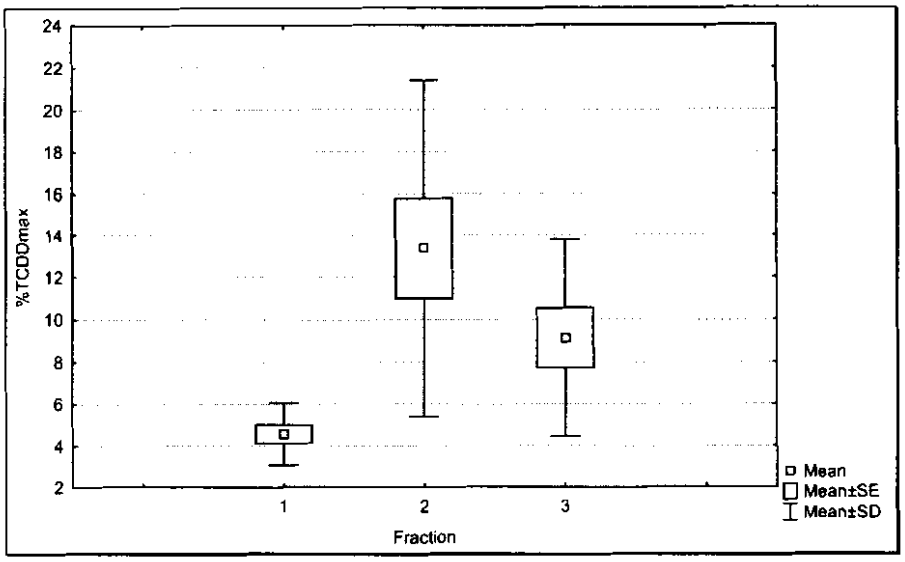

Figure 4.2. The average % TCDD maximum values elicited by each of the three fractions.

4.1.2. Determination of bio-accumulation

One of the aims of the study was to investigate the bio-accumulation of PCDD/Fs and PCBs, reported for by these chemicals in literature. This was done by comparing the quantities of dioxin-like pollutants in sediment and fish tissue to one another. For this purpose, sediment and fish samples were collected, on the same day, from the Suikerbosrand River (1F) and Blesbok Spruit (2F) (Fig 3.2 and Table 3.2). The samples were analysed with the H4IIE bio-assay.

4.1.2.1. Sediment samples

Once again, three data points greater than 20% TCDD maximum are needed to calculate TCDD-equivalents, in order to quantify the amount of dioxin-like substances in a sample. Again, the responses from raw extracts, as well as the fractions, were so low that no TCDD-equivalents could be calculated (Table 4.4). The response of the two sites can be compared by referring to the TCDD maximum values of each site. This is allowable because the same mass (40.0 g) of sediment was extracted for both sites.

The TOC values for Site 1F and Site 2F were found to be 5.11% and 8.01%, respectively. Results from the MTT-viability assay indicated that all of the cells dosed with raw and fractionated sediment extracts did not inhibit cell viability (Table 4.4). The sum of the three fractions' %TCDD maximum values was higher than the %TCDD maximum of the raw samples for both Site 1F and Site 2F (Table 4.4).

Table 4.4. Information regarding sediment samples collected from Site 1F and Site 2F.

Site nr.	% TOC	Cell viability	Response elicited by highest dosing concentration of the sample (% TCDD maximum)	
1F Suikerbosrand River	5.11			
Raw sample		N	11.84	
Fraction 1		N	5.57	*sum = 31.73
Fraction 2		N	18.97	
Fraction 3		N	7.19	
2F Blesbok Spruit	8.01			
Raw sample		N	14.52	
Fraction 1		N	27.07	*sum = 42.11
Fraction 2		N	10.73	
Fraction 3		N	4.31	

Cell viability: N = normal (above 80%).

* Sum. The %TCDD max values of fraction 1, 2 and 3 were totalled to compare it to the raw sample.

4.1.2.2. Fish tissue samples

A total of 18 fish were caught at Site 1F (Suikerbosrand River), of which nine belonged to the species *L. umbratus* (6 females, 3 males) and nine to *L. capensis* (nine males). From the Blesbok Spruit sampling site (2F), only ten fish were sampled, of which eight belonged to *L. umbratus* (6 females, 2 males) and two to *L. capensis* (females). The fish were caught during spawning season and most of the fish were sexually mature.

To minimise intra-species variation, it would have been ideal to have ten individuals per composite group, with three replicates of each group (Heath et al., 2004). However, since the amount of fish caught were a limiting factor, fish were divided into composite groups consisting of two to three individuals each. Some composite groups were alike (Group 1 and 2 and Group 6 and 7) containing individuals belonging to the same species, gender and size class (Table 4.5).

4.1.2.2.1. Fish health assessment

During the study, a limited Fish Health Assessment was performed in order to link possible POPs exposure to the general health of fish. According to the health assessment performed during the study, the lowest possible FHAI-value a fish could obtain was 0 and the highest possible score was 190 (Table 3.3). Compared to lower FHAI scores, higher scores indicate that fish had possibly been subjected to water of poorer quality (Table 4.5).

Table 4.5. Fish health assessment indexes and other information regarding fish sampled from the Blesbok Spruit and Suikerbosrand River.

Blesbok Spruit										
Composite group	**Fish ID**	**Eyes**	**Skin**	**Fins**	**Opercula**	**Gills**	**Liver**	**Bile**	**Spleen**	**FHAI score**
Group 1 *L. umbratus* Females	1	0	10	20	0	0	0	0	0	30
	2	0	10	20	0	0	0	0	0	30
	3	0	10	10	0	0	0	0	0	20
Group 2 *L. umbratus* Females	4	0	10	10	0	0	0	0	0	20
	5	0	10	10	0	0	0	0	0	20
	6	0	10	20	0	0	0	0	0	30
Group 3 *L. umbratus* Males	7	0	0	10	0	0	0	0	0	10
	8	0	10	10	0	0	0	0	0	20
Group 4 *L. capensis* Females	9	0	10	10	0	0	0	0	0	20
	10	0	0	10	0	0	0	0	0	10
Average										**21.00**
Standard deviation										**7.38**
Suikerbosrand River										
Group 5 *L. umbratus* Males	11	0	10	0	0	0	30	0	0	40
	12	0	0	0	0	0	0	0	0	0
	13	0	30	20	0	0	0	0	0	50
Group 6 *L. umbratus* Females	14	0	10	10	0	0	0	0	0	20
	15	0	10	20	0	0	0	0	0	30
	16	0	10	10	0	0	0	0	0	20
Group 7 *L. umbratus* Females	17	0	10	20	0	0	0	0	0	30
	18	0	0	20	0	0	0	0	0	20
	19	0	10	20	0	0	0	0	0	30
Group 8 *L. capensis* Males (small)	20	0	0	10	0	0	0	0	0	10
	21	0	10	0	0	0	30	0	0	40
	22	0	0	0	0	0	30	0	0	30
Group 9 *L. capensis* Males (medium)	23	0	0	10	0	0	30	0	0	40
	24	0	10	10	0	0	0	0	0	20
	25	0	10	10	0	0	0	0	0	20
Group 10 *L. capensis* Males (large)	26	0	10	20	0	0	0	0	0	30
	27	0	10	10	0	0	0	0	0	20
	28	0	10	20	0	0	0	0	0	30
Average										**26.67**
Standard deviation										**11.89**

FHAI-values = Fish Health Assessment Index values

The eyes of all 28 fishes were normal and only mild skin aberrations were observed in most of the fish, except for fish 13, which suffered severe skin aberrations. Twenty four of the 28 fish had badly damaged fins with active erosion, haemorrhage and/or secondary infection occurring in eight of these cases. Both the opercula and gills were in good condition. The bile and spleen of fish were normal, but four of the fish had fatty livers (Table 4.5).

A mean FHAI score of 21.00 was calculated for Blesbok Spruit (2F) and 26.67 for Suikerbosrand River (1F). The FHAI-value of individuals varied between 10 and 30 for Site 2F, and between 0 and 50 for Site 1F (the standard deviations of Site 1F and 2F were 11.89 and 7.38, respectively) (Table 4.5).

4.1.2.2.2. CFs of fish

The total length and mass of fish ranged from 350 mm – 510 mm and 350 g – 1400 g at Site 2F, and from 170 mm – 510 mm and 54.3 g – 1500 g at Site 1F, indicating greater variance in fish collected from Site 1F (Table 4.6).

The CF values of fish sampled from Blesbok Spruit (Site 2F) varied from 0.816 – 1.635, with a mean CF of 1.059. These values were similar to the CF values calculated for the reference site (Site 1F). Fish collected from the Suikerbosrand River had CF values distributed between 0.843 – 3.52, with a mean value of 1.222. The standard deviation for Site 2F was 0.220 and Site 1F was 0.574 (Table 4.6).

Although the values calculated for the majority of fish collected from the Suikerbosrand River were generally similar, one individual (fish nr. 24) had a high CF, which affected the mean value of Site 1F (Table 4.6).

Table 4.6. The CFs calculated from the length and mass of fish sampled from Blesbok Spruit and Suikerbosrand River.

Blesbok Spruit				
Composite group	**Fish ID number**	**Total length (mm)**	**Mass (g)**	**CF**
Group 1	1 2 3	510 460 510	1400 1000 1200	1.055 1.027 0.905
Group 2	4 5 6	430 480 495	1300 1000 1400	1.635 0.904 1.154
Group 3	7 8	390 510	600 1200	1.011 0.905
Group 4	9 10	440 350	1000 350	1.174 0.816
Average **Standard deviation**				**1.059** **0.221**
Suikerbosrand River				
Group 5	11 12 13	460 490 500	1000 1300 1200	1.027 1.105 0.960
Group 6	14 15 16	480 510 510	1200 1500 1500	1.085 1.131 1.131
Group 7	17 18 19	520 460 510	1500 1000 1300	1.067 1.027 0.980
Group 8	20 21 22	180 180 170	76.8 64.3 54.3	1.317 1.103 1.105
Group 9	23 24 25	230 250 390	113 550 500	0.929 3.52 0.843
Group 10	26 27 28	490 410 460	1200 800 1450	1.019 1.161 1.489
Average **Standard deviation**				**1.222** **0.574**

4.1.2.2.3 Bio-analysis results

Since the sediment samples had shown no detectable TCDD equivalent values (Table 4.1), it was not surprising to have found no detectable TCDD-equivalents in any of the fish tissue samples either. In order to compare TCDD maximum values, the same mass of tissue had to be extracted. This was not the case for gonadal and hepatic tissue. Equal masses of fillet tissue (10.0 g) were extracted for both of the sites (Table 4.7 and 4.8).

In order to facilitate the comparison of fish tissues to one another, and fish tissues to sediment extracts, extrapolation of % TCDD maximum values potentially produced by 40 grams of each tissue was attempted. Since none of the assayed liver and gonad samples produced responses in cells beyond 20% TCDD maximum, these values could not be used as reliable indications of PCDD/F or PCB presence in extracts, and must be treated with caution. Even cells dosed with solvent only (solvent control wells) may have produced % TCDD maximums such as these. The % TCDD maximum values of fish tissue could not be compared to that of the sediment, because of the difference in mass of extracted matrices.

Even if it was not possible to calculate TCDD-equivalents for any of the fish tissues, at least one of the fillet composite groups (group 10) demonstrated a tendency of increasing cell response at higher concentrations of sample extracts. The % TCDD maximum values calculated for this group's fillet tissue were also slightly higher than that of the other tissue composite groups. Extrapolations of these values were used in a scenario-based risk assessment (Section 4.1.2.2.4), but since only limited data was available, and since extrapolations are based on assumptions, the data was not used to determine bio-accumulation.

The detection limit for fish samples ranged from 3 - 7 ng TEQ/kg for fillet tissues, 3 - 165 ng TEQ/kg for livers, and 2 – 23 ng TEQ/kg and 3 – 60 ng TEQ/kg, for female and male gonads, respectively. The detection limit is dependent of the extracted mass, and very small masses of liver tissue were extracted (Table 4.7 and 4.8). The smaller the mass of the tissue extracted, the bigger the limit of detection.

The fish tissue samples were not fractionated, since the amount of dioxin-like substances in fish tissue was extremely low, eliciting cell responses ranging from 4.82 – 14.47% TCDD maximum (Table 4.7 and 4.8). The lipid content of tissue samples ranged from 5.72 – 13.03% for fillet tissues, 6.05 – 27.29% for livers, and 3.46 – 89.29% for gonads (Table 4.7).

Table 4.7. A summary of the lipid content determination, MTT-cell viability assay and the H4IIE bio-assay results for fish collected from Blesbok Spruit (2F).

Composite group nr.	Dry mass of tissue extracted (g)	Lipid content (%)	Cell viability	Highest response elicited (%TCDD max)
1. *L. umbratus* females				
Fillet tissue	10.02	5.89	N	8.16
Liver	9.95	7.61	N	7.15
Gonads	19.49	3.46	N	5.28
2. *L. umbratus* females				
Fillet tissue	10.03	6.51	N	6.63
Liver	10.07	6.05	N	5.13
Gonads	18.84	3.56	N	4.82
3. *L. umbratus* males				
Fillet tissue	10.01	5.82	N	9.11
Liver	2.93	17.45	N	5.77
Gonads	9.19	5.73	N	6.60
4. *L. capensis* females				
Fillet tissue	10.00	5.95	N	7.30
Liver	2.72	23.74	N	7.86
Gonads	1.36¤	45.88	N	6.60

Cell viability: N = Normal

¤ = A part of the sample was lost because of accidental spillage during extraction.

Table 4.8. A summary of the lipid content determination, MTT-cell viability assay and the H4IIE bio-assay results for fish collected from Suikerbosrand River (1F).

Composite group nr.	Dry mass of tissue extracted (g)	Lipid content (%)	Cell viability	Highest response elicited (%TCDD max)
5. *L. umbratus* males				
Fillet tissue	10.03	6.52	N	5.34
Liver	3.76	14.95	N	6.86
Gonads	10.43	5.88	N	8.03
6. *L. umbratus* females				
Fillet tissue	10.08	6.16	CT (72%)	14.47
Liver	4.38	11.29	N	7.82
Gonads	10.37	5.70	N	12.17
7. *L. umbratus* females				
Fillet tissue	10.01	5.85	N	10.05
Liver	5.64	10.39	N	8.45
Gonads	13.86	4.59	N	12.71
8. *L. capensis* males (small)				
Fillet tissue	4.3	13.03	N	8.62
Liver	0.19	27.29	N	11.06
Gonads	0.56	89.29	N	8.79
9. *L. capensis* males (medium)				
Fillet tissue	10.18	5.72	N	6.13
Liver	0.52	10.23	N	7.48
Gonads	2.13	24.59	N	8.76
10. *L. capensis* males (large)				
Fillet tissue	10.00	5.96	N	10.21
Liver	4.81	10.82	N	9.89
Gonads	6.21	9.70	N	13.34

Cell viability: N = Normal, CT = cytotoxic - % viability indicated in brackets.

Most of the sample extracts had no effect on cell viability, but the extract from composite group 6's fillet tissue sample was cytotoxic to cells, resulting in a 72% viability of cells.

4.1.2.2.4. Scenario-based risk assessment

When humans consume dioxin-contaminated fish, they are potentially exposed to PCDD/Fs and PCBs, accumulated in these fish tissues. Once present in the human body, PCDD/Fs and co-planar PCBs may cause several health effects, including cancer (US EPA, 2002a). To estimate the probability of cancer developing in humans exposed to dioxin-like substances via fish ingestion, a scenario-based risk assessment was done. Six scenarios and three different concentrations of dioxin-like substances were used for this purpose (Table 4.9).

Table 4.9. Six different scenarios to calculate possible dioxin-related human health risk.

Scenario 1: A 70 kg adult eating 100 g of fish each day for 10 years.	**Scenario 4:** A 35 kg child eating 100 g of fish each day for 10 years.
Scenario 2: A 70 kg adult eating 100 g of fish 3 times a week, for 10 years.	**Scenario 5:** A 35 kg child eating 100 g of fish 3 times a week, for 10 years.
Scenario 3: A 70 kg adult eating 100 g of fish once a week, for 10 years.	**Scenario 6:** A 35 kg child eating 100 g of fish once a week, for 10 years.

The scenario-based risk assessment was based on the following assumptions:

- The section of fish most commonly consumed by humans is fillet tissue (Isosaari, Hallikainen, Kiviranta, Vuorinen, Parmanne, Koistinen & Vartiainen, 2006), therefore bio-assay results of fish fillets were extrapolated and used to calculate possible human health risks.

- Since health risk estimations are calculated using wet weights of fish, a wet-dry tissue ratio of 1 g :0.314 g was calculated for *Labeo* species and potential % TCDD maximum values for 100 grams of wet fillet tissue, were extrapolated.

- Bio-assay results of fish in composite group 10's fillet tissues were used to extrapolate results, since this sample produced the best dose-response graph: the line of the dose-response graph was linear (R^2 = 0.96) and had a tendency of increasing cell response (% TCDD maximum) at higher concentrations of fillet tissue extract.

- According to the extrapolation, a concentration of 71.03 ng/kg dioxin-like substances might have been present in these fillet tissues. Since dioxin-like substances in fish tissues may be broken down by food preparation processes such as cooking, the risk assessment values were also calculated at concentrations of 7.1 and 0.71 ng/kg. The limit of detection for fish fillets was re-calculated for the extrapolated data, at 0.99 ng/kg.

- The following calculations were used to estimate the cancer risk of PCDD/F and PCB exposure (Heath *et al.*, 2004). Since no reference dose values are available for dioxin-like substances, the non-cancerous effects of these substances could not be estimated.

Firstly, the average daily dose (ADD), in mg/kg/day, was calculated with the following formula:

$$ADD = \frac{C_m \times IR_m \times ED}{BM \times AT}$$

Where C_m was the average concentration of pollutant in the food substance (mg/kg), IR_m was the average intake rate (kg/day), and ED was the exposure duration (days). In the risk assessment done, the ED was equal to the amount of days on which contaminated fish were consumed, over a 10 year period (eg. ED = 365 days x 10 years, if contaminated fish were eaten every day). BM was body mass (kg) and AT was averaging time (days). AT was the time period of the risk assessment, in this case 10 years (eg. AT = 10 years x 365 days, for each of the six scenarios.

The ADD, as well as the exposure duration (ED) and the expected lifetime (LT), both expressed as the amount of days, were then used to calculate the lifetime average daily dose (LADD) (mg/kg/day), with the following formula. An average life expectancy of 70 years was used.

$$LADD = \frac{ADD \times ED}{LT}$$

Finally, to establish the potential dioxin-related cancer risk (CR) of consuming contaminated fish, the following calculation was done:

$$CR = Sf \times LADD$$

The slope factor (Sf) of dioxin-like substances is 1.56×10^5 mg/kg/day (US EPA, 2003). The slope factor is pollutant-specific, and can be defined as the part of a 95% confidence interval extrapolated to represent effects at low doses.

The slope factor for dioxins and furans, recommended by the US EPA (2003), is that of 2,3,7,8-TCDD, the most carcinogenic dioxin congener. Thus, the cancer risks estimated are generally expected to be higher than would be expected for the other, less toxic dioxin, furan and PCB congeners.

Table 4.10. A summary of each risk associated value for each of the six scenarios calculated for a TCDD equivalent of 71.03×10^{-6} ng/mg.

	Average daily dose (ADD) (mg/kg/day)	Lifetime average daily dose (LADD) (mg/kg/day)	Cancer risk (CR)
Scenario 1	9.73×10^{-8}	1.30×10^{-8}	*208: 100 000
Scenario 2	4.50×10^{-9}	2.86×10^{-9}	44.5: 100 000
Scenario 3	1.50×10^{-8}	3.17×10^{-10}	4.95: 100 000
Scenario 4	1.95×10^{-7}	2.69×10^{-8}	416: 100 000
Scenario 5	9.01×10^{-8}	5.71×10^{-9}	89.1: 100 000
Scenario 6	3.00×10^{-8}	6.35×10^{-10}	9.9: 100 000

*A CR of e.g. 208: 100 000 would mean that 208 out of 100 000 exposed humans would have the risk of contracting cancer when dioxin-contaminated fish are consumed.

Table 4.11. A summary of each risk associated value for each of the six scenarios calculated for a TCDD equivalent of 7.10×10^{-6} ng/mg.

	Average daily dose (ADD) (mg/kg/day)	Lifetime average daily dose (LADD) (mg/kg/day)	Cancer risk (CR)
Scenario 1	9.73×10^{-9}	1.30×10^{-9}	20.8: 100 000
Scenario 2	4.50×10^{-10}	2.86×10^{-10}	4.45: 100 000
Scenario 3	1.50×10^{-9}	3.17×10^{-11}	0.50: 100 000
Scenario 4	1.95×10^{-8}	2.69×10^{-9}	41.6: 100 000
Scenario 5	9.01×10^{-9}	5.71×10^{-10}	8.91: 100 000
Scenario 6	3.00×10^{-9}	6.35×10^{-11}	0.99: 100 000

Table. 4.12. A summary of each risk associated value for each of the six scenarios calculated for a TCDD equivalent of 0.71×10^{-6} ng/mg.

	Average daily dose (ADD) (mg/kg/day)	Lifetime average daily dose (LADD) (mg/kg/day)	Cancer risk (CR)
Scenario 1	9.73×10^{-10}	1.30×10^{-10}	2.08: 100 000
Scenario 2	4.50×10^{-11}	2.86×10^{-11}	0.45: 100 000
Scenario 3	1.50×10^{-10}	3.17×10^{-12}	0.05: 100 000
Scenario 4	1.95×10^{-9}	2.69×10^{-10}	4.16: 100 000
Scenario 5	9.01×10^{-10}	5.71×10^{-11}	0.89: 100 000
Scenario 6	3.00×10^{-10}	6.35×10^{-12}	0.1: 100 000

4.2. Comparison of chemical analysis and bio-analysis results

Some sediment samples were chemically analysed to confirm the results of the H4IIE bio-assay. Sediment was collected from Site 15, 16, 17 and 20 during June 2006. The motivation for choosing these four sites for GC/MS analysis was the following: Site 15 and 16 were selected as the reference sites for minimal dioxin-like pollution, and Site 17 and 20 were chosen due to their locality in close proximity of potential dioxin-producers. Selection of samples to be sent away for chemical analysis was done prior to H4IIE-analysis of the initial sediment samples. For this reason, Site 9, the only site with quantifiable amounts of dioxin-like substances, was not targeted for chemical analysis. The samples were shipped to the Norwegian Institute for Air Research (NILU) to be analysed with GC/MS. Only a few samples were analysed by GC/MS, since chemical analysis is very expensive. The methods for analysis employed by NILU were accredited according to ISO/IEC – 17025.

The same samples were also extracted and analysed with the H4IIE-*luc* bio-assay, allowing for comparison of results produced by these methods.

4.2.1. Bio-analysis results

Bio-analysis of the four samples produced the following results:

Table 4.13. The amount of dioxin-like substances in samples analysed with the H4IIE bio-assay.

Site nr.	Cell viability	TCDD-equivalents (ng TEQ/kg sample)	Highest response elicited by cells (% TCDD max)
15	Normal	Too low to calculate	14.52
16	Normal	Too low to calculate	14.90
17	Normal	Too low to calculate	14.20
20	Normal	Too low to calculate	15.96

The amount of dioxin-like substances in sediment samples was too low to calculate. Three data points greater than 20% TCDD maximum are needed to calculate TCDD equivalent values. None of the samples elicited cell responses beyond this point and the highest % TCDD maximum values, produced by these samples, ranged from 14.20 – 15.96 (Table 4.13). The limit of detection of the assay for these sediments was 1 ng TEQ/kg.

4.2.2. Chemical analysis results

GC/MS analysis is a sensitive method which can accurately measure the concentrations of each individual congener. The TEQ values of each of the dioxin-like congeners were calculated for mammals, fish and birds, by multiplying the concentration of each congener by its WHO TEF value (Table 4.2) (Table 4.14 – 4.16). To determine the total TEQ of each site's dioxin-like compounds, the TEQ values of the congeners were totalled, assuming an additive effect.

Table 4.14. The concentration and TEQs of PCDD/F and PCB congeners in samples analysed by GC/MS analysis. TEQs were calculated according to WHO TEF-values for mammals/humans.

Congener	Site 15		Site 16		Site 17		Site 20	
	Concentration (ng/kg)	TEQ (ng/kg)	Concentration (ng/kg)	TEQ (ng/kg)	Concentration (ng/kg)	TEQ (ng/kg)	Concentration (ng/kg)	TEQ (ng/kg)
Dioxins								
2,3,7,8-TCDD	0.02	0.02	0.03	0.03	0.03	0.03	0.01	0.01
1,2,3,7,8-PeCDD	0.04	0.04	0.03	0.03	0.03	0.03	0.04	0.04
1,2,3,4,7,8-HxCDD	0.04	0.00	0.03	0.00	0.02	0.00	0.04	0.00
1,2,3,6,7,8-HxCDD	0.09	0.01	0.04	0.00	0.03	0.00	0.05	0.01
1,2,3,7,8,9-HxCDD	0.11	0.01	0.03	0.00	0.03	0.00	0.05	0.00
1,2,3,4,6,7,8-HpCDD	0.96	0.01	0.25	0.00	0.13	0.00	0.45	0.00
OCDD	7.34	0.00	2.64	0.00	0.72	0.00	2.15	0.00
Total	**8.60**	**0.09**	**3.05**	**0.08**	**0.99**	**0.06**	**2.79**	**0.07**
Furans								
2,3,7,8-TCDF	0.07	0.01	0.03	0.00	0.03	0.00	0.07	0.01
1,2,3,7,8/1,2,3,4,8PeCDF	0.14	0.01	0.06	0.00	0.04	0.00	0.09	0.00
2,3,4,7,8-PeCDF	0.06	0.03	0.03	0.02	0.02	0.01	0.06	0.03
1,2,3,4,7,8/1,2,3,4,7,9-HxCDF	0.12	0.01	0.03	0.00	0.02	0.00	0.09	0.01
1,2,3,6,7,8-HxCDF	0.07	0.01	0.02	0.00	0.02	0.00	0.05	0.01
1,2,3,7,8,9-HxCDF	0.22	0.02	0.09	0.01	0.05	0.01	0.03	0.00
2,3,4,6,7,8-HxCDF	0.08	0.01	0.03	0.00	0.02	0.00	0.04	0.00
1,2,3,4,6,7,8-HpCDF	0.71	0.01	0.12	0.00	0.08	0.00	0.29	0.00
1,2,3,4,7,8,9-HpCDF	0.07	0.00	0.05	0.00	0.02	0.00	0.03	0.00
OCDF	0.76	0.00	0.24	0.00	0.11	0.00	0.41	0.00
Total	**2.30**	**0.10**	**0.7**	**0.04**	**0.14**	**0.03**	**1.16**	**0.07**
PCBs								
3,3',4,4'-TeCB (PCB-77)	2.22	0.00	0.77	0.00	0.77	0.00	5.12	0.00
3,4,4',5-TeCB (PCB-81)	0.11	0.00	0.06	0.00	0.04	0.00	0.21	0.00
3,3',4,4',5-PeCB (PCB-126)	0.44	0.04	0.05	0.01	0.04	0.00	0.39	0.04
3,3',4,4',5,5'-HxCB (PCB-169)	0.10	0.00	0.03	0.00	0.02	0.00	0.03	0.00
Total	**2.87**	**0.04**	**0.91**	**0.01**	**0.87**	**0.00**	**5.75**	**0.04**
Σ (PCDD, PCDF, PCB)	**13.77**	**0.23**	**4.66**	**0.13**	**2.27**	**0.09**	**9.70**	**0.18**

Table 4.15. The concentration and TEQs of PCDD/F and PCB congeners in samples analysed by GC/MS analysis. TEQs were calculated according to WHO TEF-values for fish (Table 2.4).

Congener	Site 15		Site 16		Site 17		Site 20	
	Concentration (ng/kg)	TEQ (ng/kg)	Concentration (ng/kg)	TEQ (ng/kg)	Concentration (ng/kg)	TEQ (ng/kg)	Concentration (ng/kg)	TEQ (ng/kg)
Dioxins								
2,3,7,8-TCDD	0.02	0.02	0.03	0.03	0.03	0.03	0.01	0.01
1,2,3,7,8-PeCDD	0.04	0.04	0.03	0.03	0.03	0.03	0.04	0.04
1,2,3,4,7,8-HxCDD	0.04	0.02	0.03	0.02	0.02	0.01	0.04	0.02
1,2,3,6,7,8-HxCDD	0.09	0.00	0.04	0.00	0.03	0.00	0.05	0.00
1,2,3,7,8,9-HxCDD	0.11	0.00	0.03	0.00	0.03	0.00	0.05	0.00
1,2,3,4,6,7,8-HpCDD	0.96	0.00	0.25	0.00	0.13	0.00	0.45	0.00
OCDD	7.34	0.00	2.64	0.00	0.72	0.00	2.15	0.00
Total	**8.60**	**0.08**	**3.05**	**0.08**	**0.99**	**0.07**	**2.79**	**0.07**
Furans								
2,3,7,8-TCDF	0.07	0.00	0.03	0.00	0.03	0.00	0.07	0.00
1,2,3,7,8/1,2,3,4,8PeCDF	0.14	0.01	0.06	0.00	0.04	0.00	0.09	0.00
2,3,4,7,8-PeCDF	0.06	0.03	0.03	0.02	0.02	0.01	0.06	0.03
1,2,3,4,7,8/1,2,3,4,7,9-HxCDF	0.12	0.01	0.03	0.00	0.02	0.00	0.09	0.01
1,2,3,6,7,8-HxCDF	0.07	0.01	0.02	0.00	0.02	0.01	0.05	0.01
1,2,3,7,8,9-HxCDF	0.22	0.02	0.09	0.00	0.05	0.00	0.03	0.00
2,3,4,6,7,8-HxCDF	0.08	0.01	0.03	0.00	0.02	0.00	0.04	0.00
1,2,3,4,6,7,8-HpCDF	0.71	0.01	0.12	0.00	0.08	0.00	0.29	0.00
1,2,3,4,7,8,9-HpCDF	0.07	0.00	0.05	0.00	0.02	0.00	0.03	0.00
OCDF	0.76	0.00	0.24	0.00	0.11	0.00	0.41	0.00
Total	**2.30**	**0.09**	**0.7**	**0.03**	**0.14**	**0.02**	**1.16**	**0.06**
PCBs								
3,3',4,4'-TeCB (PCB-77)	2.22	0.00	0.77	0.00	0.77	0.00	5.12	0.00
3,4,4',5-TeCB (PCB-81)	0.11	0.00	0.06	0.00	0.04	0.00	0.21	0.00
3,3',4,4',5-PeCB (PCB-126)	0.44	0.00	0.05	0.00	0.04	0.00	0.39	0.00
3,3',4,4',5,5'-HxCB (PCB-169)	0.10	0.00	0.03	0.00	0.02	0.00	0.03	0.00
Total	**2.87**	**0.00**	**0.91**	**0.00**	**0.87**	**0.00**	**5.75**	**0.00**
Σ (PCDD, PCDF, PCB)	**13.77**	**0.18**	**4.66**	**0.11**	**2.27**	**0.09**	**9.70**	**0.13**

Table 4.16. The concentration and TEQs of PCDD/F and PCB congeners in samples analysed by GC/MS analysis. TEQs were calculated according to WHO TEF-values for birds (Table 2.4).

Congener	Site 15		Site 16		Site 17		Site 20	
	Concentration (ng/kg)	TEQ (ng/kg)	Concentration (ng/kg)	TEQ (ng/kg)	Concentration (ng/kg)	TEQ (ng/kg)	Concentration (ng/kg)	TEQ (ng/kg)
Dioxins								
2,3,7,8-TCDD	0.02	0.02	0.03	0.03	0.03	0.03	0.01	0.01
1,2,3,7,8-PeCDD	0.04	0.04	0.03	0.03	0.03	0.03	0.04	0.04
1,2,3,4,7,8-HxCDD	0.04	0.00	0.03	0.00	0.02	0.00	0.04	0.00
1,2,3,6,7,8-HxCDD	0.09	0.01	0.04	0.00	0.03	0.00	0.05	0.01
1,2,3,7,8,9-HxCDD	0.11	0.00	0.03	0.00	0.03	0.00	0.05	0.00
1,2,3,4,6,7,8-HpCDD	0.96	0.00	0.25	0.00	0.13	0.00	0.45	0.00
OCDD	7.34	0.00	2.64	0.00	0.72	0.00	2.15	0.00
Total	**8.60**	**0.08**	**3.05**	**0.06**	**0.99**	**0.06**	**2.79**	**0.05**
Furans								
2,3,7,8-TCDF	0.07	0.07	0.03	0.03	0.03	0.03	0.07	0.07
1,2,3,7,8/1,2,3,4,8PeCDF	0.14	0.01	0.06	0.01	0.04	0.00	0.09	0.01
2,3,4,7,8-PeCDF	0.06	0.06	0.03	0.03	0.02	0.02	0.06	0.06
1,2,3,4,7,8/1,2,3,4,7,9-HxCDF	0.12	0.01	0.03	0.00	0.02	0.00	0.09	0.01
1,2,3,6,7,8-HxCDF	0.07	0.01	0.02	0.00	0.02	0.00	0.05	0.01
1,2,3,7,8,9-HxCDF	0.22	0.02	0.09	0.01	0.05	0.01	0.03	0.00
2,3,4,6,7,8-HxCDF	0.08	0.01	0.03	0.00	0.02	0.00	0.04	0.00
1,2,3,4,6,7,8-HpCDF	0.71	0.01	0.12	0.00	0.08	0.00	0.29	0.00
1,2,3,4,7,8,9-HpCDF	0.07	0.00	0.05	0.00	0.02	0.00	0.03	0.00
OCDF	0.76	0.00	0.24	0.00	0.11	0.00	0.41	0.00
Total	**2.30**	**0.20**	**0.7**	**0.08**	**0.14**	**0.07**	**1.16**	**0.16**
PCBs								
3,3',4,4'-TeCB (PCB-77)	2.22	0.1	0.77	0.03	0.77	0.04	5.12	0.25
3,4,4',5-TeCB (PCB-81)	0.11	0.01	0.06	0.01	0.04	0.00	0.21	0.02
3,3',4,4',5-PeCB (PCB-126)	0.44	0.04	0.05	0.01	0.04	0.00	0.39	0.04
3,3',4,4',5,5'-HxCB (PCB-169)	0.10	0.16	0.03	0.00	0.02	0.00	0.03	0.00
Total	**2.87**	**0.44**	**0.91**	**0.05**	**0.87**	**0.04**	**5.75**	**0.31**
Σ (PCDD, PCDF, PCB)	**13.77**	**0.72**	**4.66**	**0.20**	**2.27**	**0.17**	**9.70**	**0.53**

For mammals/humans, fish and birds, Site 15 and Site 17, had the highest and lowest total TEQ values, respectively (Fig 4.3 – 4.5). The total TEQs of the four sites ranged from 0.09 – 0.23 ng/kg for mammals, 0.09 – 0.18 ng/kg for fish, and 0.17 – 0.72 ng/kg for birds (Table 4.14 – 4.16). The TEQs calculated for birds, were generally the highest (Fig 4.5), implying that the same concentration of dioxin-like substances are more toxic to birds than to mammals and fish.

Table 4.17. The total concentrations and TEQ-values of the PCDD/F and PCB congeners for mammals, fish and birds, collected at Sites 15, 16, 17 and 20.

Site number	Congener group	Total concentration (ng/kg)	TEQ (ng/kg)		
			Mammals/Humans	Fish	Birds
15	PCDD	8.60	0.09	0.08	0.08
	PCDF	2.30	0.10	0.09	0.20
	PCB	2.87	0.04	0.00	0.44
16	PCDD	3.05	0.08	0.08	0.06
	PCDF	0.7	0.04	0.03	0.08
	PCB	0.91	0.01	0.00	0.05
17	PCDD	0.99	0.06	0.07	0.06
	PCDF	0.14	0.03	0.02	0.07
	PCB	0.87	0.00	0.00	0.04
20	PCDD	2.79	0.07	0.07	0.05
	PCDF	1.16	0.07	0.06	0.16
	PCB	5.75	0.04	0.00	0.31

The total TEQs of each congener group, for mammals, fish and birds, were calculated, and the four sites compared to one another. Except for Site 15, where the TEQ of PCDF was slightly higher, PCDD was responsible for the highest TEQ through-out the other three sites' TEQ values for mammals/humans and fish (Fig 4.3 and 4.4). For birds, PCBs and PCDFs were responsible for the highest TEQ values at Sites 15 and 20, and Sites 16 and 17, respectively (Fig 4.5 and Table 4.17). PCDFs were the most toxic congener group to mammals, fish and birds, yielding a higher TEQ relative to its concentration, when compared to PCDDs and PCBs (Table 4.17). Generally, PCDFs and PCBs are more toxic to birds than to mammals/humans and fish, producing higher total TEQ-values for birds (Table 4.17).

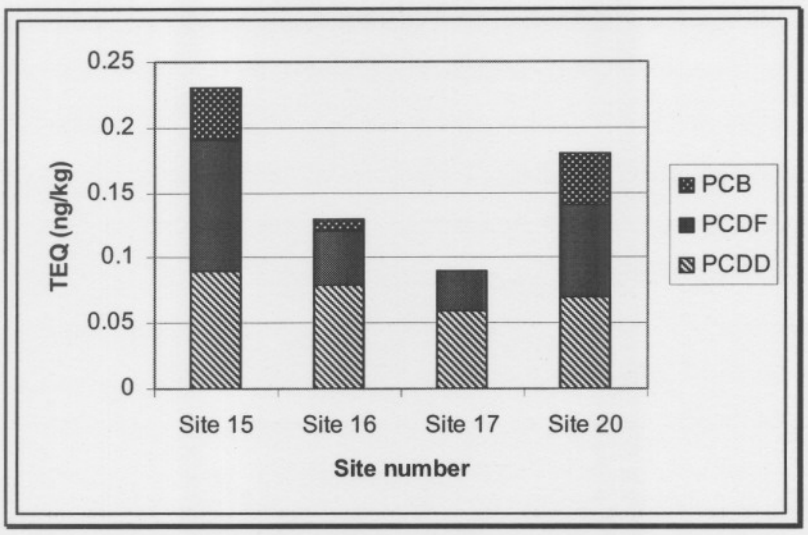

Figure 4.3. The total TEQs of dioxin-like substances for humans/mammals, for the sediment sites.

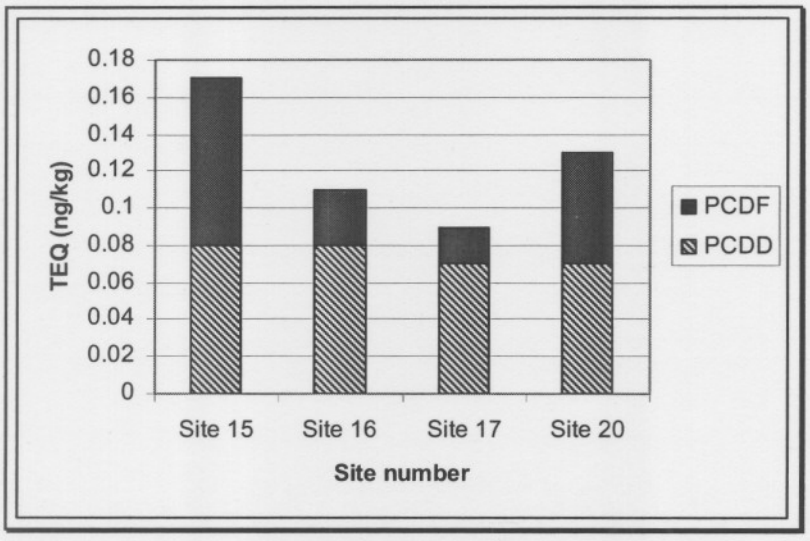

Figure 4.4. The total TEQs of dioxin-like substances for fish, for the four sediment sites.

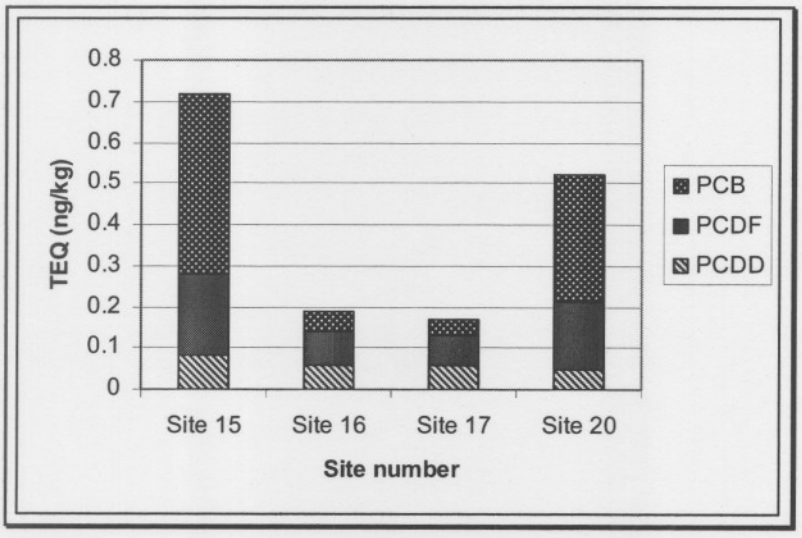

Figure 4.5. The total TEQs of dioxin-like substances for birds, for the four sediment sites.

4.3. Meteorological data

Because seasonal and meteorological changes can be linked to the amount of dioxin-like chemicals in the environment (Moon, Lee, Choi & Ok, 2005), the average monthly maximum and minimum temperatures, amount of precipitation and wind direction were taken into consideration for this project.

Vereeniging was chosen as a reference site to report the climatic conditions of the Vaal Triangle, since it is located centrally. Daily meteorological data of Vereeniging for the period of 1996 to 2006 were obtained from the South African Weather Service and used to calculate average monthly values for each variable (Table 4.18 – 4.21).

4.3.1. Ambient temperatures

Table 4.18. The average monthly maximum temperatures (°C) of Vereeniging recorded from 1996 to 2006 (South Africa Weather Services, 2006).

	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	Average
January	27.6	27.1	27.5	28.3	25.7	30.4	29.0	29.8	27.0	26.0	27.1	**27.8**
February	25.8	29.5	27.2	30.3	26.0	27.4	27.8	29.0	26.0	26.9	26.6	**27.5**
March	26.0	23.4	28.2	29.1	26.1	27.7	28.1	28.4	23.7	25.7	24.1	**26.4**
April	22.2	21.9	27.5	26.3	22.6	24.2	26.7	26.9	22.9	23.1	22.9	**24.3**
May	20.0	18.7	22.6	21.9	20.1	22.2	21.7	21.6	22.0	22.6	18.9	**21.1**
June	18.9	18.2	21.9	19.9	19.1	18.5	17.4	18.5	17.3	21.4	18.9	**19.1**
July	16.3	18.5	20.5	19.7	18.5	18.5	18.5	19.5	16.7	20.7	21.3	**19.0**
August	19.9	22.9	22.1	21.8	22.9	23.5	22.4	20.4	21.7	24.0	19.5	**21.9**
September	25.3	23.6	26.2	24.1	24.9	24.2	24.5	26.1	22.2	28.2	24.9	**24.9**
October	27.4	25.4	25.8	26.6	27.3	26.7	28.4	27.6	26.3	29.1	27.9	**27.1**
November	25.5	26.9	26.4	32.3	25.8	25.8	28.8	27.8	29.6	28.5	NDA	**27.7**
December	27.8	28.8	26.5	28.7	NDA	27.0	27.6	30.4	26.6	28.8	NDA	**28.0**

NDA = No data available.

The Vaal Triangle area has warm summers (December – February) with average maximum temperatures ranging from 27.5 – 28 °C and average minimum temperatures ranging from 14.6 – 15.0 °C. The winter months (June – August) also have relatively high maximum temperatures in the order of 19.1 – 21.9 °C, but the minimum temperatures are much lower, dropping to as low as 0.7 °C in July. Spring (September – November) and autumn (March – May) have moderate temperatures (Table 4.18 and 4.19).

The temperatures of the Vaal Triangle are generally high, which may lead to the removal and degradation of PCDD/Fs and PCBs in environmental compartments.

Table 4.19. The average monthly minimum temperatures (°C) of Vereeniging recorded from 1996 to 2006 (South Africa Weather Service, 2006).

	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	Average
January	15.3	15.2	14.9	15.6	14.9	15.2	15.9	16.5	15.5	9.4	16.8	**15.0**
February	15.1	15.0	14.7	15.2	16.0	15.3	15.5	17.1	14.0	8.6	16.2	**14.8**
March	11.0	13.9	14.3	14.3	15.6	13.8	13.4	12.3	11.4	11.2	12.8	**13.1**
April	8.0	6.7	9.3	10.2	9.4	10.7	9.8	10.2	6.8	9.8	8.9	**9.1**
May	5.1	2.8	2.8	5.3	3.8	5.2	4.5	4.1	1.5	4.1	2.2	**3.8**
June	-0.2	0.6	-0.6	0.6	3.0	1.4	2.9	0.7	-2.0	2.5	-0.2	**0.8**
July	0.4	1.9	1.7	2.1	0.6	1.4	0.1	-0.3	-2.3	0.1	2.2	**0.7**
August	4.2	3.7	3.6	3.1	3.7	4.6	7.6	3.1	3.6	5.6	3.8	**4.2**
September	7.2	9.5	10.4	7.6	9.5	8.2	8.8	9.4	5.6	8.9	7.3	**8.4**
October	13.0	11.0	12.5	11.0	13.3	13.2	12.1	12.6	9.4	12.8	12.9	**12.1**
November	12.9	12.7	14.0	15.2	13.0	14.6	12.9	14.4	12.3	14.4	NDA	**13.6**
December	14.5	15.0	14.5	16.1	NDA	15.1	15.5	14.8	11.0	14.7	NDA	**14.6**

NDA = No data available.

4.3.2. Average rainfall

Since PCDD/Fs and PCBs in the atmosphere reach the earth by means of precipitation (Corsolini *et al.*, 2002), the average monthly rainfall of the area was also calculated.

Table 4.20. The average monthly rainfall (mm) of Vereeniging recorded from 1996 to 2006.

	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	Average
January	154.0	39.4	147.2	77.6	101.6	27.6	149.6	45.0	123.4	173.2	154	**108.4**
February	112.8	27.4	25.2	29.8	133.6	112.2	52.6	68.8	85.0	41.6	99.2	**71.7**
March	35.8	176.2	94.2	45.8	123.0	33.0	13.4	57.0	22.2	120.8	89.8	**73.7**
April	46.2	34.6	0.8	28.0	28.6	27.8	24.0	6.0	26.6	73.4	38.7	**30.4**
May	9.4	89.4	0.0	26.4	41.2	26.4	36.4	0.0	0.0	5.2	5.2	**21.8**
June	0.0	7.2	0.0	2.0	7.4	0.0	22.2	4.2	10.6	0.0	0.0	**4.9**
July	1.2	0.2	0.0	0.6	0.0	0.0	0.0	0.0	5.0	0.0	0.0	**0.6**
August	6.2	7.8	0.0	0.0	0.0	5.8	21.4	11.8	23.8	0.2	22.2	**9.0**
September	2.2	60.2	66.0	20.2	32.6	27.6	11.4	2.2	0.0	0.2	3.8	**20.6**
October	67.4	48.0	103.2	31.6	103.0	116.6	14.2	36.6	28.2	12.6	32.0	**53.9**
November	69.0	198.6	190.8	46.4	62.0	93.4	15.4	67.8	28.4	99.8	NDA	**87.2**
December	149.4	62.4	115.8	79.4	NDA	NDA	151.4	45.0	125.6	52.6	NDA	**97.7**

The highest average monthly rainfall of 87.2 – 108.4mm occurred during the summer months (November – February) and the lowest average monthly rainfall of 0.6 – 9.0 mm occurred during the winter (June – August) (Table 4.20).

4.3.3. Wind direction

Because PCDD/Fs and PCBs can be carried by winds for several kilometres from their emission source (Lohman & Signeur, 2001), the wind's directional prevalence had to be established.

Table 4.21. Wind directional prevalence (%) of Vereeniging, calculated from average monthly data from 1992 – 2004 (South African Weather Service, 2006).

	Wind frequency from each direction (%)																
	Calm	**N**	**NNE**	**NE**	**ENE**	**E**	**ESE**	**SE**	**SSE**	**S**	**SSW**	**SW**	**WSW**	**W**	**WNW**	**NW**	**NNW**
January	6	11	8	10	11	12	7	3	2	2	2	2	3	5	4	5	7
February	7	10	7	11	12	13	6	4	2	2	2	3	3	4	3	4	7
March	12	11	6	11	10	8	5	3	2	3	2	3	3	4	3	5	9
April	13	14	6	6	5	5	2	2	2	3	3	4	4	6	4	7	14
May	19	14	3	3	3	3	2	2	2	3	3	5	5	6	4	7	16
June	13	15	3	4	3	3	1	1	2	3	3	6	5	7	5	9	17
July	13	13	4	4	4	4	2	2	2	3	3	5	4	6	5	9	17
August	10	16	6	6	4	3	1	1	1	3	4	5	5	5	5	9	16
September	9	16	7	6	5	4	1	1	1	2	3	4	4	7	5	10	15
October	5	15	9	8	7	6	2	2	2	3	3	4	3	6	6	8	11
November	5	16	9	9	8	6	3	2	2	2	2	3	4	6	5	7	11
December	4	14	10	10	8	8	5	2	1	2	2	2	3	5	6	8	10
%Frequency	**9.6**	**13.7**	**6.5**	**7.3**	**6.6**	**6.2**	**3.1**	**2.1**	**1.7**	**2.6**	**2.6**	**3.8**	**3.8**	**5.5**	**4.6**	**7.3**	**12.5**

N = North	NNE = North North-East	NE = North-East	ENE = East North-East
E = East	ESE = East South-East	SE = South-East	SSE = South South-East
S = South	SSW = South South-West	SW = South-West	WSW = West South-West
W = West	WNW = West North-West	NW = North-West	NNW = North North-West

According to the data in Table 4.21, calm wind conditions prevailed only 9.6% of the time, therefore winds will certainly play a role in the transport of PCDD/Fs and PCBs. Northerly (13.7%) and North North-Westerly winds (12.5%) were most prevalent, implying that winds could distribute dioxin-like substances mainly to the South.

A radar graph (Fig 4.6) was drawn to indicate the percentage frequency of each wind direction and to illustrate the possible direction of distribution of PCDD/Fs and PCBs, when carried by winds.

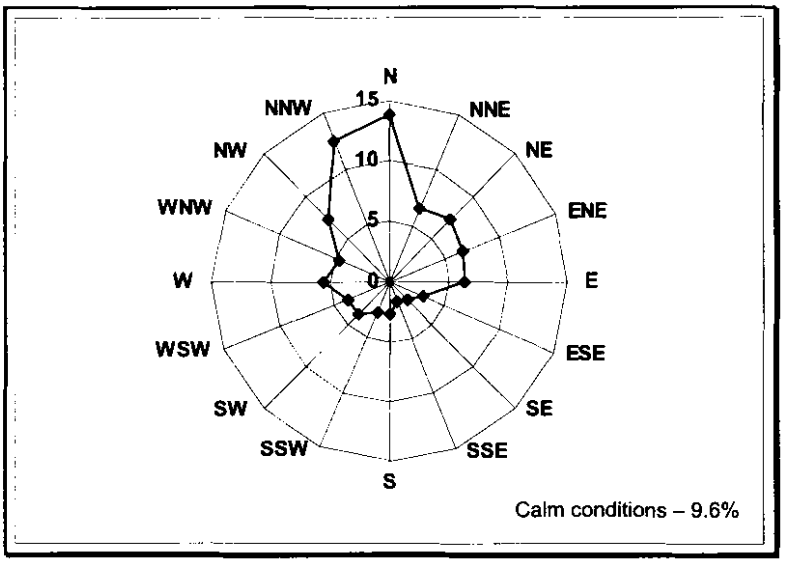

Figure 4.6. The percentage frequency of each wind direction.

Chapter 5. Discussion

5.1. Bio-analysis

The levels of PCDD/Fs and PCBs in sediments and fish tissue, measured by means of the H4IIE bio-assay, were extremely low, except for Sample 9 where high concentrations of these substances were recorded (Table 4.1).

5.1.1. Sediment samples

The low levels of dioxin-like substances measured in sediments were not anticipated, since all of the sites, except for the Suikerbosrand River reference sites (Site 16, 17 and 18), were located in close proximity to many potential sources of dioxin-like substances.

5.1.1.1. Sediment sites with unquantifiable amounts of PCDD/Fs and PCBs

A high dioxin-load was expected for Sites 1, 12, 15, 20 and 22, as these sites were situated near industries such as ferrous and non-ferrous metal producers, power generation plants, paper and pulp- producers and treatment plants, and an oil and gas refinery. All of the above-mentioned industries are classified as possible dioxin- and furan producers by UNEP (Table 2.3) (UNEP, 2003).

As stated in Section 2.2.2, of the major industries located in the Vaal Triangle, ferrous and non-ferrous metal producers are responsible for the largest contribution of PCDD/F and PCB emissions (32%). Oil and gas refineries and power generation plants also play a significant role, contributing 12% and 6% of dioxin-like emissions, respectively (Fig 2.2). These sources discharge their effluent gases through high smokestacks. According to Lohman & Signeur (2001), only about 10% of PCDD/F and PCB emitted by these types of sources are deposited locally, the other 90% of emissions are transported and deposited beyond 100 kilometres from the source. This might explain why very low concentrations of dioxin-like substances were present in sediments at sites located near these highly industrialised areas. Other possible motivations for the low dioxin-levels measured at these sites, will be discussed in Section 5.3.

As the amount of organic carbon in sediments affects the adsorbing capacity of the sediment for dioxin-like substances, the percentage TOC of each sample had to be taken into consideration too (Schumacher, 2002).

Since only Site 9 elicited a quantifiable result in cells, the correlation between percentage TOC and the amount of dioxins in sediments could not be established for this study. Site 9 had a high carbon content compared to the other sites, almost three times as high as the second highest TOC (14.58% versus 5.06%) (Table 4.1). This implies that sediments from this site would have the ability to bind to larger amounts of PCDD/Fs and PCBs, than the sites with lower TOC contents. The amount of organic carbon in sediments is not the only factor determining the dioxin concentration of a site, and sediments with small amounts of organic carbon (Site 20) may have larger dioxin loads than sites with larger fractions of organic carbon (Site 1) (Table 4.1), depending on the time and size of dioxin deposition. Site 9 was the only site in a wetland (Grootvaly Blesbok Spruit Wetland Nature Reserve), explaining the high organic carbon content.

The low % TCDD maximum responses elicited by Sites 1 and 11 (Table 4.1) were due to cell deaths. The highest concentrations of these extracts were cytotoxic and only 43% and 46%, respectively, of cells were viable. Cell populations in wells with viabilities lower than 80%, could not respond accurately to PCDD/Fs and PCBs, as explained in Chapter 4. The highest % TCDD maximum responses will therefore be lower than would be expected for wells with 100% viable cell populations, since cytoxicity masks possible dioxin-like presence in extracts.

Generally, elemental sulphur present in sediments may be toxic to cells (US EPA, 1986), but since a copper treatment was performed to remove the sulphur from the extracts, it is likely that other compounds were responsible for cell necrosis. The samples were extracted only once, but the bio-assay was performed twice or thrice to confirm the consistency of results. These samples produced the same cytotoxic results in each of the bio-assay repetitions. However, the compound/s in the sediment extracts that caused the cell deaths, is/are unknown.

It is known that dichloromethane (DCM), one of the solvents used in the extraction, may be toxic to cells and could have been considered as a possible reason for cell necrosis (Arietta, Ontiveros, Li, Garcia, Denison, McDonald, Burchiel & Washburn, 2003). It was for this reason that the extracts were evaporated to almost dryness, after which they were reconstituted with hexane. At the low volumes of hexane used in the wells, it had no toxic effects on cells, as was proven by the solvent control wells. If DCM was the cause of toxicity, it would have caused cell necrosis in all of the assays, which it did not (Table 4.1).

5.1.1.2. Sediment sites with quantifiable amounts of PCDD/Fs and PCBs

The only quantifiable TCDD equivalent value of 52.35 ng/kg (EC50) was recorded for Blesbok Spruit, Site 9 (Table 4.1). Because the EC80 was extrapolated, the EC50 TCDD equivalent value was used to report data. This value was exceptionally high when compared to the other eleven samples of which the amount of dioxin-like substances was undetectable. The Agency for Toxic Substances and Disease Register of the USA advises further investigation into a site if the amount of dioxins in soils or sediments exceeds 50 ng/kg (Fiedler, 2003). Other proposed environmental quality guidelines for aquatic sediments are listed in Table 5.1. The TCDD equivalent value of Sample 9 exceeds the limits of six out of the eight countries' advised quality guidelines. Taking the extrapolated TCDD equivalent value of 106.19 ng/kg (EC80) into consideration, the large amount of dioxin-like substances in sediments from Site 9, is reason for concern.

Table 5.1. Proposed environmental quality guidelines for aquatic sediments (adapted from Ianuzzi, Bonnevie & Wenning, 1995).

Source	TEQ guideline values
United States Army Corps of Engineers	1000 ng/kg
United States Environment Protection Agency (US EPA)	60 – 100 ng/kg
New York State Department of Environmental Conservation	10 – 100 ng/kg
Wisconsin Department of Natural Resources	1 ng/kg
International Joint Commission, Great Lakes Science Advisory Board	10 ng/kg
Environment Canada/Pacific Yukon Region	10 ng/kg
Hamburg Department of Environment, Germany	5 – 10 ng/kg
Proposed Dutch guidelines	15 – 100 ng/kg

Site 9 was located approximately 5 km North-East of Springs in the Grootvaly Blesbok Spruit Wetland Nature Reserve (Table 3.1). Although not as many major dioxin-producing industries were observed in the direct vicinity of this sampling site as in the more industrialised areas of Vereeniging, Vanderbijlpark and Sasolburg, there were several mines and tailings dams, as well as a sewage works and informal residential settlements located within a 10 km radius from Site 9 (Table 3.1).

Mining activities may lead to the production of dioxins, furans and PCBs (US EPA, 2002b). Numerous gold and coal mines, and tailings dams from these mines, are located in Welgedacht, Geduld, Brakpan, Springs and Daveyton, surrounding this nature reserve (Fig 3.2 and Fig 5.1). In fact, this sampling site was approximately 500 m removed from an old tailings dam, probably of a gold mine.

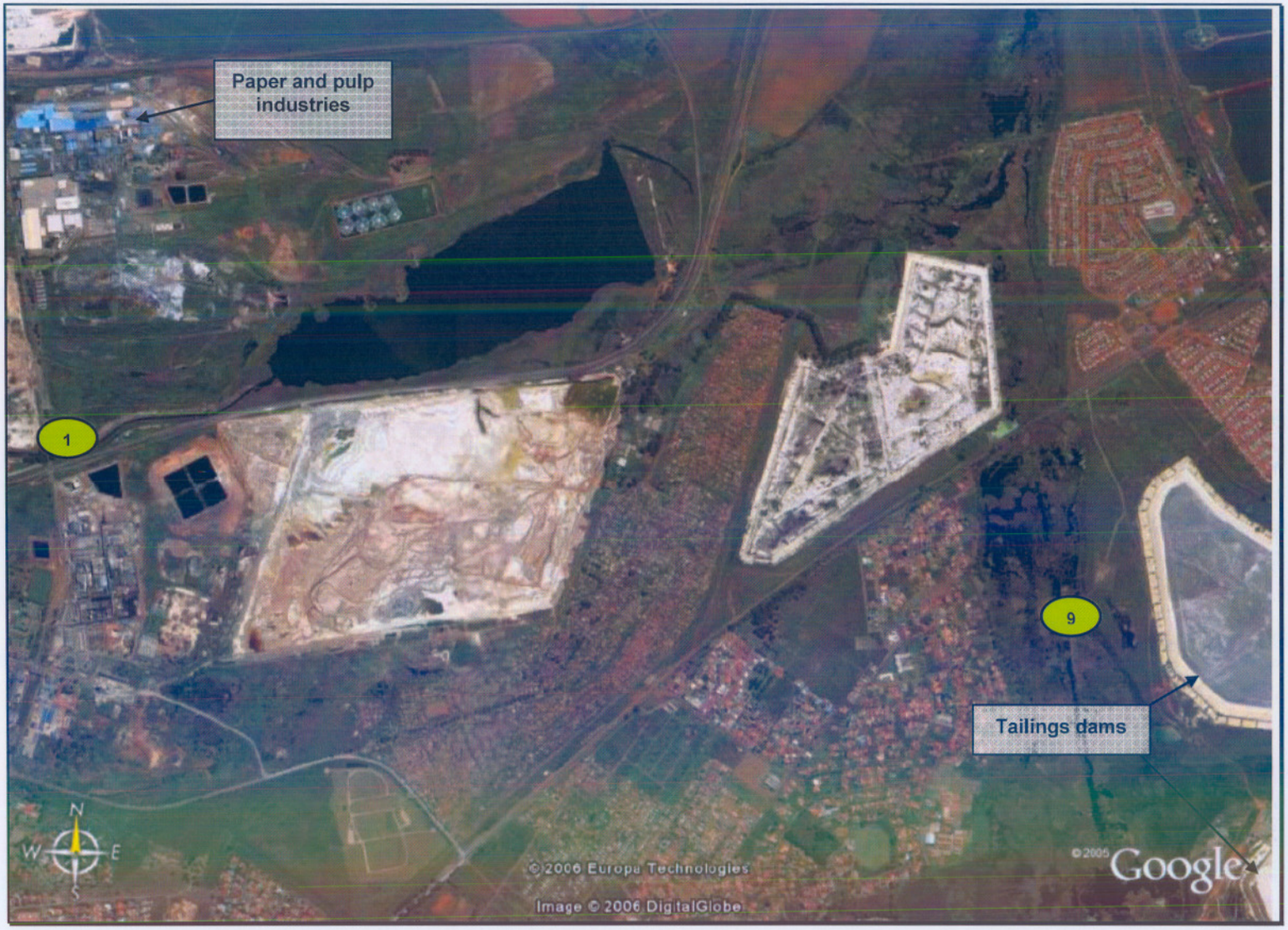

Figure 5.1. Satellite image of the locations of Site 1 and Site 9 (Blesbok Spruit) (Google Earth, 2006).

Mining activities might have contributed to the high dioxin load. Site 1 was also located in close proximity to the same mines, but the amount of dioxin-like substances at this site was too little to measure with the H4IIE bio-assay (Table 4.2). The highest concentration of Sample 1 was cytotoxic and most probably affected the results produced by these cells (Table 4.2). Another reason for the higher levels of PCDD/Fs and PCBs at Site 9, is that Site 9 is located down-stream relative to Site 1 (Fig 5.1).

The tailings dam situated near the site was not the sole source of possible dioxin pollution; many informal residential settlements were located close to the sampling area. Since many of these informal types of settlements are not supplied with electricity and municipal services, residents are dependant on open burning for preparing food, supplying warmth and incinerating waste. The burning of wood and incineration of waste containing various substances, such as plastics, rubber and other compounds, may produce large concentrations of dioxin-like substances (UNEP, 2003). PCDD/Fs and PCBs produced by these emission sources are generally deposited locally (Lohman & Signeur, 2001), and it is possible that the sources from the informal settlement contributed to the high dioxin load of Site 9. Since it is unknown if the sewage works emitted its effluent up-stream from the site, it was not certain if this possible source contributed to dioxin-like pollution at this site.

Sources farther away from the site, especially sources situated North or North North-West from the site, could also have been responsible for high levels of dioxin-like substances at Site 9, since the wind blew predominantly from these directions (Table 4.21). Located within a 20 km radius from the site, were the following probable dioxin-producing industries: a paper and pulp producer, five informal residential settlements, two gold mines, and manufactures of mining equipment and ceramic products. Other sources could also be responsible for dioxin pollution. These sources include vehicle exhaust emissions, power plants, incineration processes, landfills, as well as other categories of pollution, as listed in Table 2.3.

Many PCDD/F and PCB sources were also to be found close to the other eleven sites. In fact, many of the sites were located even closer to possible dioxin-producers. Why would Site 9 have such high levels of dioxin-like pollutants, while the amounts of PCDD/Fs and PCBs at the other sites were undetectable, if all the sites were located in close proximity to highly industrialised dioxin-producing areas? The answer most probably lies in the nature of Site 9. Site 9 was the only site situated in a wetland, while the locations of the other sites were in streams.

The water in wetlands is more stationary when compared to faster flowing river streams, which has a huge impact on the movement of sediments (Davies & Day, 1998). It implies that sediments from Site 9 are not subjected to constant river flow, which could wash away sediments with PCDD/Fs and PCBs bound to it, as in the other eleven sites (Section 5.3.3). The hydrology of wetlands allows these water bodies to function as water purification systems, by trapping and filtering pollutants through sediments (Davies & Day, 1998, Maltby, 1991). This means that the water flowing from wetlands would be "purified", whereas the sediments of the wetland would have higher loads of dioxin-like substances.

Wetlands are generally very rich in flora. The abundance of plants in wetlands helps to stabilise sediments, increasing sediment capture rates (Davies & Day, 1998). Plants may also have the additional function of protecting sediments from UV-radiation, which may degrade dioxin-like pollutants present in sediment (Section 5.3.3). These characteristics of Site 9 might have been responsible for the higher levels of PCDD/Fs and PCBs present at this site.

5.1.1.3 The reference sites

The Suikerbosrand River sites (Sites 16, 17 and 18) were selected on the grounds of its geographical location, outside of the Vaal Triangle area. These sites were selected to be the reference sites, since minimal dioxin-like pollutants were expected to be found at these sites. The results corresponded to what we expected, since the concentrations of PCDD/Fs and PCBs present at these sites were too low to quantify. Samples 17 and 18 elicited responses in cells of 22.84 and 29.97 % TCDD maximum, respectively. When the % TCDD maximum values of these two reference sites were compared to all of the other Vaal Triangle sites, Sites 18 and 17 had the second and fourth largest dioxin loads, respectively. The highest TCDD maximum value produced by Site 16's sediment was 11.82%. This value was relatively low when compared to the other two sites in the same river, but since only 72% of the cells were viable, this low grade cytotoxicity could have masked PCDD/Fs and PCBs present in the sediment extract.

As the samples were collected in the vicinity of smallholdings and farms, and no dioxin-producing industries were located in close proximity to the sampling sites, it is expected that natural sources, such as veld fires, might have been responsible for the dioxin-like contamination of these sediments. It is also a possibility that PCDD/Fs and PCBs were transported to these sites by winds, as discussed earlier.

Although the Suikerbosrand River was initially considered as an ideal reference site, it seemed that this was not the case, since the levels of dioxin-like substances measured at Site 17 and 18 were generally higher than at the other sites. Since no quantifiable dioxin-like responses were detected in eleven of the sites, and the responses from the reference sites were lower than that of Site 9 (Table 4.2), the Suikerbosrand River sites could be considered as valid for this study.

5.1.2. Fractionated samples

Although only one of the twelve samples produced a quantifiable result, all of the samples (except for Sample 13 which had extremely low levels of PCDD/Fs and PCBs) were fractionated into three different parts, and each part, or fraction, was analysed with the H4IIE bio-assay. The purpose of the fractionation procedure was to separate compounds from each other, to determine the contribution of each individual fraction to the total pollutant load of a sample. Mixtures of compounds in raw, unfractionated samples can interact with one another to mask the true AhR binding capability of samples (Safe, 1995).

Because none of the fractionated samples produced quantifiable responses in cells, the highest % TCDD maximum value, produced by each fraction, was used to report results. For each site, the % TCDD maximum values of fraction 1, 2 and 3 were totalled, assuming additive effects between compounds (Table 4.2). Comparing this totalled % TCDD maximum value to the % TCDD maximum value produced by the raw sample, the following tendencies were noted:

- **Sites 9 and 12:** The % TCDD maximum value produced by the unfractionated sample was nearly twice as high as the totalled value for the three fractions, in both Sample 9 and Sample 12. This implies that the compounds in the mixed sample had greater AhR binding capabilities than the separated compounds in each individual fraction. This is referred to as synergistic action between compounds, where the presence of one chemical enhances the toxicity (in this case AhR binding capability) of a second chemical (Safe, 1995). An additional reason for lower cell responses produced by fractionated samples might have been that some of the compounds remained bound to the Florosil® column, and were not eluted with solvent. This means that fewer compounds would have been present in the fractions, than were initially present in the raw extract.

- **Sites 1, 8, 11 and 15:** The sum of % TCDD maximum value of the fractionated compounds of each of these sites was higher than the response elicited by compounds in raw samples. In other similar studies, where sediment samples were fractionated and subjected to the H4IIE bio-assay, the same tendencies were observed. Koh and co-workers (2005) analysed sediments collected from three different water bodies in Korea: Lake Shihwa, Mason Bay and Kwangyang Bay. In all of the study areas, the sum of the response elicited by fractionated extracts was greater than responses elicited for the corresponding raw sample. For the majority of the samples, the totalled % TCDD maximum value was two to three times higher than that of the unfractionated extract. Results obtained from a study done by Khim, Lee, Villeneuve, Kannan, Giesy & Koh (2001) also supported the findings of this study. When AhR-mediated responses elicited by fractionated and unfractionated samples were compared, 23 of the 31 fractionated sediment samples had higher cell responses than the raw, unfractionated samples.

  This might be an indication of antagonistic interactions among compounds in the unfractionated sample. The presence of one chemical can reduce the toxicity, or in this case the AhR binding capability, of another chemical by competing with it for a binding site on the AhR. The AhR has greater affinity for some compounds than for others, with the implication that compounds with lesser AhR affinities do not bind with the receptor and therefore do not exert their effects on cells (Safe, 1995). This leads to a lowered % TCDD maximum value, although many compounds might be present in a sample (Safe, 1995). When these "competing" compounds were separated from one another by the fractionation process, the antagonistic effects among chemical substances were less, and more dioxin-like substances could bind to the AhR. In this way fractionated chemicals produce a higher % TCDD max, than raw, unfractionated compounds (Villalobos, Anderson, Denison, Hinton, Tullis, Kennedy, Jones, Chang, Yong & Kelly, 1996).

- **Sites 16, 17, 18, 20 and 22:** In the remaining five samples the % TCDD maximum value produced by the raw sample, and the totalled value of the three fractions, were similar. In this regard, the assumption of additivity of compounds, (as is done when calculating TEQ-values with GC/MS data) is a reasonably good reflection of the true AhR binding capabilities of compounds in raw samples (Villalobos *et al.*, 1996).

Since each bio-assay was repeated two to three times to ensure that results were consistent, the data was considered as reliable. It was therefore assumed that interactions between chemicals, as stated above, did indeed take place.

The average % TCDD maximum values for each of the three fractions of the 12 sites were calculated and compared to each other. The non-polar fraction, fraction 1, had the lowest average % TCDD maximum value of 4.58 (Table 4.2) and fractions 2 and 3, the more polar fractions, were responsible for the highest contribution of dioxin-like substances at 13.39 and 9.12 % TCDD maximum, respectively (Table 4.2). This indicated that compounds with intermediate and high polarity were mainly present in the sediment samples. Once again, these results were supported by the study performed by Koh *et al.* (2005), where higher dioxin-like activity was also observed for fractions 2 and 3, when compared to fraction 1. According to their study, fraction 1 extracts caused minimal responses in cells, ranging from 0 – 8 % TCDD max. Fraction 2 and fraction 3 were responsible for the most significant dioxin-like responses in cells. TCDD maximum values varied between 15 and 120 %, and 12 and 69 % for fraction 2 and fraction 3 extracts, respectively. Although the levels of dioxin-like substances were generally much higher in sediment extracts analysed by Koh *et al.* (2005), the tendencies observed during our study were similar.

Dioxin-like compounds present in the highest dosing concentration of Sample 1 and 11's fraction 1, and in Sample 16's fraction 2, caused cell necrosis. This was consistent with the cytotoxicity observed in the corresponding raw samples. This meant that non-polar compounds were responsible for the cell deaths observed in Samples 1 and 11, and compounds with intermediate polarity contributed to the cytotoxicity of Sample 16.

5.1.3. Bio-accumulation samples

Bio-accumulation refers to the build up of substances, in this case dioxin-like substances, in the body of an organism, so that the concentration of the substance in the organism would be higher than the concentration of the substance in the organism's surrounding environment (Brouwer *et al.,* 1999). According to this definition, it is expected that if PCDD/Fs and PCBs were present in the aquatic environment, the concentration of these substances would be higher in fish tissues, than in aquatic sediments.

To determine if bio-accumulation of PCDD/Fs and PCBs did occur, sediment and fish samples collected from Site 1F (Suikerbosrand River) and Site 2F (Blesbok Spruit) were biologically analysed and the results were compared to one another. The Suikerbosrand River was used as the reference site, once again.

5.1.3.1. Sediment samples

The levels of PCDD/Fs and PCBs in sediments from Site 1F and Site 2F were too low to quantify. As with the sediment samples discussed in Section 5.1.1 and 5.1.2, the highest % TCDD maximum values will be used to discuss the results and to compare the sites to one another.

Site 2F's raw sediment sample elicited a response in cells equal to 14.52% TCDD maximum. This value was not much higher than the 11.84% TCDD maximum value produced by the reference site (Site 1F) (Table 4.4). Considering the location of the Suikerbosrand River reference site, minimal dioxin-like pollutants were expected to be found at this site. The sampling site was located on a cattle farm, far away from potential dioxin-producing industries. Thus, the low levels of dioxin-like substances measured at this site, corresponded well to what we anticipated.
The total organic carbon content of only 5.11% indicated that these sediments had a relatively low dioxin-binding capability (Table 4.4).

The Blesbok Spruit sampling site (2F) was situated down-stream of potential dioxin-producing industries, such as paper mills, ferrous metal producers and power stations. There were especially many gold and coal mines located within a 20 km radius from the site. The sediment was collected from a recreational park in the residential area of Heidelberg (Fig 3.2) (Table 3.2). The road close to the site carried heavy traffic, and exhaust emissions might have been an additional source of dioxin-like substances. However, the bio-assay results indicated that the highest dosing concentration of this site's sediment extract, elicited a low response of only 14.52% TCDD maximum (Table 4.4).

The low carbon content of sediment (8.01%) could have contributed towards the minute quantities of PCDD/Fs and PCBs present at Site 2F. There is also a possibility that other factors, such as meteorological conditions and degradation processes, were responsible for the low levels of dioxin-like substances in sediments of Site 2F (Table 4.4).

The sediment samples collected from Site 1F and 2F were also fractionated and each fraction was assayed. The totalled % TCDD maximum values of the three fractions were higher than the % TCDD maximum value elicited by the raw samples, at both of the sites. The totalled value of Site 1F's three fractions was 31.73%, compared to the raw sample's value of 11.84%. For Site 2F, the totalled fractions yielded a TCDD maximum value of 42.11%, and the raw sample produced a response of 14.52% (Table 4.4).

The totalled % TCDD maximums at both of the sites were three to four times higher than that of the raw extracts. This implied that antagonistic interactions occurred among compounds in the raw sample (explained in Section 5.1.2) and as soon as the compounds were separated into fractions, they elicited higher responses in cells.

When the fraction compositions of the samples were examined, it was apparent that fraction 2 was responsible for the highest contribution of dioxin-like substances in sediment from Site 1F. It seems that PCDD/Fs and PCBs with intermediate polarity were the most abundant dioxin-like substances at this site (Table 4.4). This corresponded well with the initial fractionated sediment samples of the Suikerbosrand River (Sites 16, 17 and 18), where fraction 2 was also responsible for the highest dioxin contribution (Table 4.2).

Sample 2F's fraction composition differed from the other fractionated samples, with fraction 1 having the highest TCDD maximum value of 27.07%. Fractions 2 and 3 produced % TCDD maximum values of 10.73% and 4.31%, respectively. Thus, the majority of the dioxin-like substances present at this site had low polarity. These fractions differed from the initial Blesbok Spruit sediment sites (Site 1, 8, 9, 11, 12, 13 and 15), where fractions 2 and 3 were responsible for the highest TCDD maximum values. None of the raw or fractionated extracts had any effects on the viabilities of cells, and in this regard the results were perceived as reliable (Table 4.4).

5.1.3.2. Fish tissue samples

Fish were caught at the same sites where sediment was collected (Site 1F and 2F) for the purpose of determining bio-accumulation. The fish were separated into groups of two to three individuals, according to the criteria in Section 3.3.3, and a total of six composite groups were analysed for Site 1F and four for Site 2F.

5.1.3.2.1. General fish health

Because the general health and physical condition of fish is an indication of stress, including POPs exposure, a limited Fish Health Assessment was done and the CFs of fish were calculated. The majority of fish from both Site 1F and Site 2F were in good condition, indicating that neither of the water bodies were extensively polluted, however the reference site appeared to be in a poorer state than the industrialised site. Since the amount of dioxin-like substances present at both of the sites were barely detectable, the high FHAI-values may probably be attributed to other than dioxin-related factors. Numerous factors may lead to injury or bad health of fish: availability of food, presence of predators, or variables in the external environment (e.g. temperature, pH, etc).

In comparison to other variables, the fins and skin of fish were the most severely injured. It has to be taken into account that fish were caught by means of gill nets, which might have caused extensive damage to the skin. When trapped in gill nets, fish are immobile and vulnerable to attacks from predators, such as larger fish or crabs, which may also lead to injury. Thus, taking the method of sampling and the environmental conditions into account, the damage to fish was within the usual variance expected for wild populations (Table 4.5).

CFs of both Site 1F and 2F indicated that fish were generally in good condition: The majority of fish had high body masses relative to their lengths (Table 4.6). The CF values might not be accurate reflections of the fish's condition, as the fish were collected during spawning season, and the extensively developed gonads would have contributed considerably to the mass of each fish.

One would gain a more accurate reflection of the fish's condition if fish were caught before or after spawning season, eliminating any contributions to the actual mass of each individual. Both the FHAI- and CF values showed similar tendencies, with little difference in the general health of fish sampled from the industrialised site and the reference site.

5.1.3.2.2. Bio-analysis results of fish tissue samples

In general, the fish were in a good condition and it appeared as if the fish were not significantly exposed to any form of chemical pollution. This was confirmed with the bio-analysis which had shown that very low quantities of dioxin-like compounds were present in composite fillet-, liver- and gonad samples. The TCDD maximum values elicited by fish tissue samples ranged from 4.82% to only 14.47% (Table 4.7 and 4.8).
Since the masses of the tissues extracted and analysed were variable, the % TCDD maximum values produced by samples could not be compared to one another, or to sediment samples, to determine bio-accumulation.

To facilitate the comparison of dioxin-like presence in samples, the probability of extrapolating % TCDD maximum values, which would have been produced by 40 grams of each tissue, were investigated. But because only small sample masses were extracted, samples contained such low concentrations of dioxin-like substances, that the cell responses produced by sample extracts, were barely distinguishable from cell responses produced by pure solvent. Especially cell responses produced by livers and gonads were extremely low and irregular, and the extrapolated values would have been invalid reflections of dioxin-like activity in these samples.

The lipid content of fish-tissues ranged from 3.46 to 89.29%. Liver- and gonadal tissues had the highest lipid contents, which suggested that these tissues had high capabilities of binding to PCDD/Fs and PCBs, if these substances were present in aquatic environments (Table 4.7 and 4.8). Low levels of dioxins were expected to be found in sediments and fish tissues collected from the Suikerbosrand River (1F), since this site was used as a reference site. However, it was expected that higher levels of dioxin-related compounds would be present in sediments and fish tissue of the Blesbok Spruit site (2F), as the site was located near to, and down-stream of, many potential sources of dioxins (Table 3.2).

With the very low levels of PCDD/Fs and PCBs in the sediment, one would not expect to find very high levels of these substances in bottom-feeding fish. The two fish species collected, *L. umbratus* and *L. capensis*, are both bottom-feeding species and therefore they may ingest dioxin-contaminated sediment particles. They are also in direct physical contact with potentially contaminated sediments (Carey *et al.*, 1998). Therefore, the expectation was to find levels slightly higher than that of the sediment.

Although higher levels of PCDD/Fs and PCBs were anticipated in sediments as well as fish tissues collected from the Blesbok Spruit (2F), the amounts of these substances were very low at both sites. Because the levels of PCDD/Fs and PCBs in sediments, as well as in fish tissues, were too low to quantify, sediment and biota samples could not be compared to each other, and bio-accumulation could not be determined.

5.1.3.2.3. The estimated cancer risk of contaminated fish consumption

The US EPA (2001) categorises dioxin-like substances as "probable human carcinogens". This classification is based on the fact that certain dioxin-like congeners may have the capability to cause cancer in humans. To estimate the probable cancer risk of consuming dioxin-contaminated fish, a scenario-based risk assessment was done. For this purpose, the % TCDD maximum values produced by one of the composite fillet samples (Composite group 10) was extrapolated, and the concentration of PCDD/Fs and PCBs present in the fillet tissue was expressed as if 100 g of wet tissue were extracted and analysed (Section 4.1.2.2.4). These results based on extrapolations should be treated with caution.

Six different scenarios were used (Table 4.9) to estimate the probability of cancer developing in an adult or in a child consuming dioxin-contaminated fish fillets on a regular basis (daily, three times a week, or once a week), over a period of ten years.

Since food preparation processes, such as cooking the fish, might reduce the levels of PCDD/Fs and PCBs present in the fillets, the cancer risk estimations were calculated for three possible concentrations of dioxin-like substances: the extrapolated concentration of 71.03 ng/kg, and concentrations of ten times (7.10 ng/kg) and hundred times (0.71 ng/kg) less than the extrapolated value.

The estimated potential cancer risk, associated with the consumption of contaminated fish fillets, was high. The slope factor of dioxin-like compounds, for estimating probable cancer risks, is 1.56×10^5 mg/kg/day. This value was calculated according to 2,3,7,8-TCDD's potential to cause cancer (US EPA, 2003).

It is known that 2,3,7,8-TCDD is the most toxic and most carcinogenic dioxin congener. By using this congener's slope factor to calculate cancer risk, the assumption is made that all of the dioxin-like congeners, present in the fish tissue (measured as TCDD-equivalents in this study), have the same carcinogenic potency as 2,3,7,8-TCDD. This is not the case, since the toxic potency of different dioxin-congeners varies, and some congeners are less likely to cause cancer than other congeners (US EPA, 2003). The calculated risks may therefore be an overestimation.

All of the scenarios produced high cancer risks associated with consuming dioxin-contaminated fish, especially for persons consuming fish on a daily basis. The cancer risk associated with consuming 100 g of fish each day for ten years, was twice as high for a child (416 out of 100 000) as for an adult (208 out of 100 000), consuming the same amount of fish (Table 4.10 – 4.12). The reason for this is that the body mass of a child is only half that of the adult, and this implies that the same amount of dioxin-like substances would affect a child more severely. It was also observed that persons who consumed fish more frequently, and were exposed to higher concentrations of dioxin-like substances, had a greater risk of developing cancer (Table 4.10 – 4.12). Once again, these results should be treated with caution.

5.2. Comparison of chemical analysis and bio-analysis results

During June 2006, sediment samples were collected again at the same locations as Sites 15, 16, 17 and 20, so that the concentrations of PCDD/F and PCB congeners could be determined with GC/MS. These samples were chemically analysed with the intention of comparing the results to that of the bio-assay, as an additional measure to ensure the accuracy of data obtained from the bio-assay.

The amounts of PCDD/Fs and PCBs were undetectable with the bio-assay. The TCDD maximum values were similar for all four of the sites, ranging from 14.20% – 15.96% (Table 4.13). Because the % TCDD maximum values of the four sites were so much alike, the sites could not be distinguished from each other in terms of the highest and lowest dioxin-loads.

According to literature (Safe, 1995) GC/MS analysis is a very sensitive technique, which can measure the exact concentrations of individual dioxin congeners. It was therefore expected that this method would perhaps have detected compounds, which the bio-assay was not sensitive enough to detect. However, this was not the case, and very low quantities of PCDD/Fs and PCBs were measured for the four sites, with this technique as well. The total TEQ-values of the sites ranged from only 0.09 ng/kg to 0.23 ng/kg. These values are smaller to the detection limit of the bio-assay, which was 1 ng/kg for 40 g of sediment. For this reason the small amounts of PCDD/Fs and PCBs, present in these sediments, were undetectable with the H4IIE bio-assay.

Generally, PCDDs were responsible for the highest TEQs and PCBs were responsible for the lowest TEQs in mammals/humans and fish. The low PCB TEQs can be ascribed to the fact that these compounds were not very abundant at these sites, and because these compounds are less toxic to mammals and fish. PCBs were responsible for higher TEQ values in birds, because PCB's TEF-values are larger for birds than the other two animal groups, and are more toxic to birds (Table 4.17).

Both of the analytical methods measured low quantities of dioxin-like substances in sediments of the four sites, and chemical analysis thus confirmed that very low concentrations of PCDD/Fs and PCBs were in fact present in sediment samples.

5.3. Possible reasons for low levels of PCDD/Fs and PCBs in aquatic sediments

During the study, extremely low levels of dioxin-like substances were recorded at the majority of the sites. Quantifiable amounts of PCDD/Fs and PCBs were present at only one of the samples, collected from Site 9 (Blesbok Spruit), and the TCDD-equivalent value of this site was high (EC50 = 52.35 ng/kg). Since all of the sites were selected because of their close proximity to highly industrialised areas, it was surprising to find such low concentrations of these substances in both sediments and fish. Factors responsible for the low levels of dioxin-like substances in aquatic sediments will be discussed in this section.

5.3.1. Seasonal and meteorological conditions

Seasonal and meteorological changes have been linked to the occurrence of dioxin-like chemicals in environmental compartments. Moon *et al.* (2005) established that the concentrations of dioxin-like substances in soils and sediments were higher during winter months than in the summer, for Korea, which is a summer rainfall region. The low ambient temperatures of winter months might be responsible for the higher dioxin-loads in sediments and soils. It is known that high temperatures facilitate the volatilisation of dioxin-like substances from surfaces, leading to lowered concentrations of PCDD/Fs and PCBs in the environment (Moon *et al.*, 2005).

To be deposited onto surfaces, many dioxin-like substances, especially the more chlorinated dioxin-like congeners, are dependant on wet deposition (Meneses, Schumacher, & Domingo, 2003). The amount of precipitation therefore affects the quantities of PCDD/Fs and PCBs that are deposited in an area. High ambient temperatures may lower the amount of PCDD/Fs and PCBs available for deposition, because chemical degradation processes such as OH^- radical reactions can remove these chemicals during their movement into the atmosphere.

During summer months when sufficient precipitation occurs, the high temperatures of the area would contribute towards the breakdown of PCDD/Fs and PCBs. Conversely, during the colder winter months, when conditions are favourable for the deposition of dioxin-like substances, there is very little or no precipitation to facilitate deposition. Thus, the climatic conditions of the Vaal Triangle do not favour PCDD/F and PCB deposition, and although many of these substances may be produced and released in this area, we found little in the sediments and bottom-feeding fish. There are, however, indications from other studies in South Africa, that high levels of dioxin-like substances might be present in soil.

5.3.2. Photodegradation of dioxin-like compounds

The Vaal Triangle area has very hot summers, with long sunny days, receiving approximately 12 to 13 hours of sunlight on average (South African Weather Service, 2006). This implies that the environment is exposed to solar rays for long periods of time.

Dioxin-like compounds have optimal ultra violet (UV) absorption wavelengths ranging from shorter than 270 nm to 290 nm. Therefore, sunlight can act as a radiation source to degrade these compounds via photolysis (Isosaari, 2004). It appears that photolysis is one of the few environmentally significant degradation mechanisms for PCDD/Fs and PCBs in water, air, soils and sediments. The major degradation pathways of photolysis are cleavage of the carbon-chlorine bond, known as photodechlorination, or cleavage of the carbon-oxygen bond (Isosaari, 2004).

UV-light irradiation leads to the preferential loss of chlorines from the para positions (positions 1, 4, 6 and 9), rather than from the lateral positions (positions 2, 3, 7 and 8). Carbon-oxygen cleavage is a very important degradation pathway for dioxin-like substances containing four or less chlorines. The end-products of C-O bond cleavage are dihydroxybiphenyls or hydroxydiphenylethers and lower chlorinated PCDD/Fs. Hydrobenzoic acid is the ultimate aromatic photodegradation product of PCDD/Fs (Kim & O'Keefe, 2000).

Photolysis in aquatic environments:

Many experiments have been conducted on the photodegradation of dioxin-like compounds in pure water and in natural waters, such as rivers and ponds. It has been found that the degradation half-lives of these compounds in natural waters (4 – 6 hours) are much shorter than in pure water (1.2 – 6.5 days). This can be attributed to the presence of natural organics in natural waters acting as sensitisers, facilitating degradation processes (Isosaari, 2004).

Although sunlight easily penetrates water, solar rays only penetrate the top few millimetres of sediments and only the compounds in this layer can be degraded via photolysis. Photodechlorination in sediments is similar to that in water, since para-substituted chlorines are also preferentially cleft and lateral chlorines are less reactive. However, the degradation of PCDD/Fs and PCBs in sediments takes place at a slower rate, occurring over a period of five to eight days (Isosaari, 2004).

Since the aquatic sediments in the area were subjected to much UV-radiation, it is possible that PCDD/Fs and PCBs present in the sediments were degraded by photodechlorination or cleavage of the carbon-oxygen bond. During this study, sediment samples were collected from the upper sediment layer and since sediments were collected by hand, the samples were taken at locations that were easily accessible. This implied that sediment was collected at locations, within the sampling area, where the water level was about knee-deep. At this depth, sunlight can easily penetrate water to irradiate the top sediment layer (Isosaari, 2004). Thus, PCDD/Fs and PCBs in the upper sediment layers, of which we sampled from, could have been affected by UV-light degradation.

5.3.3. The dilution effect

Although dioxin-like substances are deposited through precipitation, large amounts of rainfall may lead to lowered levels of dioxin-like substances in sediments. During periods of high precipitation, large amounts of water may enter a river. These large quantities of water may wash away sediments, as they flow down-stream (Davies & Day, 1998). This implies that the dioxin-like substances bonded to sediments are also carried down-stream, decreasing the concentrations of PCDD/Fs and PCBs in the up-stream parts of rivers.

The sediment samples were collected during a three-month period, extending from April to June 2005. The majority of the samples (1, 8, 9, 11, 12, 13 and 15) were taken during April, the month following the rainy season of the Vaal Triangle. Since the largest amount of rainfall occurred during November to March, it is possible that the large quantities of precipitation could have affected the amounts PCDD/Fs and PCBs in sediments through the dilution effect. During months when precipitation was minimal, it is less likely that dilution had an effect on PCDD/Fs in sediments.

5.3.4. Degradation by microorganisms

Microorganisms may also be responsible for the degradation of dioxin-like compounds. The bio-degradation and bio-transformation of PCDD/Fs by microorganisms have even been considered as bio-remediation options for polluted environments. Certain aerobic bacteria, containing aromatic hydrocarbon dioxygenases, have broad substrate specificity, and they can degrade the ring structures of dioxins and related compounds. At least two other modes of dioxin bio-transformation have been recognised, in addition to this type of bio-degradation. These modes include reductive dechlorination by anaerobic microorganisms and fungal degradation (Halden, Halden & Dwyer, 1999).

Microbial reductive dechlorination of PCDD/Fs has been demonstrated in sediments and soils (Wittich, 2004). This dechlorination process of dioxin-like substances may degrade highly chlorinated congeners, which are generally hardly attacked by aromatic hydrocarbon dioxygenases. *Dehalococcoides* and *Dehalococcoides ethenogenes* are two strictly anaerobic strains of bacteria that have been shown to be able to dechlorinate selected PCDD/F congeners. It is also presumed that members of the *Dehalococcoides* group are capable of dechlorinating a tetrachlorinated congener of PCBs, chlorobenzene and trichlorodibenzo-*p*-dioxin. Members of the *Dehalococcoides* group are widely dispersed in nature, and play a major role in the transformation of chlorinated substances (Adrian & Lechner, 2004).

Sphingomonas wittichii is another bacterial strain with the unique ability to mineralise PCDD/Fs by the key enzyme, dioxin dioxygenase (Halden *et al.*, 1999). Many other microorganisms and fungi may be responsible for the degradation of dioxins. Although water and sediments were not analysed for the presence of microorganisms during the study, it has to be taken into account that dioxin-like compounds may possibly be degraded by this pathway.

Site 9, the wetland, had more favourable conditions for anaerobic microbial degradation of dioxin-like substances, than the other site located in rivers. Rapid currents continuously supply river sediments with dissolved oxygen (SEED, 2006). In wetlands, oxygen initially present in sediment, is rapidly utilised by biological activities, and because of the stationary nature of the water in wetlands, this oxygen is not replaced (Collins, 2005). For this reason, the sediment in wetlands is less oxygenised, than the sediment in rivers.

Another factor contributing to decreased amounts of oxygen in wetland sediments is decomposition processes. When plants in wetlands die, or when leaves are dropped, they accumulate on the surface of sediments, because water movement is limited. Microorganisms utilise oxygen when they decompose plant material, leading to an increase of the anaerobic nature of sediments (Collins, 2005).

Compared to the other sites, Site 9 was optimal for the anaerobic degradation of PCDD/Fs and PCBs by microorganisms. However, this site was the only site with detectable amounts of dioxin-like substances. It could be that the microbial activity at Site 9 plays a smaller role in the breakdown of PCDD/Fs and PCBs, than breakdown by UV radiation and removal of sediments by water currents at the other sites.

The rate of dioxin breakdown by microbial decay at Site 9 may be slower than the rate of dioxin deposition at the same site, giving an explanation as to the measured TCDD-equivalents.

Although it is well-known that microorganisms can degrade PCDD/Fs and PCBs, it is also speculated that some bacteria may produce dioxin-like substances from chlorinated phenols (Hoekstra, De Weerd, De Leer & Brinkman, 1999). Limited research has been done on this subject, and it is therefore uncertain if the production of dioxin-like substances by microorganisms plays a role in aquatic sediments of South Africa.

This highlights that the knowledge regarding factors that influence the presence of dioxin-like chemicals under South African conditions is very limited.

Chapter 6. Conclusions and Recommendations

6.1. Conclusions

This study established that PCDD/Fs and PCBs were either absent, or present at below the LOD, in the aquatic environments of the Vaal Triangle area. The results obtained with the H4IIE-*luc* bio-assay, were confirmed by GC/MS data. Only one of the twelve sites that were investigated, Site 9, had concentrations of dioxin-like substances that were high enough to quantify. This value exceeded 50 ng/kg, which is the level of action proposed by the USA. Although the amount of PCDD/Fs and PCBs at this site produced a relatively high TCDD equivalent value, the pollution appeared to be restricted to this area. Since the pollution was isolated to only one site, it was difficult to thoroughly characterise dioxin-like pollution in the aquatic environments of the Vaal Triangle region.

It seemed that the climatic conditions of the area, which include summers with high temperatures and long days, and winters with minimal precipitation, worked against the deposition of dioxin-like chemicals in aquatic sediments. PCDD/Fs and PCBs available in the sediments might have been broken down by UV-radiation. Some dioxin-like chemicals, which would be volatilised because of the high ambient temperatures, could have adhered to OH^-, therefore minimising the number of dioxin-like compounds that may return to the earth as precipitation.

Sampling from the upper sediment layer had advantages and disadvantages. Because it is the part of sediment which aquatic biota is mostly in contact with, it may be a useful indication of the concentrations of PCDD/F and PCBs to which living organisms are exposed. However, it is also the part of sediment which is exposed to microorganisms, and to UV-radiation in shallow waters, which both have the capability of degrading dioxin-like substances. Rapid flowing river currents may also have an impact on upper sediment layers, by moving sediments, with dioxin-like substances bound to it, to down-stream localities. It appeared that the stationary water and the lush flora of Site 9, the wetland, contributed to the high dioxin-loads present in these sediments, by preventing water currents from washing away dioxins, and by protecting the dioxin-like compounds from UV-degradation.

Since the concentrations of PCDD/Fs and PCBs were very low in sediments, as well as in fish tissues, bio-accumulation could not be determined.

However, extrapolated TCDD-equivalents of fillet tissues were used to estimate cancer risks associated with the consumption of contaminated fish tissues. It was established that the consumption of dioxin-contaminated fish might lead to the development of cancer in humans. This provisional conclusion is based on limited data, and a more extensive risk assessment has to be performed, before any substantiated assumptions can be made.

Although the concentrations of dioxin-related compounds in sediments of the industrialised sites were generally low, similar or even slightly higher cell responses were recorded for sediments collected from the reference sites. It was therefore apparent that the Suikerbosrand River sites were not pristine. Its dioxin-like levels were still lower than the single site (Site 9) that did have measurable TCDD-equivalents, and therefore a valid reference site.

6.2. Recommendations

- To minimise the effects of river currents on the movement of sediment to down-stream localities, it is suggested that sediment is sampled from stationary water bodies, such as wetlands, instead of river streams.

- Floods are mainly responsible for shifts in sedimentation. It is recommended that sediment samples are collected during the time of the year when rainfall will have a minimal effect on the distribution of sediments, for instance during the winter for regions receiving summer rainfall, and vice versa.

- Although it initially seemed as though the Suikerbosrand River was a good choice for a reference site, the levels of dioxin-like compounds at the reference sites were relatively high when compared to the other, more industrialised sites. Ideally, reference sites should be chosen in localities where the sites are removed from possible sources of dioxin-like pollution, but are still subjected to similar environmental conditions. The question remains: Are pristine reference sites still available? POPs are transported globally and finding a pristine spot would seem impossible. This means that in the future, a reference site's samples will have to be analysed before the commencement of a research project, making sure that a site is selected that truly has the lowest background TCDD-equivalents.

- The amount of dioxin-like substances was undetectable in the small masses of tissue samples that were extracted. In order to determine bio-accumulation of PCDD/Fs and PCBs, larger composite groups of five to ten individuals should be pooled, and larger masses of fish tissue (in the order of 50 – 100 g) should be extracted and analysed.

- The number of individuals, belonging to the same species, was a limiting factor at some sites. It is therefore recommended that other more abundant species of aquatic biota, such as fresh water molluscs and crustaceans, should also be considered for analysis, to determine bio-accumulation. However, these species are not commonly consumed by humans, and for this reason, they cannot be used for human risk assessments.

- It is also advised that more sampling locations should be targeted, to determine bio-accumulation of dioxin-like substances in aquatic biota. It would probably be most effective to perform a pilot screening study, to establish the presence of dioxin-like substances in sediments, prior to fish sampling. This would facilitate site selection, and ensure that the time and resources spent to collect fish are not futile.

- The risk assessment performed during this study, was based on extrapolated concentrations of dioxin-like substances in fish fillets. More detailed investigations are needed to efficiently characterise the potential risk of cancer development in humans, associated with the consumption of dioxin-contaminated fish.

- Other cell-based bio-assays can be performed to determine if sediments are capable of eliciting oestrogenic and androgenic responses. MVLN- and MDA bio-assays, where stably transfected human breast cancer cell lines are employed, are recommended.

- It is suggested that samples should be analysed chemically for other POPs such as the chlorinated pesticides, PAHs and industrial chemicals.

- Since birds are abundant at the Grootvaly Blesbok Spruit Wetland Nature Reserve (Site 9), it is suggested that bird eggs are collected from this site, and analysed for the presence of dioxin-related substances.

- It is recommended that the possible sources of POPs pollution are characterised, by making use of GC/MS analysis. This is known as "finger printing".

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