ENVIRONMENTAL WATER QUALITY MANAGEMENT OF GLYPHOSATE-BASED HERBICIDES IN SOUTH AFRICA

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PAUL KOJO MENSAH

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ABSTRACT

Although the use of pesticides is necessary to meet the socio-economic needs of many developing countries, especially in Africa, side effects of these bio-active chemicals have contributed to contaminating aquatic and terrestrial ecosystems. Environmental water quality degradation by pesticides interferes with ecosystem health and poses numerous risks to aquatic life. In South Africa, glyphosate-based herbicides are frequently used to control weeds and invading alien plants, but ultimately end up in freshwater ecosystems. However, there are no South African-based environmental water quality management strategies to regulate these bio-active chemicals. Therefore, this study sought to provide a sound scientific background for the environmental water quality management of glyphosate-based herbicides in South Africa, by conducting both laboratory and field investigations. In the laboratory investigations, aquatic ecotoxicological methods were used to evaluate responses of the freshwater aquatic shrimp *Caridina nilotica* exposed to Roundup® at different biological system scales, and the responses of multiple South African aquatic species exposed to Roundup® through species sensitivity distribution (SSD). In the field investigations, the effect of Kilo Max WSG on the physicochemical and biological conditions of three selected sites in the Swartkops River before and after a spray episode by Working for Water were evaluated through biomonitoring, using the South African Scoring System version 5 (SASS5) as a sampling protocol. Both Roundup® and Kilo Max WSG are glyphosate-based herbicides. All the data were subjected to relevant statistical analyses. Findings of this study revealed that Roundup® elicited responses at different biological system scales in *C. nilotica*, while SSD estimates were used to derive proposed water quality guidelines for glyphosate-based herbicides in South Africa. The biomonitoring revealed that using glyphosate-based herbicides to control water hyacinth within the Swartkops River had a negligible impact on the physicochemical and biological conditions. Based on these findings, a conceptual framework that can be used for the integrated environmental water quality management of glyphosate-based herbicides in South Africa was developed as part of integrated water resource management (IWRM). The combined data sets contribute to a sound scientific basis for the environmental water quality management of glyphosate-based herbicides in South Africa.

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DEDICATION

To my childhood friends, represented by Ike.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

This chapter provides background information about the world around us: the biosphere, with a particular emphasis on aquatic ecosystems. Globally, aquatic ecosystems are experiencing all kinds of pollution, especially pollution caused by anthropogenic activities. In this context, the chapter reviews literature on water resources management in South Africa by briefly discussing functions of the National Water Act (NWA) of 1998 and National Water Resource Strategy (NWRS). The combined use of water physicochemistry, biomonitoring and ecotoxicology, as well as the use of water quality guidelines (WQGs) as management tools for environmental water quality were also reviewed. Other areas of importance that were reviewed included pesticide pollution of aquatic ecosystems, focusing on herbicides in general and glyphosate in particular; the use of crustaceans as model organisms in ecotoxicity testing, focusing specifically on the indigenous freshwater shrimp *Caridina nilotica*; life-history stages toxicity tests; endpoints used to measure responses of aquatic organisms to biological stressors, and environmental assessment. The motivations behind this study, overall aim and specific objectives, are summarised early in the chapter to clarify direction. The chapter concludes in the light of the literature, with a reiteration of the aims, together with the guiding research question and an outline of the whole thesis.

1.1.1 Background: a polluted world

The Earth's environment has been considerably affected by rapid human population growth; extensive use of natural resources such as water, land, minerals and fossil fuels; consequent production processes, and technological progress that have characterised the accelerating industrial revolution since the mid-twentieth century. This has caused anthropogenic disturbances such as climate change, acid rain, nutrient enrichment of aquatic environments, pollution by metals, and synthesised toxic substances on local, regional and global scales.

Freshwater ecosystems are among the most at risk (Moiseenko, 2008; Vorosmarty *et al*., 2010), as studies investigating aquatic ecosystem responses to anthropogenic stresses have shown that freshwater resources are highly vulnerable to contamination (Moiseenko, 2008). Agricultural, industrial and domestic activities are the major sources of this contamination (Schwarenbach *et al.*, 2006). These activities use more than one-third of the Earth's accessible renewable freshwater resources and have contaminated water with numerous synthetic and geogenic compounds (Schwarenbach *et al.,* 2006). For instance, about 300 billion kilograms of synthetic compounds used in industrial, consumer and agricultural products find their way into natural freshwater systems every year (Jurado *et al*., 2011). Ten percent of the globally accessible runoff is used, generating a stream of wastewater, which flows or seeps into groundwater, rivers, lakes, or the oceans (Jurado *et al*., 2011).

The use of agrochemicals is necessary to control pests and increase yields in order to produce adequate food for the global population, estimated at 6.8 billion in 2009 (Jurado *et al*., 2011), and recently reported to have reached 7 billion (PRB, 2012). Underdeveloped countries, where 1.02 billion people (15%) are undernourished and 1.3 billion people (19%) live on an inadequate diet (Jurado *et al*., 2011), need an adequate food supply. However, the agricultural sector's annual application of over 140 billion kilograms of fertilizers and large amounts of pesticides creates massive sources of diffuse pollution of freshwater systems (Schwarenbach *et al.,* 2006).

The presence of these toxic chemicals in both aquatic and terrestrial ecosystems has become an important issue globally. Growing research-based evidence shows that many industrial chemicals, metals, and pesticides interfere with the health and normal functioning of the endocrine systems of a wide range of organisms, including humans (LeBlanc, 2007; Correia *et al.*, 2010; Pedersen *et al*., 2010). It is believed that the effects of these chemicals on the normal functioning of the endocrine system are responsible for a number of developmental anomalies in a wide range of species, from invertebrates to higher mammals (e.g. Abel, 2002; London *et al.,* 2005; Mihaich *et al.,* 2009; Benstead *et al.,* 2011).

Available information on pesticides indicates that they pose serious risks to a range of non-target organisms, including aquatic invertebrates. Although there is extensive northern hemisphere literature and substantive data for Australasia (Kegley *et al,* 2010), there has been relatively little research on the general impact of pesticides on South African freshwater ecosystems (London *et al*., 2005).

In South Africa, Roundup® and other glyphosate-based herbicides are used extensively, and specifically by Government in the poverty relief and environmental programme "Working for Water", to control aquatic weeds. This provides a focus area for the investigation of formulated herbicides in a context where non-target aquatic organisms might be at risk. The overall aim of this study was therefore to provide sound scientific background for the environmental water quality management of glyphosate-based herbicides in South Africa. In order to achieve this aim, the following specific objectives were set:

- To evaluate the exposure-response relationship of *C. nilotica* exposed to Roundup® under different laboratory exposure conditions.

- To evaluate the species sensitivity distribution (SSD) of South African aquatic organisms exposed to Roundup®.

- To evaluate the field effect of a glyphosate-based herbicide on physicochemical and biological conditions of the Swartkops River through biomonitoring.

- To develop water quality guidelines (WQGs) for the management of glyphosate-based herbicides as part of integrated water resource management (IWRM) in South Africa.

- To develop a conceptual framework for the integrated environmental water quality management of glyphosate-based herbicides in South Africa.

In the light of this aim and objectives, a detailed literature-based consideration follows and leads on to their reiteration in a fuller context.

1.1.2 The general South African context

South Africa is a semi-arid, water-stressed country so management of water pollution is of paramount concern (DWAF, 2004). The climate varies from desert and semi-desert in the west, to sub-humid along the eastern coastal area. The average annual rainfall is 450 mm, well, below the world's average of 860 mm per annum, while evaporation is comparatively high (DWAF, 2004). As a result, South African rivers have a low stream flow compared to other rivers in the sub-region. For example, the average annual stream flow of all the rivers in South Africa is about 49 000 million cubic metres, which is less than half the annual flow of the Zambezi River to the north. The Orange River carries only 10 percent of the volume of water flowing down the Zambezi River and one percent of that in the Congo River. The Limpopo, Inkomati, Pongola and Orange Rivers, which together drain 60 percent of the country's land area and contribute 40 percent of its total river flow, are shared with other southern Africa countries. Furthermore, only about 20 percent of groundwater in major aquifers can be used on a large scale, due to the hard nature of South African rocks (DWAF, 2004). All these factors limit water availability in South Africa. This means that dilution, which is the most common and cheapest pollution management approach, can only be used to a limited extent before it impacts negatively on other water uses and users.

In this context, pollution poses an acute water resource management problem. Major sources of pollution of surface water are: agricultural drainage and wash-off (irrigation return flows; fertilizers, pesticides and runoff from feedlots); urban wash-off and sewage treatment effluent return flows (bacteriological contamination, salts and nutrients); industries (chemical substances); mining (metal ions, acids and salts), and areas with inadequate sanitation services (microbial contamination). Groundwater has been polluted by mining activities, fertilizers (particularly nitrogen), agrochemicals, leachate from landfills, human settlements and intrusion of sea water (DWAF, 2004). This has implications on ecosystem health, and on people living in South Africa. For instance, recent studies of such phenomena include changes in phytobenthos and phytoplankton communities upon exposure to a sunflower oil spill in a protected freshwater wetland (Oberholster *et al*., 2010); evaluations of air pollution as a threat to the endangered blue swallow (*Hirundo atrocaerulea*) (Kylin *et al*., 2011); and changes in urinary dialkyl phosphate levels amongst farm workers in the Western Cape before and after first season chlorpyrifos spraying (Dalvie *et al*., 2011).

Therefore, to protect aquatic life from pollution and to reduce human health risks, it is necessary to determine the levels of contamination in aquatic ecosystems, as well as the responses of organisms to a variety of chemicals and mixtures (Zhou *et al.*, 2008).

There is a wide range of chemicals that pollute South Africa's waters, but there is little information on the environmental impact of pesticides. Of these, herbicides, especially glyphosate-based herbicides, are among the most commonly used in South Africa. Therefore, it is necessary to devise a means of protecting water resources against chemical pollution. In this context, South Africa has two important documents which form the basis of managing the country's water resources. These are the National Water Act (Act number 36 of 1998) and the National Water Resource Strategy (NWRS) (DWAF, 2004).

1.2 Water Resources Management in South Africa

The South African National Water Act (Act number 36 of 1998) (hereafter referred to as the NWA) recognises sustainability and equity as the central guiding principles in the protection, use, development, conservation, management and control of water resources. The NWA was promulgated to ensure that the entire South African population has access to clean, safe water for their basic needs, without compromising water resources' health so that future generations can also benefit from them. This means that any management approach for the country's water resources must ensure their sustainable, equitable, and efficient use. Therefore, the NWA provided for the development of a National Water Resource Strategy (NWRS), which recommends an integrated and adaptive approach to managing the country's water resources (DWAF, 2004). The NWRS, which is currently under review, acts as the national implementation framework for the NWA. It outlines the goals and objectives of water resources management for the country. It also provides plans, guidelines, and strategies on how to achieve these goals and objectives.

The NWRS identified four regulatory activities in order to facilitate its implementation, as follows:

- Resource-directed measures (RDM), which involve co-operatively defining the appropriate level of protection for a water resource, and on that basis, setting clear numerical and/or descriptive goals for the resource quality i.e. Resource Quality Objectives (RQOs). RQOs are required for flow (volume, velocity, and distribution through time) and for quality (Palmer *et al*., 2004a; DWA, 2010).

- Source-directed controls (SDC), which aim to control impacts on the water resource through the use of regulatory measures such as registration, permits, directives, and prosecution, and economic incentives such as levies and fees, in order to ensure that the RQOs are met. Typical SDCs include discharge and abstraction permits.

- Managing water resource demands in order to keep water utilisation within the limits required for protection. This can be achieved through a range of approaches, including water pricing, water-use restrictions, institutional negotiations, and public programmes.

- Continual monitoring, which involves monitoring the status of the country's water resources in terms of flow and quality on a continual basis, in order to ensure that the RQOs are being met, and to enable DWAF to modify programmes for resource management and impact control as and when necessary.

Water quality of an aquatic ecosystem deteriorates when pollutants are present. In order to incorporate water quality into the overall management of a water resource, the toxicity of chemicals and the effects of their by-products on aquatic animals need to be determined. This can be done using the environmental water quality (EWQ) management approach (Palmer *et al.,* 2004b).

1.2.1 Environmental water quality (EWQ)

Water quality refers to the physical, chemical and selected biological characteristics of water. It also describes closeness of the water to its natural state, or for use by different users (Palmer *et al.,* 2004b). Different ecosystems and different user groups may have variable water quality requirements, just as different water bodies vary in their natural water quality (Dallas and Day, 2004). It is therefore critical to determine the concentration of any pollutant that will be harmless or have varying impacts on aquatic organisms (Dallas *et al.*, 1994). Such levels of concentration can then be used as thresholds to monitor the specific or related toxicant in question.

In order to understand the mechanism of aquatic ecosystems' deterioration by both organic and inorganic pollutants, it is necessary to know the eco-physiology and basic biology of aquatic biota. From a biological point of view, any toxic effect is significant if it negatively influences, or is likely to influence the physiology or behaviour of an organism in such a way as to alter capacity for growth, reproduction, mortality, or to influence its pattern of dispersal, since these are major determinants of the distribution, abundance and survival of species (Abel, 2002). Thus, in order to take account of water quality in the overall management of a water resource, the toxicity of pollutants and their by-products to aquatic animals needs to be determined, since any compound manufactured and used in substantial quantities is likely to contaminate watercourses (Abel, 2002).

Water physicochemistry, biomonitoring and ecotoxicology are tools employed to investigate environmental water quality (EWQ) (Palmer *et al.,* 2004b). Water physicochemistry only measures and monitors physical and chemical variables in order to understand the magnitude of specific abiotic conditions without considering the aquatic biota, whereas biomonitoring methods assess aquatic ecosystem health based on the presence, absence and abundance of species in various taxa so as to provide a time-integrated indication of the consequences of exposure (Palmer *et al.,* 2004b). Depending on the objective of an EWQ investigation, water physicochemistry and biomonitoring can be used separately or together to assess aquatic ecosystem health under different experimental systems and conditions, and at different times. Ecotoxicological studies add the capacity to identify causal relationships. A combination of water physicochemistry, biomonitoring and ecotoxicology provides an integrated assessment of aquatic ecosystem water quality.

Water physicochemistry

The Earth's hydrological cycle, mainly driven by evaporation and gravity, is a complex system through which all freshwater bodies are interconnected to the oceans, the atmosphere, and aquifers. Water bodies such as rivers, streams, wetlands, aquifers, estuaries, oceans and icecaps participate in the continuous circulation of water on the planet. Ecosystems and human societies depend on the hydrological cycle, but growing human populations have put stresses on natural waters by impairing both the quality of the water and the hydrological budget (Bellingham, 2009). However, the fate and transport of many anthropogenic pollutants are determined not only by hydrological cycles, but also through physicochemical processes.

In order to mitigate the impact of anthropogenic activities on natural waters, it is becoming increasingly important to implement comprehensive monitoring regimes (Bellingham, 2009). Measuring and monitoring water physicochemical parameters such as pH, dissolved oxygen, electrical conductivity, temperature, turbidity, total nitrogen, nitrate, ammonia, ammonium, phosphate and total phosphorus may be used to quantify water quality, identify impairments, and help policy makers make water-use decisions that will not only preserve natural areas, but improve the quality of life (Palmer *et al*., 2004b).

Biomonitoring

Biomonitoring (biological monitoring) is central to environmental water quality, and is defined as the regular and systematic use of living organisms to evaluate changes in environmental water quality (Rand *et al.* 1995). It is a valuable assessment tool that is increasingly being used in environmental water quality monitoring programmes. There are two types of biomonitoring. One type of biomonitoring involves assessment before and after a project is complete, or before and after a toxic substance enters the water. The other type of biomonitoring ensures compliance with regulations and guidelines for maintenance of water quality. Biomonitoring involves the use of indicators, indicator species or indicator communities.

Although benthic macroinvertebrates, fish, and/or algae are generally employed in biomonitoring, macroinvertebrates are most frequently used. Biochemical, genetic, morphological, and physiological changes in certain organisms have been used as response indicators to particular environmental stressors. The presence or absence of the indicator, an indicator species or indicator community reflects environmental conditions. Absence of a species might not necessarily be the result of pollution but rather other reasons such as competition, predation and/or geographic barriers which prevented it from ever being at the site. However, absence of multiple species belonging to different orders (or families) with similar tolerance levels that were present previously at the same site is more indicative of pollution than absence of a single species. Therefore, it is necessary to know which species should be found at a particular site or in the ecosystem in order to properly monitor the system's health.

Ecosystem health may be defined as the level of functional efficiency of the system. An ecosystem has good health (resilience) if it is capable of self-restoration of the harmony existing within and across its biotic and abiotic factors after suffering external perturbations. However, introduction of new factors or a dramatic rise in one or more of them can disrupt the normal functioning of the ecosystem. This may result in ecocide (i.e. "death" of the ecosystem) and, therefore, inability of the system to perform its ecological services. Generally, symptoms of poor ecosystem health (i.e. damage to the system's integrity) include build-up of waste material and the proliferation of simpler life forms (e.g. bacteria and insects) that thrive on it but no consequent population growth in those species that normally prey on them; loss of keystone species, often a top predator, causing smaller carnivores to proliferate, very often overstressing herbivore populations; higher rate of species mortality due to disease rather than predation, climate, or food scarcity; migration of whole species into or out of a region, contrary to established or historical patterns; and proliferation of a bio-invader or even a monoculture where previously a more bio-diverse species range existed.

The River Health Programme (RHP), introduced in 1994 by the South African government, has been a successful biomonitoring program that monitors the ecosystem health of the country's rivers (Roux, 1997). The RHP is based on the principle that the ecological integrity or health of the biota inhabiting the river ecosystems provides a direct and integrated measure of the health of a river as a whole.

The RHP uses information obtained through biomonitoring the in-stream and riparian environments, using species such as fish, macroinvertebrates and riparian vegetation as indicators, to characterise the response of the aquatic environment to stress or perturbations. The objectives of the RHP are: to measure, assess and report on the ecological state of aquatic ecosystems; to detect and report on the spatio-temporal trends in the ecological state of aquatic ecosystems; to identify and report on emerging problems regarding aquatic ecosystems; and to ensure that all reports provide information that is not only scientifically relevant, but also suitable for managing the national aquatic ecosystem.

Aquatic Ecotoxicology

Ecotoxicology is a branch of toxicology that studies the toxic effects of natural and artificial substances on living organisms that constitute the biosphere, taking into account the complex interaction within an ecosystem. This may include the interaction of toxic substances with the physical environment (Rand *et al.,* 1995). Aquatic ecotoxicology is therefore a branch of ecotoxicology that deals with the study of the effects of toxic agents or substances on aquatic organisms at various levels of organisation, from sub-cellular, through individual organisms, to community and ecosystem levels (Rand *et al.,* 1995).

These effects may include mortality or survivorship; changes in behaviour, growth, development, tissue structure and reproduction (organismal level); induction or inhibition of an enzymatic activity (sub-organismal level), or changes in relative abundance and physiological condition of a specific species (supra-organismal level). Aquatic ecotoxicology, as a scientific field of study, not only plays a very important role in understanding the impact of anthropogenic activities on aquatic ecosystems, but also helps to preserve and protect an aquatic ecosystem's health through prognostic effect assessments (Moiseenko, 2008).

A toxicity test may be used to evaluate acute or chronic effects. An acute effect occurs rapidly due to short-term exposure of an aquatic organism to a chemical. The effect can be noticed in a matter of a few hours or days (often up to four days) and is usually measured as the concentration that resulted in 50% mortality of the exposed organisms (LC50) (Rand *et al.,* 1995). A chronic effect occurs from long-term (weeks, months or years) or repeated exposure of the test organisms to lower concentrations of toxic chemicals (Rand *et al.,* 1995).

Chronic toxicity tests primarily evaluate the potential for reproductive effects and the effects on offspring. Aquatic toxicity tests are necessary to detect and evaluate the potential toxicological effects of chemicals on aquatic organisms, especially at relatively low exposure concentrations. Thus, aquatic toxicity tests provide a database that can be used to assess the risk associated with a situation in which the chemical agent, the organism and the exposure conditions are well defined (Rand *et al.,* 1995).

Environmental water quality (EWQ) was used in the present study as a management tool of glyphosate-based herbicides by conducting laboratory and field evaluation of glyphosate exposure effects. The laboratory studies involved ecotoxicological studies using *C. nilotica* as a test model in single-species test bioassays of the herbicide Roundup®. Specific life history stages of the shrimp were exposed to different Roundup® concentrations in order to evaluate the suitability of using *C. nilotica* as a test organism for glyphosate-based herbicides. The aim was to develop acute (lethal) and chronic (sub-lethal) endpoint measures that can be used in routine ecotoxicological studies. Another aim was to assess the possibility of deriving a water quality guideline with detailed ecotoxicity data from *C. nilotica*. This study is reported in Chapter Three of this thesis.

Another aspect of the laboratory studies based on ecotoxicology, which is reported in Chapter Four of this thesis, is the responses of multiple South African aquatic species in terms of species sensitivity distribution (SSD) after exposure to Roundup®. A global SSD was also constructed with data extracted from the ECOTOX database (USEPA, 2012). The SSD uses a statistical extrapolation method based on the variability in sensitivity (LC50s and NOECs) between various test species to derive the risk levels for ecosystems. The variability in sensitivity of test species is assumed to be representative for the variability of species in the environment (Knoben *et al*., 1998). Currently, SSDs are the preferred international method of deriving water quality guidelines (WQGs). The method is used in America, Australia and the Organization for Economic Coorporation and Development (OECD) countries (Browne, 2005). These methods have been used extensively in the development of similar guidelines for South Africa because of limited indigenous organism response data and the absence of a standard national toxicological database (DWAF, 1996a; Browne, 2005).

In constructing SSDs for this study, representative organisms of various taxonomic groups, including plants, insects, molluscs, crustaceans and plants were exposed to Roundup® and their LC50s and NOECS were estimated. The SSDs for the LC50s and NOECs were used to derive short-term and long-term WQG values, respectively.

The biomonitoring part of EWQ was undertaken through field evaluation of glyphosate in the Swartkops River, in the Eastern Cape Province of South Africa. This was achieved by assessing the impact of glyphosate-based herbicide on macroinvertebrate communities before and after chemical control of invasive plant species. Quantitative biomonitoring with the South African Scoring System version 5 (SASS5), and multivariate statistical techniques (MST) were also employed in this study. The SASS provided indices in terms of SASS Scores of the various taxa and average score per taxon (ASPT) that formed the macroinvertebrate community of the river. The MST was used to link water quality variables in terms of physicochemical parameters and nutrients with macroinvertebrates, and these results are reported in Chapter Five of this thesis.

1.2.2 Water quality guidelines (WQGs)

Water quality guidelines (WQGs) are important tools in the management of water resources in South Africa (Palmer *et al*., 2005) and other parts of the world, including Australia and New Zealand (ANZECC and ARMCANZ, 2000), United States (USEPA, 2005). Canada (CCME, 2007) and China (Yan *et al*., 2012; Yang *et al.*, 2012). Data from water physicochemistry, biomonitoring and aquatic ecotoxicological studies aid the derivation of WQGs, which are then used to protect aquatic ecosystems.

South Africa has a history of deriving water quality criteria and associated management guidelines for freshwater ecosystems, and the first South African water quality guidelines, contained in an eight-volume series, was published in 1996 (DWAF, 1996a). *The South African Water Quality Guidelines* remain the primary source of information for determining the water quality requirements of different water uses and for protecting and maintaining aquatic ecosystems' health.

Besides providing information on the water quality requirements for managing and protecting aquatic ecosystems, the guidelines also provide background information to help users make informed judgments on the likely impacts of water quality on the health and integrity of aquatic ecosystems (DWAF, 1996a). The eight volumes that comprise *The South African Water Quality Guidelines* are (listed from volumes 1 to 8): guidelines for domestic water use; recreational water use; industrial water use; agricultural water use: irrigation; agricultural water use: livestock watering; agricultural water use: aquaculture; aquatic ecosystems; and field guide. *The South African Water Quality Guidelines for Aquatic Ecosystems* covers the following essential subjects (DWAF, 1996a):

- The quality of surface water required to protect freshwater aquatic ecosystems.

- Quantitative and qualitative criteria for chronic and acute toxic effects for toxic constituents.

- Quantitative and qualitative criteria for non-toxic constituents and system variables to protect the structure and functioning of ecosystems.

- Quantitative and qualitative criteria to protect aquatic ecosystems against changes in trophic status in the case of nutrients.

- Modifications that can be made to water quality criteria on a site-specific basis, whilst still providing the same level of protection as the original criteria (DWAF, 1996a).

The South African Department of Water Affairs (formerly Department of Water Affairs and Forestry) uses *The South African Water Quality Guidelines for Aquatic Ecosystems* as the primary source of reference information and for supporting decisions required to manage and protect aquatic ecosystems. However, the guidelines do not address the limitations associated with in-stream biological monitoring, direct assessment of whole-effluent-toxicity, or biotoxicological assessment of sediment quality (DWAF, 1996a).

In developing WQGs for South Africa, policy-makers and regulators have paid relatively little attention to the effects of pesticide pollution of freshwater resources on public health (London *et al.,* 2005). The *South African Water Quality Guidelines* do not include standards to protect aquatic ecosystems from Roundup® and other glyphosate-based herbicide pollution (DWAF, 1996a). A South African aquatic macroinvertebrate ecotoxicological database is being developed and curated by the Unilever Centre for Environmental Water Quality (UCEWQ) division of the Institute for Water Research (IWR), Rhodes University, South Africa, based primarily on macroinvertebrate tolerances to salinity, salts and complex effluents (Palmer *et al.,* 2004a). The purpose of this database is ultimately to expand into a national database, incorporating data from a wider variety of toxicants and organisms. This would form the basis of any revision of the South African WQG. The outcome of this research will contribute directly to such an ecotoxicological database.

1.3 Herbicides

Weeds are plants that grow in places people do not wish them to grow because they compete with "beneficial and desirable" plant species. Until the last century, much of the energy used in farming went into removing weeds to provide suitable conditions for efficient cropping. However, during the industrial revolution, more people moved to work in factories, thus creating a shortage of labour on farms and it became necessary to develop more efficient ways to control weeds (Jurado *et al*., 2011).

Herbicides are chemical substances used to suppress or kill unwanted vegetation (weeds). They are only one of the many types of pesticides that include insecticides, fungicides, rodenticides and nematocides (Jurado *et al*., 2011). Herbicides may be classified based on the time of application: pre-plant herbicides are applied to the soil before the crop is planted; pre-emergence herbicides are applied to the soil after the crop is planted, but before the crop or weeds emerge; post-emergence herbicides are applied to both crop and weeds after they have germinated and emerged from the soil. Herbicides may also be classified by the way they kill or suppress plants. These include hormone inhibitors, cell division inhibitors, photosynthesis inhibitors, pigment synthesis inhibitors, lipid synthesis (cell membrane) inhibitors, or cell metabolism (e.g. amino acid biosynthesis) inhibitors (Radosevich *et al.,* 2007).

All herbicide products have chemical properties that influence their ability to suppress growth or kill plants. While some of these properties are inherent in the chemical nature of the herbicides, others are added to enhance their efficacy. The following are some chemical properties of herbicides that influence their use:

- *Chemical structure*: The biologically active portion of a herbicide product is the *active ingredient*. It is the fundamental molecular composition and configuration of the herbicide. The physical and chemical properties of a herbicide can also determine the method of application and use.

- *Water solubility and polarity*: Herbicides that are produced as salts dissolve quite well in water and are usually formulated to be applied in water, while non-polar herbicide sources are not. Water is the main substance used to disperse (spray) herbicides, and hence the water solubility of a herbicide influences the type of product that is formulated, how it is applied and the movement of the herbicide in the soil profile.

- *Volatility*: Herbicides with a high vapour pressure volatilise easily, while those with a low vapour pressure are relatively non-volatile. The volatility of a herbicide can determine the mode of action and the herbicide's fate in the environment.

- *Formulations*: Commercial herbicide products contain an active ingredient and "inert" ingredients. An "inert" ingredient could be a carrier that is used to dilute and disperse the herbicide (e.g. water, oil, certain types of clay, vermiculite, plant residues, starch polymers, certain dry fertilizers) or an adjuvant (e.g. activator, additive, dispersing agent, emulsifier, spreader, sticker, surfactant, thickener, wetting agent) that enhances the herbicide's performance, handling, or application (Radosevich *et al.,* 2007). In recent years, carriers and adjuvants have been implicated in adding to the toxicity of the active ingredients, and in some cases, have been even more toxic than the active ingredient alone (Radosevich *et al.,* 2007).

Before herbicide products are registered for use, the registration authorities require experimental information on their toxicology, biology, chemistry, and biochemical degradation in addition to their effect on air and water quality, soil microorganisms, and wildlife. Although commercial herbicide products contain several different ingredients, toxicity tests are usually conducted only on the active ingredient, which is the component of the product believed to actually affect the target organism (Radosevich *et al.,* 2007). The criteria for assessing the possible effects of herbicides on the safety of humans, animals and the environment are the herbicide's toxicity (including carcinogenicity, mutagenicity, endocrine disruption, reproduction and developmental abnormalities), biomagnification, and persistence in the environment (Radosevich *et al.,* 2007).

Given the scarcity of water resources in South Africa, aquatic herbicides are of special interest. The potential of an aquatic herbicide to adversely affect aquatic organisms depends on its inherent toxicity to the specific organism and the organism's exposure to the compound in terms of concentration and duration (Wilson, 2009). The inherent toxicity of the pesticide, which is due to its mode of action, is a specific relationship between the organism and the chemical, whereas factors such as application rates and techniques, chemical and physical properties of the pesticide, and environmental conditions at the time of application can make exposures highly variable.

Herbicides now lead all other pesticide groups in terms of amount produced, total acreage treated, and total value from sale. Over the past decades, public awareness of the worldwide increase in the use of herbicides and their adverse effects on aquatic ecosystems has been growing (Pérez *et al*., 2011). Herbicides may reach water bodies directly by overhead spray of aquatic weeds, or indirectly through processes such as agricultural runoff, spray drift and leaching. Potential problems associated with herbicide-use include injury to non-target vegetation, injury to crops, residue in soil or water, toxicity to non-target organisms, and concerns for human health and safety (Radosevich *et al.,* 2007).

Herbicides can decrease environmental water quality and ecosystem functioning by, for example, reducing species diversity, changing community structure, modifying food chains, altering patterns of energy flow and nutrient recycling, and reducing resilience of ecosystems (Pérez *et al*., 2011). In this study, Roundup®, a commercially formulated glyphosate herbicide, was used as the test chemical.

1.3.1 Glyphosate-based herbicides

Glyphosate is an aminophosphonic analogue of the natural amino acid glycine (Pérez *et al*., 2011).The International Union of Pure and Applied Chemistry's (IUPAC) name for glyphosate is N-(phosphonomethyl) glycine and the Chemical Abstracts Service (CAS) registry number is 1071-83-6. The glyphosate molecule has several dissociable hydrogens, especially the first hydrogen of the phosphate group (Figure 1.1). Thus, a typical glyphosate molecule is an acid, and is often referred to as the technical grade glyphosate.

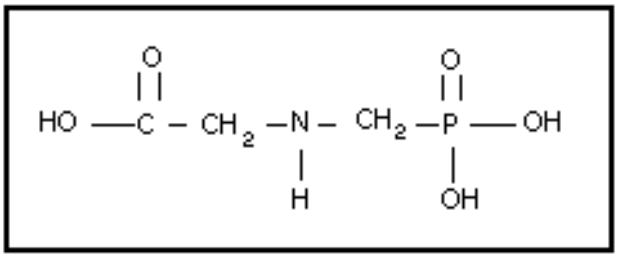


Figure 1.1: Molecular structure of N-(Phosphonomethyl) glycine

Technical-grade glyphosate has a relatively low solubility in water (12 g/L at 25° C and 60 g/L at 100° C), and is insoluble in other solvents because of strong intermolecular hydrogen bonds that stabilise the crystal lattice (WHO, 1994). For this reason, commercial herbicide formulations contain glyphosate in the form of salt, which has much higher solubility but still maintains the herbicidal properties of the parent compound (Pérez *et al*., 2011). Formulations of glyphosate in salt form include monoammonium salt, diammonium salt, isopropylamine salt, potassium salt, sodium salt, and trimethylsulfonium or trimesium salt. Of these, the isopropylamine, sodium, and monoammonium salt forms are commonly used in formulated herbicide products (Miller *et al*., 2010).

The isopropylamine salt is the most commonly used in commercialised formulated products (e.g. Roundup®). The physicochemical properties of glyphosate acid and two of its salt forms are listed in Table 1.1. The concentration of glyphosate is commonly expressed as mg a.i./L (active ingredient/Litre) or mg a.e./L (acid equivalents/Litre) (Pérez *et al*., 2011). Acid equivalent is the theoretical percent yield of parent acid from a pesticide active ingredient, which has been formulated as a derivative (usually esters, salts or amines) (Nordby and Hager, 2011).

Table 1.1: Physical and chemical properties of glyphosate acid, glyphosate isopropylamine salt, and glyphosate ammonium salt (Miller *et al*., 2010).

Active ingredient	Form	Vapour pressure	Henry's constant	Molecular weight	Solubility in water	Log K_{ow}	K_{oc}
Glyphosate acid	Odourless, white solid	1.31×10^{-2} mPa (25° C); 1.84×10^{-7} mmHg (45° C)	4.08×10^{-19} atm·m^3/mol	169.07 g/mol	pH 1.9: 10,500 mg/L; pH 7: 157,000 mg/L	< -3.2	300 - 20,100
Glyphosate Isopropylamine salt	Odourless, white solid	2.1×10^{-3} mPa (25° C); 1.58×10^{-8} mmHg (25° C)	6.27×10^{-27} atm·m^3/mol	228.19 g/mol	pH 4.1: 786 ,000 mg/L	-3.9 or -5.4	300 - 20,100
Glyphosate ammonium salt	Odourless, white solid	9×10^{-3} mPa (25° C); 6.75×10^{-8} mmHg (25° C)	1.5×10^{-13} atm·m^3/mol	186.11 g/mol	pH 3.2: 144,000 mg/L	-3.7 or -5.3	300 - 20,100

Glyphosate-based herbicides are the world's leading post-emergent, organophosphonate systemic, broad-spectrum and non-selective herbicides for the control of annual and perennial weeds (Pérez *et al*., 2011). Roundup® is the major glyphosate-based herbicide in which glyphosate (the active ingredient) is formulated as isopropylamine (IPA) salt, polyoxyethylene amine (POEA) (a surfactant), and water. Other formulations (e.g. Rodeo®) contain the IPA salt of glyphosate without POEA, and in some countries are primarily used for controlling aquatic weeds (Tsui and Chu, 2003; Zhou *et al*., 2004). Other trade names of glyphosate-based herbicides include Roundup®, Roundup Ultra®, Roundup Pro®, Accord®, Honcho®, Pondmaster®, Protocol®, Rascal®, Expedite®, Ranger®, Bronco®, Campain®, Landmaster®, Fallow Master® and Aquamaster® by Monsanto; Glyphomax®, Glypro® and Rodeo® by Dow Agrosciences; Glyphosate herbicide by Du Pont; Silhouette® by Cenex/Land O'Lakes; Rattler® by Helena; MirageR® by Platte; JuryR® by Riverside/Terra; and Touchdown® by Zeneca (Tu *et al*., 2001).

1.3.2 Mode of action of glyphosate

As a systemic herbicide, glyphosate is readily translocated through the phloem to all parts of the plant. Glyphosate molecules are absorbed from the leaf surface into plant cells where they are symplastically translocated to the meristems of growing plants (Pérez *et al*., 2011). Glyphosate's phytotoxic symptoms usually start gradually, becoming visible within two to four days in most annual weeds, but may not occur until after seven days in most perennial weeds. Physical phytotoxic symptoms include progress from gradual wilting and chlorosis, to complete browning, total deterioration and finally, death (Pérez *et al*., 2011). The primary mode of action of glyphosate is confined to the shikimate pathway aromatic amino acid biosynthesis, a pathway that links primary and secondary metabolism.

Shikimate (shikimic acid) is an important biochemical intermediary in plants and microorganisms, such as bacteria and fungi. It is a precursor for the aromatic amino acids phenylalanine, tryptophan and tyrosine. Other precursors of the shikimate pathway are indole, indole derivatives (e.g. indole acetic acid), tannins, flavonoids, lignin, many alkaloids, and other aromatic metabolites. The biosynthesis of these essential substances is promoted by the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), the target enzyme of glyphosate (Figure 1.2). This enzyme is one of the seven enzymes that catalyse a series of reactions, which begins with the reaction between shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP). The shikimate pathway accounts for about 35 % of the plant mass in dry weight and therefore any interference in the pathway is highly detrimental to the plant. Glyphosate inhibits the activity of EPSPS, preventing the production of chorismate, the last common precursor in the biosynthesis of numerous aromatic compounds in bacteria, fungi and plants. This causes a deficiency in the production of the essential substances needed by the organisms to survive and propagate (Stenersen, 2004; Pérez *et al*., 2011). The pathway is absent in animals, which may account for the low toxicity of glyphosate to animals.

However, acute effects in animals, following intraperitoneal administration of high glyphosate doses suggest altered mitochondrial activity, possibly due to uncoupling of oxidative phosphorylation during cellular respiration (WHO, 1994). In summary, glyphosate ultimately interrupts various biochemical processes, including nucleic acid synthesis, protein synthesis, photosynthesis and respiration, which are essential life processes of living things.

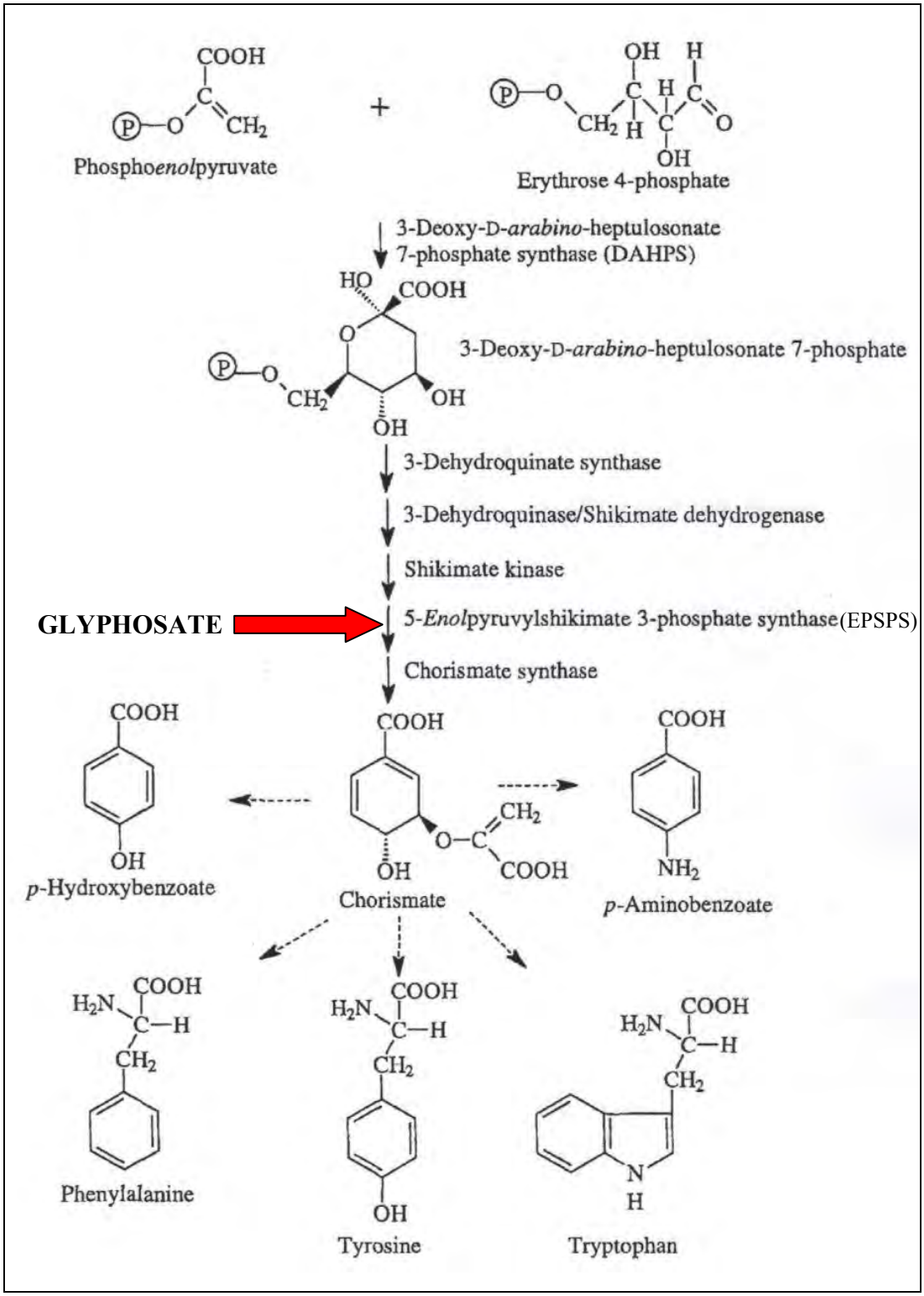


Figure 1.2: Mode of action for glyphosate in plants (red arrow pointing to the glyphosate targeting enzyme 5-enolpyruvylshikimate 3-phosphate synthase [EPSPS]) (Modified from Herrmann and Weaver, 1999)

1.3.3 Environmental fate of glyphosate

Glyphosate has a strong soil adsorption capacity, which limits its movement in the environment. The average half-life of glyphosate in soil is two months, but can range from weeks to years (Tu *et al*., 2001). The presence of glyphosate in water systems may be due to runoff from vegetation surfaces, spray drift, and intentional or unintentional direct overspray, with an average half-life of two to ten weeks (Tu *et al*., 2001). Glyphosate is susceptible to chemical and photo-degradation, although microbial degradation is the primary dissipation mechanism in soils. The rate of degradation in water is generally slower than in most soils because of fewer microorganisms in water than in soils (Schuette, 1998). When glyphosate degrades, it produces aminomethylphosphonic acid (AMPA) and carbon dioxide (Meyer *et al*., 2009), both of which reduce pH when dissolved in water. However, pH is known to affect the stability of glyphosate in water. For instance, glyphosate did not undergo hydrolysis in buffered solution with a pH of 3, 6, or 9 at 35° C, while insignificant photodegradation has been recorded under natural light in a pH 5, 7, and 9 buffered solutions (Miller *et al*., 2010). In natural water systems, glyphosate dissipates through degradation, dilution, and adsorption on organic substances, inorganic clays and the sediment (the major sink for glyphosate in water bodies) (Schuette, 1998; Tu *et al*., 2001). With its long half-life and its ability to cause the death of organisms in aquatic systems, it is recommended that glyphosate should be used as an aquatic herbicide to treat only one-third to half a water body at any one time (Tu *et al*., 2001).

1.3.4 Toxicology of glyphosate

Ecotoxicologists are greatly concerned about the exposure of non-target aquatic organisms to glyphosate formulations because of its high water solubility and the extensive use of glyphosate-based herbicides in the environment, especially in shallow water systems (Tsui and Chu, 2003). The surfactant polyoxyethylene amine (POEA) is thought to be responsible for the relatively high toxicity of Roundup® to several freshwater invertebrates and fishes, although isopropylamine (IPA) salt of glyphosate is also contributes its share (Giesy *et al*., 2000; Tsui and Chu, 2003). Technical grade glyphosate is slightly to very slightly toxic, with reported LC50 values of greater than 55 mg/L and a 21-day NOEC value of 100 mg/L.

Conversely, formulations of glyphosate are moderately to very slightly toxic with 2-day EC50 values of 5.3-5600 mg/L and 21-day MATC values of 1.4-4.9 mg/L reported (WHO, 1994). The LC50 values also determine which glyphosate formulation can be applied in aquatic systems. For example, Touchdown 4-LC® and Bronco® have low LC50s for aquatic species (<13 mg/L), and are not registered for aquatic use, while Rodeo® has relatively high LC50s (>900 mg/L) for aquatic species and is permitted for use in aquatic systems. In the same manner, Roundup® is not registered for use in aquatic systems in the United States because its 96-hour LC50 for *Daphnia* is 25.5 mg/L, while that of glyphosate alone is 962 mg/L (Tu *et al*., 2001). However, Roundup® and other glyphosate-based herbicides are commonly used in South Africa to control both aquatic and terrestrial weeds, even though there is no local water guideline to indicate the effects of such herbicides on non-target organisms.

1.3.5 The use of glyphosate-based herbicides in South Africa

On a worldwide scale, glyphosate-based herbicides, including Roundup®, are the best-selling herbicides. Monsanto first manufactured glyphosate and marketed it as a herbicide in 1971 and was the sole owner of the patent for many years, until the patent expired in 2000 (Tu *et al*., 2001). After 2000, glyphosate-based herbicides could be manufactured by several other companies under different trade names. There are now at least 69 glyphosate-based products on the market in South Africa, produced by 26 companies, including Syngenta, Dow Agrosciences, Bayer, Volcano Agroscience and Kynoch Agrochemicals (ACB, 2005). Sanachem, a South African-based company that was absorbed into Dow Agrosciences in 1997, is the world's third-largest manufacturer of generic agrochemicals, including glyphosate. Since Monsanto's patent for glyphosate expired, other manufacturers have produced cheaper glyphosate-based products that could be used instead of Roundup® but which have the same effect.

The use of glyphosate-based herbicides has increased in recent years for two reasons. Firstly, this is due to the development of a minimum and/or no-till cultivation system for row-cropping systems, a system also known as zero tillage cultivation. Zero tillage cultivation systems eliminated the need for traditional tillage such as ploughing and harrowing, allowing farmers to plant crop seeds directly into soil beneath a mulch of dead plant residue.

Farmers around the world rapidly adopted the zero tillage practice because it required less field preparation, fewer expenses linked to farm machinery, and produced higher profits. Besides these economic benefits, other significant ecological aspects, such as the prevention of soil erosion, organic matter loss, water evaporation and biodiversity have also been identified (Bayer *et al*., 2006; Pérez *et al*., 2011). Notwithstanding these benefits, the system requires large quantities and constant use of herbicides to clear unwanted plants.

The second factor that contributed to glyphosate becoming the most widely used herbicide in the world was the introduction of glyphosate-resistant (GR) crops by Monsanto, in response to the increased competition in the production of glyphosate-based herbicides, and to their promotion of these genetically-modified (GM) crops in "poverty alleviation" programmes (Pérez *et al*., 2011). The first commercially marketed GR crop was Monsanto's glyphosate-tolerant Roundup Ready® (RR) soybean, followed by GR cotton, maize, canola, alfalfa and sugarbeet (Dill *et al*., 2008). Adoption of these transgenic solutions (GR seeds + glyphosate) by conventional farmers significantly improved weed control because glyphosate could be applied before seeding and sprayed several times during growth without damaging the crop. The areas, worldwide, under GR crop cultivation increased between 1998 and 2008 from about 15 million hectares to over 130 million hectares (James, 2008; Pérez *et al*., 2011).

Of the so-called "megabiotech countries" that produce about 90% of the world's GM crops, South Africa is the only African country (FOEI, 2008). As the first country in Africa to permit commercial production of GM crops, South Africa used an area of about 300-500 thousand hectares to cultivate GM cotton, maize and soy beans in the 2004-2005 growing seasons (ACB, 2005). This increased to about 1.4 million hectares in the growing seasons that followed (FOEI, 2008). The two main traits of commercial GM crops have always been herbicide-tolerance (HT) and insect-resistance (IR) (FOEI, 2008). Farmers use more herbicides because they have herbicide-tolerant crops. This practice leads to herbicide resistance (FOEI, 2008). Herbicide-tolerant crops also allow farmers to spray a particular crop frequently and indiscriminately with more herbicide without fear of killing or damaging the crop (FOEI, 2008).

Monsanto's HT varieties of crops grown in South Africa include soya, corn, cotton and canola. About 99% of the world's HT crops are Monsanto's Roundup Ready® varieties, which are tolerant to the herbicide glyphosate (marketed by Monsanto as Roundup®). The upsurge in the reliance on glyphosate with the Roundup Ready® system has resulted in an epidemic of glyphosate-resistant weeds (FOEI, 2008; Preston *et al.,* 2009; Thompson, 2012).

Glyphosate resistance was first discovered in 1996 in Australia among rigid ryegrass populations (Preston *et al.,* 2009). Two major mechanisms of glyphosate resistance have been reported; target site mutations and non-target site alterations (Thompson, 2012). In target site mutations, glyphosate is prevented from binding to the target site 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) by substituting proline 106 within the site with a serine, alanine, or threonine amino. Target site mutation causes nearby amino acids to extend into the glyphosate binding site, which overlaps with the binding site for phosphoenolpyruvate (PEP) (Preston *et al*., 2009; Thompson, 2012). In non-target site alterations, the pattern of glyphosate translocation is altered such that glyphosate accumulates in the leaf tips instead of in susceptible, actively growing tissues such as the root and shoot meristem. This type of resistance is caused by a nuclear encoded gene with partial or complete dominance (Preston *et al*., 2009; Thompson, 2012). The target site mutations tend to provide a lower level of resistance than does the altered translocation mechanism (Preston et al., 2009). Glyphosate-resistant rigid ryegrass is a major issue in vineyards in South Africa (FOEI, 2008).

In the 1990s, Monsanto initiated a programme called 'Seeds of Hope' that was aimed at helping poor smallholder farmers in developing countries, including South Africa (ACB, 2007). Under this programme, Monsanto introduced 'Combi-Packs', which are boxes containing a package of GM maize seeds, some fertilizer, some herbicide, and pictogram instructions for illiterate users. The 'Seeds of Hope' Campaign also promotes 'no- or low-till farming' whereby farmers do not plough or till the land. Rather, they make a small furrow for the seeds and thus avoid the use of tractors, which saves the poor farmer money and time. 'No- or low-till farming' promotes the use of herbicides in large quantities, since weeds are not removed by tilling the land (ACB, 2007).

In 2002, Monsanto, with the support of the South African government, initiated a five-year project known as the *Massive Food Production Programme*, in the Eastern Cape Province (ACB, 2007). Emerging farmers from regions that have potential for agricultural activities were given subsidies to buy Roundup Ready® seeds, insect-resistant (Bt) maize seeds, Roundup® herbicides and fertilizers. Key regions that enrolled in the programme were Alfred Nzo (Mount Ayliff, Kokstad and Matatiele), OR Tambo (Umtata and Mqanduli) and Amatole (Idutywa to Bedford). Thus, large quantities of Roundup® herbicides were used and continue to be used in these areas (ACB, 2007).

Commercial farmers all over South Africa use glyphosate herbicides to control weeds during the cultivation of plant products such as almonds, avocados, bananas, citrus, coffee, deciduous fruit, grapes, guavas, hops, kiwi fruit, papayas, pecan nuts, apples, pineapples, mangoes, litchis, olives, lucerne, leguminous pastures, sugar cane, macadamia nuts, aloes, blackberries, granadilla, cactus, pears, tea, sisal, fruit tree crops, pinea, quinces and agricultural non-crops. Glyphosate herbicides are also applied to seed-beds, to macadamia, during afforestation, in home gardens, and in industrial and municipal areas (Maharaj, 2005; Jadhav *et al.,* 2008).

In addition to agriculture, glyphosate has been used widely in the Working for Water (WfW) programme initiated by the National Department of Water Affairs. The Department recommends using glyphosate-based herbicides such as Mamba, Tumbleweed, Ecoplug, Touchdown and Roundup® and Kilo Max WSG to control invasive alien aquatic and terrestrial plants species. The herbicides are often sprayed directly on these nuisance plants, and thus often directly into a water system. Currently, manufacturers of registered glyphosate-formulated herbicides in South Africa recommend between 2-4 % of the active ingredient (i.e. glyphosate) as the application rate. For example, the recommended lethal dose for Monsanto's Roundup® herbicide on water hyacinth is 3 % (Jadhav *et al.,* 2008). This is also the recommended rate by WfW's guide for foliar spray treatment with glyphosate herbicides for adults of some aquatic plants (Bold, 2007; DWA, 2009). Although these recommended application rates may serve the intended purpose of getting rid of the nuisance plants, there is currently no locally generated ecotoxicological information on the effects of glyphosate-formulated herbicides on resident non-target aquatic animals.

1.3.6 The choice of glyphosate-based herbicides

Although literature on glyphosate research in South Africa is scarce, glyphosate has been found since the 1990s in high concentrations in the Hex River Valley, an agriculturally intensive grape-farming area in the Western Cape (Maharaj, 2005; Dalvie *et al*., 2011). In recent years, the use of glyphosate-based herbicides has increased tremendously in South Africa because of their promotion by both private and public organisations, including the manufacturers, Working for Water (WfW), and commercial farmers (see Section 1.3.5). The following are the main reasons that informed the choice of glyphosate for this study:

- Even though it is generally regarded as having a low potential for contaminating surface waters due to its perceived rapid dissipation and strong adsorption to soils and sediments, it has been detected in surface waters long after being used to kill aquatic weeds (Glusczak *et al*., 2007).

- There is a growing concern among aquatic ecotoxicologists regarding its potential impact on the environment due to increased cultivation of genetically modified glyphosate-resistant (GMG-R) crops and consequent increase in herbicide use, in recent years (Kolpin *et al*., 2006).

- Its mode of action was designed to affect only plants (Stenersen, 2004), but various studies in recent years have reported adverse impact on non-target animals, which warrants study (Giesy *et al*., 2000; Tsui and Chu, 2003; El-Shebly and El-Kady, 2008).

- There is no South African water quality guideline that can be used to assess the effects of the herbicide on water resources (DWAF, 1996a).

Roundup® was selected as a representative of glyphosate-based herbicides by the virtue of it being the most popular and widely used herbicide in South Africa and most parts of the world (Bold, 2007; Romero *et al*., 2011). It is composed of isopropylamine (IPA) salt of glyphosate as the active ingredient; the surfactant polyoxyethylene amine (POEA); and water. Roundup® readily dissolves in water because of the IPA salt form of glyphosate that it contains.

1.4 Crustaceans as organisms for toxicity testing

Crustacea may be classified as a Sub-phylum of the Phylum Arthropoda of the Kingdom Animalia. Their bodies are bilaterally symmetrical and metamerically segmented, and have jointed limbs on all or some of the segments. The entire body architecture is covered with a calcium-containing exoskeleton, which is shed during ecdysis to allow for growth (Hart *et al*., 2001). There are over 40,000 species of crustaceans the world over. The majority of these live in marine and estuarine environments, with only a few freshwater species existing today (Hart *et al*., 2001). Crustaceans have unique biological characteristics, which make them suitable candidates for toxicity testing. These features include their morphology, physiology, behaviour, adaptability, life history and reproductive patterns (Rinderhagen *et al*., 2000).

1.4.1 *Caridina nilotica* (Roux 1833)

Caridean shrimps are true-freshwater crustaceans that belong to the Class Malacostraca, Sub-Class Eumalacostraca, Super-order Eucarida, Order Decapoda, Sub-Order Macrura and Family Atyidae, and are widely distributed in African inland waters (Day, 2001). *Caridina nilotica* is the most common of four indigenous freshwater caridean species found in the Southern Africa sub-region. The others are *C. typus*, *C. africana* and *C. indistinct* (Hart *et al*., 2001). *Caridina nilotica* inhabits both lentic and lotic waters of Mozambique, and the greater part of eastern and northern South Africa, from as far south as the Gamtoos River, extending westwards to the lower Orange River (Hart *et al*., 2001). They thrive in temperatures between 10 to 30° C but their oxygen tolerances are not well known (Hart *et al*., 2001). They are considered important role players in the freshwater ecosystems as they form part of most food webs. They are omnivorous-detritivorous surface scrapers that feed on periphyton scraped from hydrophytes and on plant detritus. They also scavenge on remains of animals such as fish, insects and shrimps. This mode of feeding is useful in clearing debris and epiphytic microflora from leaves of submerged macrophytes, thereby enhancing macrophyte photosynthesis and recycling organic matter (Hart, 1981, Hart *et al*., 2001). *C. nilotica,* an important member of the communities of submerged macrophyte beds and the profundal benthos, provides food for other members of the community as it is preyed upon by predators such as herons, lake-terns and the Nile perch, *Lates nilotica*. *C. nilotica* is reportedly eaten by humans as a delicacy and therefore has economic value (Budeba, 1999).

1.4.2 Suitability of *Caridina nilotica* as an ecotoxicity test organism

Caridina nilotica has been identified as a potential standard toxicity test organism for producing ecologically relevant toxicity test data for the following reasons:

- They are ecologically and economically important as they form an important component of an aquatic ecosystem's food chain.

- They are indigenous, freshwater species and widely distributed. Thus, they are easily accessible and obtainable.

- They are relatively small, making it possible to house many in a relatively small place.

- They breed throughout the year in the wild and in captivity, making them available for toxicological studies at all times.

- They can easily be cultured and maintained in the laboratory (Hart *et al*., 2001).

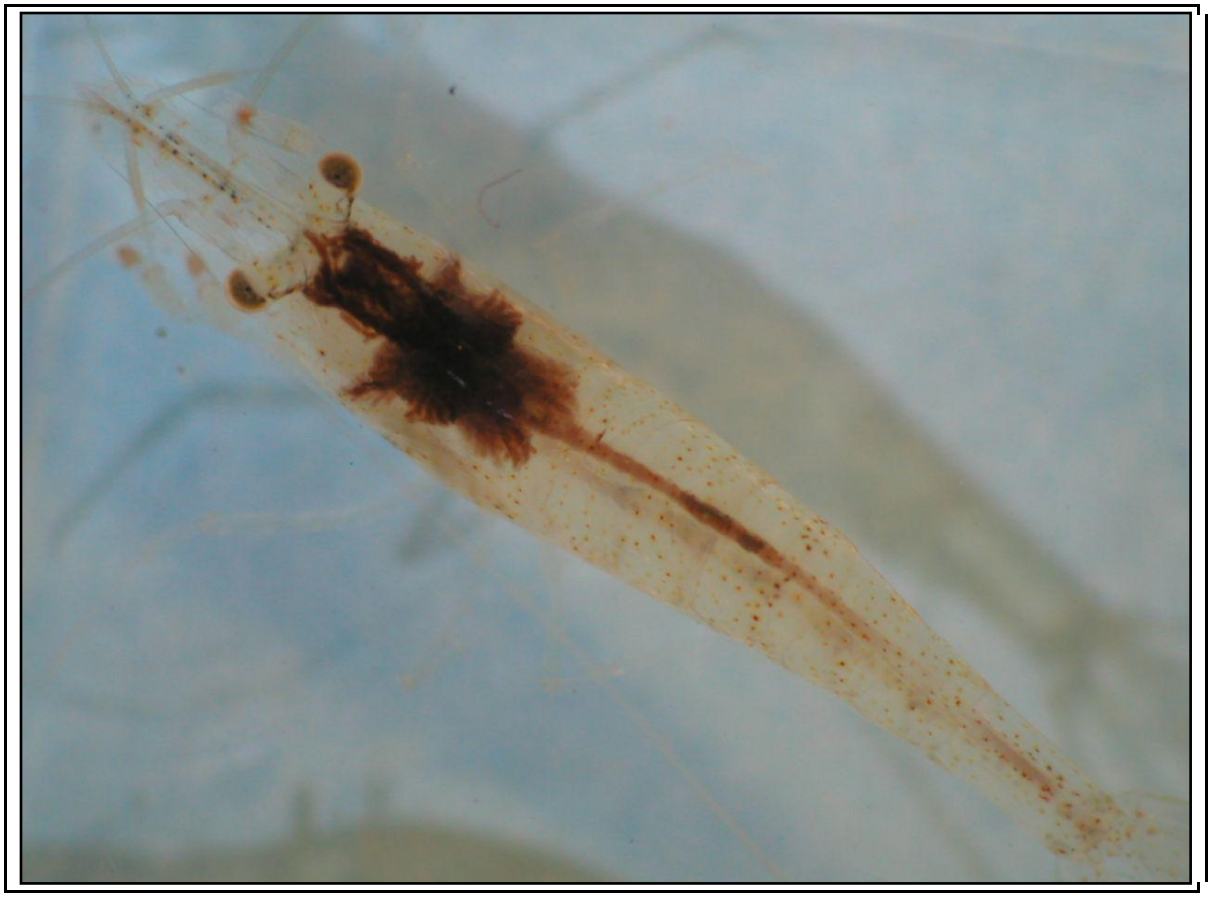


Figure 1.3: Adult *Caridina nilotica*

C. nilotica has been suggested as a good model for developing partial life-cycle, full life-cycle, or multigenerational toxicity testing protocols that can be used to assess ecologically relevant effects of chemicals on growth and reproduction (Okuthe *et al*., 2004). *C. nilotica* toxicity tests have been developed for acute toxicity tests for neonate, juvenile and adult life history stages (Scherman and Palmer, 2000). Chronic test methods for embryotoxicity and partial life-cycle tests have also been conducted (Slaughter, 2005; Ketse, 2006). In this context, this study hypothesised that each life history stage of *C. nilotica* can potentially be used in routine and regulatory testing for glyphosate-based herbicides.

1.5 Life history toxicity tests (LHTT)

Organisms in the environment are often exposed to variable concentrations of pollutants that are mostly of anthropogenic origin. Among these, pesticides are one of the major components, with strong direct and indirect impacts on both flora and fauna communities (Eraly *et al.,* 2011). Life history theory postulates that under direct and indirect environmental stress, organisms tend to have reduced growth rates, adult sizes, reproductive allocation, and production of larger offspring, and postponed or delayed reproduction (Eraly *et al.,* 2011). These trends are primarily linked to energy constraints. At the individual level, direct physiological effects may occur when pesticides interact with essential molecules and alter their structure or function. This can have an impact on biochemical processes (Paul-Pont *et al.*, 2010) such as inhibition of various enzymatic pathways and antioxidant activity. Indirect physiological effects in individual organisms may occur when reactive oxygen species are produced and they tend to disrupt lipid structure, cause lipid peroxidation or apoptosis (Paul-Pont *et al.*, 2010; Eraly *et al.,* 2011). Pesticide pollution may also impact natural populations through cascading ecological effects. For example, changes in vegetation structure or reduction in prey availability due to pollution may cause reduction in fitness due to increased intraspecific and interspecific competition for critical resources. This will result in a lower energy budget which may cause changes in predation risk, and decrease reproductive output or survival (Eraly *et al.,* 2011). Changes at population level due to environmental stress may, in turn, induce changes at community level, especially if highly sensitive species are keystone members. This may alter critical ecosystem functions (Clements and Rohr, 2009).

Studies on invertebrate populations from polluted sites have revealed multiple and often complex effects of pesticides on life history traits such as development time, survival, body size, age at reproduction, fecundity and egg size (Eraly *et al.,* 2011). These alterations in life history traits as a result of pollution can be considered adaptive if individuals from the polluted sites increase in fitness compared to individuals from less or non-polluted sites (Kawecki and Ebert, 2004). Body condition (e.g. body mass relative to body size) is considered as one of the most sensitive individual-based measures of environmental stress effects (Engert *et al*., 2012).

While body size is fixed after maturation, body mass varies with nutritional status and energy balance, and this is often correlated with fecundity in a positive manner (Aisenberg, 2009). For instance, when females are energetically stressed due to chemical pollution, they often invest in fewer but larger offspring relative to their own size so that the offspring can mature earlier, develop faster and survive better. This will help the offspring to counter the adverse effects of the pollutant that accumulate during a lifetime (Eraly *et al.,* 2011).

Non-adaptive life history may occur when tolerance mechanisms of organisms are reduced due to environmental stress. Since these mechanisms are believed to be energetically costly, life-history theory predicts that resource demanding traits such as growth and fecundity will be reduced under environmental stress. For instance, energetic constraints in polluted environments may cause non-adaptive delays in reproduction. Such a phenomenon may impact changes on body size, reproductive isolation between stressed and reference populations, and consequently on population dynamics (Eraly *et al*., 2009).

Furthermore, environmental stress due to chemical pollution may cause life history shifts such as delay change from one stage to the next in aquatic organisms. For example, some marine dinoflagellates can form temporary cysts from motile cells, which may function to resist unfavourable short-term environmental conditions (Toth *et al*., 2004). A shift in life history stage when exposed to chemical agents suggests that intraspecies chemical communication can be an important mechanism that controls life history shifts in such organisms. In this study, the impact of Roundup® herbicide on life history stages and physiological traits of *C. nilotica* under laboratory conditions was evaluated.

1.6 Endpoints used to measure responses of aquatic organisms to biological stressors

In any toxicity test, it is important that the measured response of a particular endpoint is directly related to exposure to the potential stressor and that the response is the result of physiological function interference. Endpoint measures may be lethal or sub-lethal, and may be interrelated, which tends to complicate the interpretation of toxicity test results (OECD, 2005).

For example, a chemical that causes a sub-lethal effect could reduce a test animal's ability to swim, so reducing its ability to feed, which then reduces its growth. This reduced growth may also be expressed in reduced fecundity or other measures of reproduction. Additionally, different endpoint measures may have different sensitivities to stressors (OECD, 2005). For example, reproductive parameters are usually more sensitive measures of contaminant toxicity than survival. Thus, considering these two characteristics of endpoints (i.e. the possible interconnections among potential endpoints and the different sensitivities of endpoints), it is necessary to use multiple endpoints in assessing potential chemicals.

Endpoint measures that are used to assess lethal or acute effects of chemicals on animals are mainly mortality and immobility. However, there are several endpoint measures that can be used to assess sub-lethal or chronic toxicity of chemicals to animals. At the 'physical' level, measures of survival, growth, morphological changes, and behavioural changes in test animals are used as endpoint indicators. Measures of reproductive performance that are often used to assess sub-lethal response include sexual maturity, time to first brood release, time required for egg development, fecundity, gonad histopathology, and alterations in reproductive characteristics. Biochemical measures used as possible endpoints for chemical toxicity tests include, but are not limited to, metabolic disruption, steroid metabolism, vitellogenin induction, lipid peroxidation, acetylcholinesterase activity, cytochrome P450enzymes and blood glucose levels. In the present study, it was necessary to select endpoint measures that could be included in the development of life history toxicity test protocol for *C. nilotica*. Possible lethal and sub-lethal endpoints that could be used as responses of *C. nilotica* exposure to glyphosate based herbicides were investigated. Mortality was used as the endpoint measure to show the lethal exposure response of *C. nilotica* to Roundup®. Endpoint measures reflecting sub-lethal responses of *C. nilotica* to Roundup® that were investigated included growth (moulting, length, weight), reproduction (embryo development, gonad histopathology), and biochemical (acetylcholine activity, lipid peroxidation). Each of these endpoints is briefly reviewed in the following pages, together with a description of the different experiments that were conducted to examine each measure of endpoint.

1.6.1 Lethal exposure response measures

Mortality is the most common endpoint measure when organisms are exposed to a lethal dose in ecotoxicological studies, although immobility has frequently been used. Rubach *et al*. (2011) evaluated the relevance of using mortality and immobility as endpoints to reflect the toxicity of the organophosphorous insecticide chlorpyrifos in fourteen different freshwater arthropods. Using dose response models and species sensitivity distributions (SSDs), they compared the differences in response dynamics during 96 hours of exposure with these two endpoints across the different species. Their study suggests that freshwater arthropods vary less in their immobility response than in their mortality response. They suggested immobility as the relevant endpoint for SSDs and ERA (environmental risk assessment) because they found it was a more sensitive endpoint than mortality, with less variability across the tested species. Generally, effect concentrations for immobility and mortality will converge to the same value with time, but this does not occur with the same speed for all species (Rubach *et al*., 2011). However, a good match between effective (immobility) and lethal (mortality) concentrations can exist right from the start of a toxicity test where LC50/EC50 ratios equal one, approximately. For some species, the differences between LC50 and EC50 can remain relatively constant within the 96 hours of testing. Furthermore, the extent to which LC50 and EC50 values differ for certain time points is species specific (Rubach *et al*., 2011). For example, exposure concentrations may not induce any significant incipient mortality in a particular species, but will induce immobility at very low concentrations in another species. This is due to differences in toxicokinetics and/or toxicodynamics between the species. For instance, differences in toxicokinetics may enable one species to decrease or regulate uptake and eliminate the test chemical, or detoxify it quickly, thereby significantly delaying incipient mortality. Toxicodynamic differences, such as differences in the interaction of the stressor and target enzyme, or in the ability to compensate or repair damage, may cause different species to respond differently to the test chemicals (Rubach *et al*., 2011).

Mortality was also used as an endpoint response measure by Wirth *et al.* (2001) when they studied the acute mortality of adults and sub-lethal embryo responses of *Palaemonetes pugio* to endosulfan. Their findings suggest that the insecticide endosulfan may preferentially affect male grass shrimp, and exposed female grass shrimp may produce embryos with delayed hatching times.

They suggested that the size difference between male and female grass shrimp might be the cause as mortality decreases by 25% with a corresponding increase in size of 1 mm. Key and Fulton (2006) investigated the correlation between 96-h mortality and 24-hour acetylcholinesterase inhibition in three life stages of the grass shrimp (*Palaemonetes pugio*). They found a strong positive relationship (R^2 = 0.962) between the ratio of the lowest observed effect concentration and 20% effect concentration (LOEC/EC20). Therefore, they concluded that sub-lethal endpoints could be used as a predictor of 96-h mortality for the life stages of *P. pugio*. Mortality was used as an endpoint in a 21-day nitrate exposure test with the amphipod *Gammarus pseudolimnaeus* (Stelzer and Joachim, 2010). Mortality rates of *G. pseudolimnaeus* were low during the experiment, with less than 10% mortality recorded in all concentrations. They concluded that the nitrate concentration had no effect on amphipod mortality. The authors were unable to calculate LC10 or LC50 for *G. pseudolimnaeus* because of the low levels of mortality and lack of treatment effects.

1.6.2 Growth measures used as sub-lethal responses to exposure

Body weight

Body weight and length are two direct measures of growth that may be used in the assessment of sub-lethal effects on arthropods. Simple dry weight can be determined by drying sampled animals at an average temperature of 60 ºC, and a mean drying time of 48 hours to constant weight (OECD, 2005). However, for many invertebrates, ash-free dry weight (AFDW) is often used as the appropriate weight measurement because the method reduces any inaccuracies that might be introduced by inorganic constituents in the animal's body. Inorganic components may arise from processes such as the development of skeletal components, or from feeding (the ingestion of sediment) (OECD, 2005). In small-sized crustaceans, such as caridean shrimps and mysids, the removal of ash from the dry weight measurement is unnecessary since it would have a negligible effect on the accuracy of the measurement (USEPA, 2000). Separate determinations should be made for male and female crustaceans because they might be different sizes (OECD, 2005).

Body length

Different body length dimensions of shrimp can be measured to determine growth. These may include the distance from the base of the eye-stalks to the tip of the telson or to the tip of the exopod; or from the tip of the carapace to the tip of the exopod along the midline of the body (OECD, 2005). Sometimes, it is difficult to measure preserved animals because of the body curvature that results from the fixation process. Relaxing the animal and then determining length as the sum of a series of relatively straight-line measurements prior to fixation may reduce inaccuracy. Animals may be anaesthetized in soda water to relax them prior to length measurements (Langdon *et al*., 1996; Winkler and Greve, 2002).

Reduced growth may not be a particularly sensitive endpoint, but it is the most common response to sub-lethal exposure to toxicants (OECD, 2005). Reduced growth is connected to reproductive success since the size of female crustaceans is directly related to fecundity (Verslycke *et al*., 2004). The age of test animals and the toxicant concentration are related to the effect of toxicant exposure on growth. In general, young crustaceans are more sensitive than adults to toxicant exposure. However, effects of toxicants on juvenile survival do not always lead to reductions in population growth rate since survivors may compensate for the lost individuals by increasing their own reproduction (Widarto *et al*., 2007). Similarly, effects at the individual level may sometimes run opposite to the population level effects. Moe *et al*. (2001) found that juvenile *Lucilia sericata* (blowflies) increased growth when exposed to sub-lethal cadmium concentrations, although population growth rate decreased. Indeed, their studies confirmed the complex relationship between toxicant effects on individual performance versus population dynamics. In a separate study, Widarto *et al*. (2007) evaluated the effects of nonylphenol on two life-history traits (i.e. juvenile survival and fecundity) of the parthenogenetic springtail, *Folsomia candida,* in relation to population growth rate. They reported that the presence of nonylphenol stimulated fecundity and the body-growth rate of test organisms, but did not affect population growth rate. The authors found that the effect of the test chemical on fecundity was the main contributor of the observed effect on growth rate. However, since relative sensitivity of fecundity (elasticity) was very low, large changes in fecundity resulted in a minimal effect on population growth rate. Conversely, juvenile survival had higher elasticity, but was not affected by nonylphenol, and hence did not contribute to effects on population growth rate.

The study by Widarto *et al*. (2007) revealed that increase in body size and fecundity after exposure to chemicals does not necessarily translate into increase in population growth rate. Their study also shows that effects of chemicals on individual life-history traits are attenuated at the population level and that population growth rate is an appropriate endpoint for ecotoxicological studies.

Moulting

Moulting is an important physiological process in arthropods because it allows them to grow (Lachaise *et al*., 1993; Spaziani *et al*., 1999). It is regulated by the interaction of moult stimulating hormones (MSHs, generally referred to as ecdysteroids), and nervous system secretions produced in the cephalothorax, and with moult-inhibiting hormones (MIHs) produced in the eyestalks (OECD, 2005). In higher crustaceans such as the Malacostraca, paired cephalic endocrine organs called Y-organs (absent in lower crustaceans such as Entomostraca) secrete three different ecdysteroids, namely ecdysone (E), 25-deoxyecdysone (25dE), and 3-dehydroecdysone (3DE). Usually these organs produce either E + 25dE, or E + 3DE. Activities of the Y-organ are regulated mainly by the MIH, an inhibitory neuropeptide secreted from the X-organ-sinus gland complex (Lachaise *et al.*, 1993; Spaziani *et al*., 1999). Since hormones regulate moulting in crustaceans, moulting is a clear indicator of the adverse effects of endocrine disrupting chemicals, which include many pesticides. Hormonal regulation of moulting in crustaceans makes the process vulnerable to the adverse effect of endocrine disrupting chemicals (EDCs), including many pesticides (OECD, 2005). Furthermore, since substantial growth in crustaceans can only occur as a result of moulting, any disruption in the moulting process could affect growth. Therefore, estimation of moulting frequency may be a useful endpoint.

Moult stage is a useful technique for measuring growth (OECD, 2005). If moult stages are classified based on duration of different stages under normal laboratory conditions, then the environmental effects on relative duration of stages can be evaluated, using the moult-stage technique (Gorokhova, 2002). However, moult-cycle chronology is a prerequisite for the use of moult staging in growth studies. The moult-stage technique was used to determine the main moult stages for juveniles and young adults of *Mysis mixta* and *Neomysis integer* under different temperature conditions and feeding. The technique was also used in the field to determine the moult cycle duration of *Mysis mixta* (Gorokhova, 2002).

1.6.3 Reproductive measures used as sub-lethal responses to exposure

Embryotoxicity

Embryo development time (or incubation period) in caridean shrimps is measured as the number of days between the first appearance of embryos in the brood pouch and the first release of neonates. The incubation time for *Neomysis integer* was calculated as the difference between the date of laying eggs in the brood pouch and the date of release of the juveniles from the marsupium (Winkler and Greve, 2002). In uncontaminated systems, incubation period is related to environmental temperature, salinity and an interaction between the two factors. However, the effect of most contaminants is to lengthen hatching time. In many embryotoxicity studies, either gravid females are placed in exposure containers, or fertilized eggs are removed from the female and placed in exposure containers where they develop to hatching. Winkler and Greve (2002) removed gravid females from culture jars and placed them in individual exposure containers. They reported that temperature affected the start of maturation, and that the incubation period was shorter at a higher temperature (1.5 months at 15° C) than when the temperature was lower (3 months at 10° C). They suggested that the reproduction rate increased at higher temperatures because of declining incubation periods and an increased number of neonates released per brood. Mu and Leblanc (2002) exposed both gravid maternal and isolated embryos of *Daphnia magna* to the agricultural fungicide fenarimol to evaluate embryo development and susceptibility to the anti-ecdysteroidal properties of the fungicide. They reported that exposure of either gravid maternal animals or isolated embryos to the test toxicant resulted in embryo abnormalities which ranged from early partial developmental arrest to incomplete development of antennae and shell spines. They found that such developmental abnormalities were linked to suppressed ecdysone levels in the embryos and that the abnormalities could be prevented by co-exposure to 20-hydroxyecdysone. The results also showed how environmental anti-ecdysteroids, such as fenarimol, in many agro-chemicals disrupt the normal development of crustacean embryos.

Effective embryotoxicity investigations are based on identification of specific developmental features during embryogenesis and the susceptibility of such features to chemical exposure. Ketse (2006) investigated embryonic development of *C. nilotica* under laboratory conditions and identified stages in embryonic development which could be used as quantifiable experimental endpoints in toxicity tests. She identified and described seven potential developmental stages that could be used in toxicity tests to study exposure-response relationships to stressors. In the current study, these developmental features were used as the basis for *C. nilotica* embryotoxicity, using glyphosate as the test substance.

Gonad histopathology

Histopathology is a technique that combines knowledge and experience of fundamental animal anatomy, physiology, endocrinology, pathology, and toxicology. It can enhance relevant biological information in sub-lethal exposure tests by allowing proper and more specific hazard identification, such as the organs targeted by toxic substances and mechanisms of action in aquatic ecotoxicological studies (Wester *et al*., 2002). Histopathology is relevant to an ecological assessment of toxicants because it can detect critical adverse biological effects (e.g. reproductive abnormality) and is more sensitive than the classical toxicological testing, since histological effects are visible at lower exposure concentrations than they are at toxicological endpoints, such as mortality or behavioural changes (Wester *et al*., 2002). The use of small crustaceans in practical histopathology makes it possible to embed the animals *in situ* for a quick overview of various relevant organs, making screening fast and comprehensive (Wester *et al*., 2002).

1.6.4 Biochemical measures used as sub-lethal responses to exposure

Acetylcholinesterase activity

The main physiological function of the enzyme AChE is to hydrolyze acetylcholine (ACh), a neurotransmitter of cholinergic synapses during transduction of nerve impulses. Inhibition of AChE prevents the hydrolysis of ACh in nerve synapses and neuromuscular junctions, causing accumulation of excess ACh at these sites.

This results in over-excitation of the synaptic and muscular tissues, which may lead to abnormal behaviours such as hyperactivity, asphyxia and death. AChE activity is therefore regarded as a good biomarker to detect a range of toxic compounds in aquatic animals, including insecticides, herbicides, surfactants and metals (Parvez and Raisuddin, 2005; Richardson *et al*., 2010).

In a study to evaluate AChE activity in the oyster *Crassostrea corteziensis*, Bernal-Hernandez *et al*. (2010) exposed the organisms to the pesticide dichlorvos. The results of their study revealed that AChE activity was 65% lower in oysters exposed to the pesticides than in control animals. Based on this outcome, they suggested using AChE activity in oysters as early biomarkers of effects and exposure to pesticides in aquatic environments. Similar observations and suggestions were made when the mosquito fish *Gambusia affinis* was exposed to the pesticide chlorpyrifos (Kavitha and Venkateswara, 2008).

Although AChE is used as a classical biomarker in biomonitoring studies with regard to the exposure of a number of organophosphate and carbamate pesticides, recent studies have shown the existence of sublethal effects of glyphosate-based compounds on biomarkers of neurotoxicity including AChE (e.g. Glusczak *et al*., 2007; Modesto and Martinez, 2010; Menendez-Helman, 2012)

Lipid peroxidation

Lipid peroxidation is a recognised mechanism of cellular injury in plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds which include reactive carbonyl compounds. Polyunsaturated fatty acid peroxides decompose to produce malondialdehyde (MDA) and 4-hydroxyalkenals (HAE), and the measurement of MDA and HAE is used as an indicator of lipid peroxidation. Whether cells and tissues are susceptible to oxidative stress when exposed to pesticides reflects the balance between oxidative stress and the anti-oxidant defence capability. Since free radicals and hydroperoxides are potentially harmful, toxicants that stimulate lipid peroxidation and/or weaken anti-oxidant defence capability may cause or increase cellular susceptibility to oxidative damage.

Animals exposed to pesticides may have their anti-oxidant defence capabilities directly or indirectly modified, rendering them susceptible to oxidative stress. Oxidative damage of cells and tissues of animals exposed to pesticides may be the result of insufficient anti-oxidant potential (Banerjee *et al*., 1999). Developing biomarkers of oxidative stress as a pollution-mediated mechanism of toxicity requires knowledge of how anti-oxidant biochemical systems and target molecules are influenced by test toxicants (Barata *et al*., 2005).

Different toxicants may produce different anti-oxidant/pro-oxidant responses in organisms, depending on whether the organism can produce reactive oxygen species and anti-oxidant enzymes to detoxify them. Barata *et al*. (2005) assessed changes in anti-oxidative processes in juveniles of the freshwater crustacea *Daphnia magna* after exposure to paraquat, endosulfan (pesticides), cadmium, copper (metals) and menadione (quinine) in a 48-h sub-lethal toxicity test. They evaluated lipid peroxidation and activities of key anti-oxidant enzymes including catalase, superoxide dismutase, glutathione peroxidase and glutathione S-transferases. They found that increased lipid peroxidation produced low anti-oxidant enzyme activity for menadione and endosulfan, while decreased lipid peroxidation enhanced levels of anti-oxidant enzyme activities for paraquat. Conversely, high levels of both anti-oxidant enzyme activities and lipid peroxidation were found for copper, whereas cadmium recorded low anti-oxidant enzyme responses with insignificant increases in lipid peroxidation. Hence, Barata *et al*. (2005) deduced that cadmium has little potential to alter the anti-oxidant/pro-oxidant status in *D. magna*.

Beuret *et al*. (2005) suggested that glyphosate exposure and metabolism in the liver of animals can lead to excessive production of MDA and oxidative stress through unregulated generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radical, peroxyl radicals and singlet oxygen. Excessive ROS in turn can be detrimental to cell structure through oxidative damage of lipids, proteins or DNA, and altered regulation of gene functions critical for development, differentiation, and aging.

1.6.5 Lethal and sub-lethal exposure responses of *Caridina nilotica* to Roundup® exposure

In this study, lethal (short-term and long-term) and sub-lethal (short-term and long-term) responses of the freshwater shrimp *C. nilotica* to Roundup® exposure were investigated using different endpoint measures. For the lethal tests, range-finding and definitive acute toxicity tests were conducted. The range-finding test was aimed at determining the range of glyphosate concentrations that are likely to cause a response in *C. nilotica*. This information was used to calculate the effective concentrations required for different *C. nilotica* life stages in the definitive acute and chronic toxicity tests.

The specific objective of the definitive acute toxicity test was to investigate the herbicide's effect on neonates, juvenile and adult *C. nilotica* with mortality or immobility as endpoints. This was necessary to provide ecotoxicological data, including the median lethal concentration (LC50), lower observed effect concentration (LOEC) and no observed effect concentration (NOEC) of Roundup® for *C. nilotica*. The test also formed the basis for selecting the appropriate concentrations needed for chronic toxicity tests with *C. nilotica*.

For sub-lethal investigations, the aim was to determine the effects of glyphosate on *C. nilotica* growth, reproduction and biochemical markers as endpoints. The specific objectives of growth tests were to determine the glyphosate concentrations that would suppress growth of *C. nilotica* in long-term exposure tests. The test also provided a long-term no observed effect concentration (NOEC) and no effect concentration (NEC), which were used in long-term species sensitivity data to derive water quality guidelines. Response measures investigated for growth were body weight, total length and moulting, whereas embryotoxicity and gonad histopathology were evaluated as response measures of reproductive toxicity. Furthermore, this study investigated the use of biochemical endpoints of *C. nilotica* as possible biomarkers for the pollution of South African surface water by glyphosate formulated herbicides. Acetylcholinesterase activity and lipid peroxidation were used as response measures.

1.7 Environmental assessment

Risk is a function of hazard and exposure. It expresses the possibility that a particular hazard has caused harm to a certain extent, according to the degree of exposure. There are different types of risk assessment. These include human, chemical, ecological and environmental risk assessments. Environmental risk assessment can be used as a tool to develop environmental guidelines and decision making (Finizio and Villa, 2002). Cormier and Suter (2008) proposed four different types of environmental assessment. These are condition assessment (to detect chemical, physical, and biological impairments); causal pathway assessment (to ascertain the causes and sources of such impairments); predictive assessment (to appraise environmental, economic, societal risks, and benefits associated with different possible management actions); and outcome assessments (to assess the results of the decisions of an integrative assessment) (Cormier and Suter, 2008).

Although there are frameworks that have integrated ecological and human health risk assessments (WHO, 2001), no current framework overtly includes all types of environmental assessments in an integrated manner. They attributed this to lack of common assessment terminologies, which in turn, affect effective communication of research findings. In order to standardise terminology and make communication easy between types of assessments and environmental programmes, Cormier and Suter (2008) proposed that all environmental assessments should have a common structure of planning, analysis, and synthesis. They also proposed an integrated environmental assessment framework that combines all four types of assessments. This framework recognises that the link between assessments is based on intermediate decisions that lead to another assessment or a final decision, which indicates success in resolving the problem. In this study, the framework is applied to the environmental water quality management of the Swartkops River, and formulation of water quality guidelines for glyphosate in South Africa.

1.8 Motivation, aim, objectives and guiding research question

1.8.1 Motivation

The above discussions have demonstrated that herbicides as toxic agents can have deleterious effects on aquatic ecosystems. Glyphosate-formulated herbicides are frequently used by individuals such as farmers and by organisations such as Working for Water to control terrestrial and aquatic weeds in South Africa. The use of glyphosate-based herbicides is encouraged by multinational companies, including Monsanto, who are the principal manufacturers of these herbicides. Since the primary aim of these groups is to control growth of weeds, it is necessary to investigate more about the effects of such chemicals on non-target organisms. Unfortunately, South Africa currently has no ecotoxicological data on the impact of these herbicides on indigenous freshwater organisms. Thus, motivation for this study was based on the desire to fill this gap by providing an environmental water quality assessment, including ecotoxicology and biomonitoring data for glyphosate.

1.8.2 Aim, objectives and guiding research question

Based on the literature review and the motivation, the overall aim of this study was therefore to provide a sound scientific background for the environmental water quality management of glyphosate-based herbicides in South Africa. In order to achieve this aim, the following specific objectives were set:

- To evaluate the exposure-response relationship of *C. nilotica* exposed to Roundup® under different laboratory exposure conditions.

- To evaluate the species sensitivity distribution (SSD) of South African aquatic organisms exposed to Roundup®.

- To evaluate the field effect of a glyphosate-based herbicide on physicochemical and biological conditions of the Swartkops River through biomonitoring.

- To develop water quality guidelines (WQGs) for the management of glyphosate-based herbicides as part of integrated water resource management (IWRM) in South Africa.

- To develop a conceptual framework for the integrated environmental water quality management of glyphosate-based herbicides in South Africa.

As the literature reviewed earlier in this chapter (section 1.3.3) revealed, glyphosate has a strong soil adsorption capacity, which limits its movement in the environment. However, it has been found in freshwater ecosystems. Furthermore, the rate of glyphosate degradation in water is generally slower than in most soils because there are fewer microorganisms in water than in soils. Therefore, the guiding research question underlying this study is:

Do glyphosate-based herbicides pose any risks to South African freshwater ecosystems?

This is important because without any good evidence that these herbicides are likely (at least) to pose any risk to the country's freshwater ecosystems, there will be no need to commit time and other scarce resources in managing them.

1.9 Thesis outline

This thesis consists of seven chapters. Although each chapter represents a major section of the study with various sub-sections in a chronological sequence, each chapter builds on the preceding one.

Chapter One

This chapter spells out the background for this study, including pollution of freshwater ecosystems and the need to protect aquatic life. The chapter reviews management of water resources in South Africa; tools used for environmental water quality management; herbicides as biological stressors, particularly glyphosate-based products; crustaceans as test organisms, especially the freshwater shrimp *C. nilotica*; life-history stages toxicity tests; endpoints used to measure responses of aquatic organisms to biological stressors, and environmental assessment. The chapter concludes by stating the motivations, overall aim, specific objectives and guiding research question of this study.

Chapter Two

This chapter provides insight into the general material and methods used in the study. These include maintenance and culture of test animals, test solutions, experimental systems, and general statistical methods.

Chapter Three

This chapter presents the ecotoxicological aspects of this study. Laboratory-based investigations were conducted under different conditions to describe the biological stress responses at varying biological scales in the shrimp *C. nilotica* exposed to varying concentrations of Roundup®.

Chapter Four

This chapter is also based on laboratory ecotoxicological study, but rather than responses of a single species to a biological stress, the responses of multiple South African aquatic species exposed to Roundup® were investigated through species sensitivity distribution (SSD). The outcome of the SSD was used to develop a South African-based water quality guideline for glyphosate.

Chapter Five

This chapter presents field investigations of the physicochemical and biological conditions of the Swartkops River before and after spraying with Kilo Max WSG (a glyphosate-based herbicide).

Chapter Six

This chapter presents an integrated conceptual framework for environmental management of glyphosate-based herbicides by applying the weight of evidence (WoE) method to integrate data from both laboratory and field investigations. This framework includes a newly proposed method called Confounding Factor Analysis (CFA) that can be used to assess in-stream concentration.

Chapter Seven

This chapter presents the concluding summary of the study in relation to the overall aim, specific objectives, research question and hypotheses it set out to address. Suggestions as to how to apply and implement the findings and conclusions emanating from this study are also given. The chapter concludes by giving recommendations for further studies in order to address specific issues encountered during the course of this study.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Introduction

This chapter presents general materials and methods used in both laboratory and field studies to achieve the aims and objectives of this work. These include maintenance and culture of test animals, equipment used, medium of exposure, preparation of solutions, analysis of glyphosate concentration in water, and statistical analysis. Details of specific materials and methods are described in relevant chapters that follow.

2.2 Maintenance and culture of test animals

The Unilever Centre for Environmental Water Quality of the Institute for Water Research (UCEWQ-IWR), Rhodes University, maintains a culture of *C. nilotica* in its freshwater laboratory according to a protocol developed and constantly revised and updated by the Institute since 1998. Shrimps of all ages are maintained in glass tanks filled with carbon-filtered dechlorinated tap water. Each tank is fitted with an automatic aquarium heater to provide a constant temperature of 25±1 ºC for maintenance and breeding; a thermometer; an aerator to ensure sufficient oxygen dissolution; and an external water-circulating pump to circulate water and remove debris by functioning additionally as a biological filter. The inlet of the water circulating pump is covered with a fine nylon mesh to prevent neonates from being drawn into the filter. Shrimps feed on green algae growing in the tanks, supplemented with a daily supply of fish feed flake (TetraMin Tropical Flake). A net is suspended in the middle of the tank to act as a vertical surface for the shrimps to rest on. Shrimp cultures receive natural light through transparent window panes, and this light is supplemented with a 12-hour light: 12-hour dark artificial light regime using Biolux fluorescent tubes.

Ovigerous females (females with eggs) are removed from the breeding tanks and placed into separate tanks. After about 17 days of embryo development, hatching occurs and neonates are released. Neonates are considered juveniles after seven days and adults after 40 days (Okuthe *et al*., 2004). Each life-history stage, (embryos, neonates, juveniles and adults) was used in conducting different toxicity tests.

2.3 Test solutions

2.3.1 Preparation of stock and working solutions

Liquid Roundup®, registered and distributed by Monsanto South Africa (Pty) Ltd. (Figure 2.1), was purchased from a local shop in Grahamstown, South Africa. It contains 360 g glyphosate (glycine) acid equivalence per litre (a.e./L) (480 g isopropylamine salt of glyphosate/litre) active ingredient. Since the manufacturers of glyphosate-formulated herbicides recommend 2-4% application rate, a 2% stock solution was prepared by dissolving 20 mL Roundup® with 980 distilled water (measured with a 1000-mL measuring cylinder) to obtain a concentration of 7200 mg/L a.e. This stock solution was kept in a 1000-mL Schott Duran bottle. Appropriate dilutions of the stock solution were made with dechlorinated tap water to obtain the desired nominal exposure concentrations just before the start of each experiments. Both stock and working solutions were prepared at the start of the experiments. Roundup® dissolves well in water so no solvent carrier was used. All units for Roundup® concentrations reported in this study were in mg/L a.e., just as the unit of the stock solution.

Figure 2.1: Liquid Roundup® (5 L) registered and distributed by Monsanto South Africa (Pty) Ltd.

2.3.2 High performance liquid chromatography (HPLC) analysis of glyphosate

Measured concentrations of glyphosate in a set of test samples were determined using a high performance liquid chromatography (HPLC) technique. A laboratory spiked sample method was used to evaluate recovery of the complete analytical technique, as modified and optimised from Peruzzo *et al*., 2008. A glyphosate (PESTANAL® analytical standard) of 99.2 area % (HPLC assay) was obtained from Sigma-Aldrich. Acetonitrile (CH_3CN, pesticide quality), chloroform ($CHCl_3$, analytical grade), potassium phosphate monobasic (KH_2PO_4, analytical grade), phosphoric acid (H_3PO_4, analytical grade), 9-fluorenylmethylchloroformate (FMOC-Cl) (analytical grade, Sigma-Aldrich) and deionized milliQ water were used. The HPLC system comprised a pump, UV detector, auto-injector, and degasser module. The chromatographic separations were carried out on Supelco RP 18 column (5 µm particle size; length × i.d: 250 mm × 10 mm).

Derivatisation was performed as follows: 0.25 mL of borate buffer (5 %) and 0.30 mL of FMOC-Cl 2 mM in $CHCl_3$ were added to 1 mL of sample. After 24 hours of reaction at 40º C in dark, the reaction was stopped by adding 0.30 mL of H_3PO_4 (2 %) and kept in a fridge at -4º C until analysis. The derivatised product (Gly-FMOC) was analysed by HPLC under the following chromatographic conditions: mobile phase 0.05 M phosphate buffer (pH 5.5): acetonitrile (65:35); flow: 0.8 mL/min; UV detection: 206 nm.

2.3.3 Measured glyphosate concentrations

The method used for the detection of glyphosate in the test samples (as described above) shows the following reference parameters: limit of detection (LOD) = 0.526 mg/L and limit of quantitation (LOQ) = 0.959 mg/L. Mean recoveries of 95 % were obtained for glyphosate in water by the laboratory spiked sample method. This means that the measured or actual concentration of glyphosate in the test solutions is about 95 % of the nominal concentration.

2.3.4 Sources of variability in the tested concentrations

The tested concentrations in each experimental exposure may be liable to two sources of variability: (a) the use of formulated Roundup® rather than technical grade glyphosate and, (b) the use of nominal rather than measured concentrations. Firstly, although the tested concentrations were based on the calculated amount of glyphosate in Roundup®, it is possible that the surfactant in the formulation may influence the toxicity of glyphosate, which is the active ingredient. In addition, the recorded concentration of glyphosate on the packaging may vary from what is present in the Roundup®. However, Roundup® was used and not technical grade glyphosate in order to simulate environmental "realism" since glyphosate is mostly used as a formulated herbicide (such as Roundup®) to control weeds. Secondly, it was not practically possible to test for actual glyphosate concentrations in every exposure test conducted in this study. For these reasons nominal concentrations were used as the measure, with levels of errors specified by a set of HPLC measured dilutions.

2.4 Experimental systems

2.4.1 Exposure method

Caridina nilotica swims in the water column and can rest on tank walls or on nets suspended in the water column and feeds on algae found on these surfaces. In the wild, the shrimps abound in marginal vegetation, and plant materials provide the resting and feeding surfaces. Shrimps ingest food from surroundings and often rest and feed on sediment that forms the substratum of their habitats. Thus, they can be used as sensitive indicator species to monitor the effects of pesticides through exposure to the water column, dietary uptake, or contact with sediment. Using the water column as the exposure medium involves adding the test chemical to water directly. (If exposure is through dietary uptake or sediment, the test chemical is added directly to the food or sediment respectively). Although each of these methods of exposure seems specific in theory, they are not totally exclusive of each other in practice. For example, in a short-term acute toxicity test, where feeding and sediment are not required, the medium of exposure is exclusively water. However, during longer test periods where feeding is required, the test chemical will probably attach to the food, whether the exposure medium is water or sediment. The route of exposure used in this study was the water column for both short-term and long-term exposure tests, although there is the possibility of the herbicide attaching to food and/or sediment during the 25-day long-term exposure tests as explained earlier.

The method of exposure used in the present study to deliver the test chemical to the test organisms was either static, whereby tests solutions were not changed throughout the experimental period, and was used for 24-96-hour range-finding and definitive acute toxicity tests; or static renewal whereby test solutions were replaced every other day with freshly prepared solutions during the 25-day long-term sub-lethal tests. This frequency of renewal of test solutions was based on the half-life of glyphosate in water, which is about two to ten weeks (Tu *et al*., 2001). This ensured that the glyphosate concentration in the test solution would be above 80 % of the actual measured concentration all the time.

2.4.2 Test chambers

Glass test chambers were used because glyphosate has a very low adsorption affinity to glass (Byer *et al*., 2008). In order to ensure that no glyphosate molecule or other component of Roundup® herbicide adsorbed to or remained on the test chambers before re-use, all test chambers were acid washed (Appendix A). Glass beakers (600-mL) (Figure 2.2) were used for the range-finding and definitive short-term toxicity tests, while glass tank aquaria (20-L) (Figure 2.3) were used as test chambers for the long-term toxicity tests. The tanks were filled with dechlorinated and carbon filtered tap water and supplied with aerators to ensure sufficient dissolution of oxygen. A thermostat heater, thermometer and the water circulation pump, which also served as a biological filter, were attached to each tank. Glass embryo incubation chambers were used for embryotoxicity tests (Figure 2.4) The bottoms of the chambers were cut off and replaced with nylon screen of a mesh size sufficient to retain embryos but not so small as to become easily clogged. In this way, a condition was created similar to that of the brood pouch of the shrimp, which allows for gentle agitation and sends fresh oxygen to the eggs until hatching (Ketse, 2006). The brooding chambers also made it easy to remove eggs for observations and measurements during experiments.

Figure 2.2: Test chambers (600-mL beakers) used for short-term toxicity tests

Figure 2.3: Glass tank aquaria (20-L) filled with dechlorinated tap water and supplied with aerators, thermostat heaters, thermometers and water circulation pumps used for long-term toxicity tests

Figure 2.4: Experimental brood chambers for the simulation of *C. nilotica* brood pouch used for embryo development toxicity tests

2.4.3 Water quality

Dissolved oxygen (DO), electrical conductivity (EC), and hydrogen ion concentration (pH) were monitored and recorded at the beginning and end of all short-term and long-terms toxicity tests, and weekly during long-term tests to ensure that they were not outside the set laboratory condition values of DO>5mg/L, 40mS/m<EC<60mS/m and 5<pH<8 respectively. However, temperature was measured daily to ensure that it was not outside its set laboratory condition value of 24-26° C.

2.5 General statistical methods

2.5.1 Hypothesis testing and point estimate

Statistical methods are standard tools used in ecotoxicology and environmental risk assessment (ERA) to analyse how significant an observation is, such as the effect of a toxicant (Wang and Riffel, 2011). Observed effect data may be analysed using parametric methods (normally distributed data with homogeneous variances) or non-parametric methods (data that are neither homogeneous nor normally distributed). To evaluate data distribution, parametric methods are preferred to non-parametric methods in practical ecotoxicology, such as in guidelines for pesticide regulation and in cases where sample sizes are very small (Wang and Riffel, 2011). In this study, to ensure an unbiased assignment of treatments within the experimental system and to ensure that no treatment was favoured and that observations were independent, a randomised experimental design was used. For example, test organisms were randomly selected from a cultured or stock population and randomly placed in test containers with random labels.

All statistical tests assume some assumptions and violation of these assumptions changes the research outcome. In this study, Assumption of normality of data was tested using Kolmogorov-Smirnov's test, while assumption of homogeneity of variance was tested using Levene's test. If normality of the data and equal variances were established, then analyses of variance (ANOVA) were performed to test the hypothesis that all the treatments, including the control, were equal. If assumptions of normality or homogeneity of variance were not met, the data were first transformed, using parametric techniques, before analysis.

For example, arcsine-square-root was used to transform endpoints such as the proportion surviving (percent mortality), while growth and reproduction endpoints were transformed by logarithmic methods. However, non-parametric techniques were used if data were not normally distributed or variances among treatments were not homogeneous, even after transformation. Furthermore, a Tukey or Dunnett's multiple comparison test was used in post hoc analysis to show specific pairwise comparisons of treatment groups after ANOVA had shown significant differences. Point estimates data such as mortality (LC_X) and effect concentration (EC_X) were estimated using the probit or linear regression analysis. Statistics were performed using Statistica Version 9 (StatSoft Inc., 2009) and WINKS SDA software (Texasoft, 2000), and all statistical decisions were made at alpha = 0.05. Figure 2.5 shows a decision tree of the process used to choose specific statistical methods.

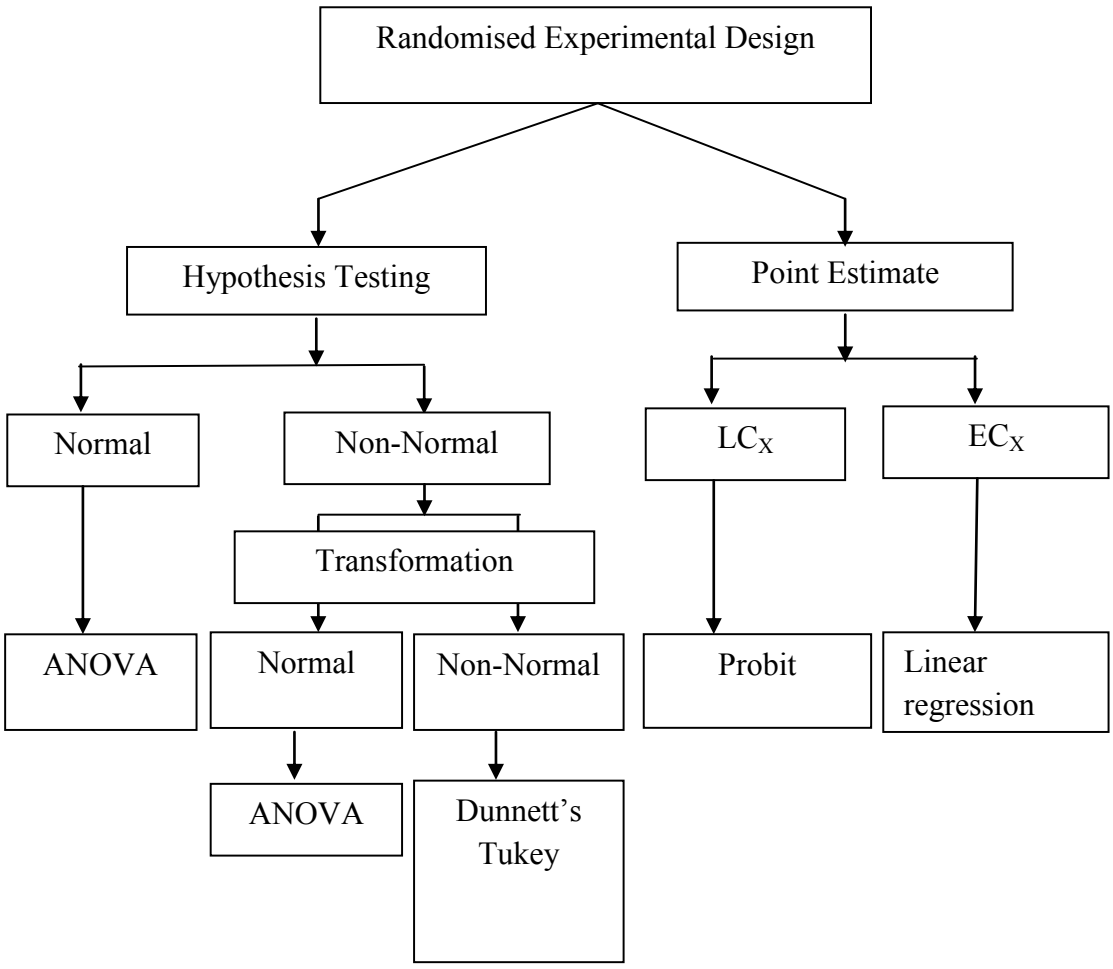


Figure 2.5: A decision tree for choosing specific statistical methods (LC_X = lethal concentration; EC_X = effective concentration)

2.5.2 Multivariate statistical techniques

Multivariate statistical techniques (MST) are applied to characterise and evaluate surface and freshwater quality, identify possible factors/sources that influence water systems, as well as to verify temporal and spatial variations caused by natural and anthropogenic factors linked to seasonality (Shrestha and Kazama, 2007). Therefore, these techniques are valuable tools for reliable management of water resources as well as for providing rapid solutions to pollution problems. In this study, different multivariate statistical techniques such as canonical correspondence analysis (CCA) and principal component analysis (PCA) were applied in the interpretation of complex data matrices to better understand the water quality and ecological status of the Swartkops River (the field studied system). The technique used studied water quality variables responsible for spatial and temporal variations in the river water quality in selected sampling sites.

The relationships among sampling sites and time (seasons and weeks) based on physical and chemical water quality variables were explained using PCA. Principal component analysis was used for the ordination of sites and seasons or weeks based on water quality variables because it is a conceptually simple method well suited for ordinations of environmental variables (Clarke and Warwick, 1994). In addition, PCA was chosen because it assigns all environmental variables equal weight irrespective of their scale of measurement so that different scales of measurement do not impact on the final results of the ordinations (Clarke and Warwick, 1994). Correspondence Canonical Analysis (CCA) is used to explain the relationships between biological community and their environment, especially determining which environmental variable(s) is/are important in structuring the biological community (i.e. (ter Braak and Verdonschot, 1995). In this study, CCA was used to explain the relationship between the macroinvertebrate community assemblage and the measured physicochemical water quality variables in order to determine which variables are responsible for the observed spatio-temporal distribution of the macroinvertebrate community. Principal component analysis were performed using Statistica Version 9 (StatSoft Inc., 2009), while Principal component analysis were performed using the computer programme Environment Community Analysis 1.33 package (ECOM) (Pisces conservation Ltd, 2000).

CHAPTER THREE

USING BIOLOGICAL STRESS RESPONSES TO MEASURE THE TOXICITY OF THE HERBICIDE ROUNDUP® TO *CARIDINA NILOTICA* (ROUX 1833)

3.1 Introduction

The conceptual framework underlying this study is environmental water quality, which uses a combination of data and insights from investigations of the chemistry of toxicants and receiving waters, biomonitoring and ecotoxicology (Palmer *et al.,* 2004b; Chapter One). This chapter presents the ecotoxicological aspects of this study, describing the biological stress responses at varying biological system scales in the shrimp, *C. nilotica,* exposed to varying concentrations of the stressor, Roundup® (Figure 3.1). The stress (Figure 3.1) is classified and explored according to different exposure lengths (short-term or long-term) and exposure type (lethal or sub-lethal) (Table 3.1). In this study, short-term exposure length was defined as not more than 96 hours (≤ 4 days), while long-term exposure length was more than 96 hours (≥ 4 days) (Table 3.1). Lethal exposure to stress can possibly cause a biological system to respond in short-term and/or long-term. Similarly, biological systems can experience sub-lethal responses to a stressor in short-term and/or long-term (Table 3.1). Lethal exposure will often result in mortality (i.e. immobility, decolouration and degeneration) of *C. nilotica*, but sub-lethal exposure normally results in a cellular level response including growth (length, weight and moulting), reproduction (embryo and gonads) and biochemical (acetylcholinesterase and lipid peroxidation) (Table 3.1).

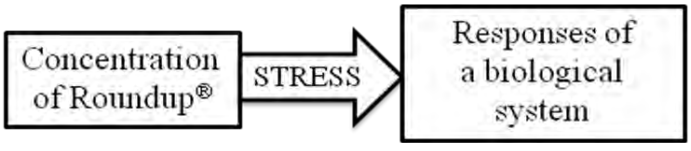


Figure 3.1: Diagrammatic representation of the herbicide Roundup® as a stressor that can cause a biological system to respond to a stress. Small concentrations of Roundup® can cause biological stress responses at different biological system scales

Table 3.1: Exposure types, exposure lengths and biological systems of *C. nilotica* explored in this study

Exposure type	**Exposure length (days)**	***C. nilotica* biological system**
Lethal exposure	2	Neonate
		Juvenile
		Adult
	4	Neonate
		Juvenile
		Adult
	21	Adult
Sub-lethal exposure	4	Adult acetylcholinesterase
		Adult lipid peroxidation
	21	Adult acetylcholinesterase
		Adult lipid peroxidation
	25	Adult length
		Adult weight
		Adult moulting
		Embryo
		Adult gonad

The aim of this chapter is therefore to provide as comprehensive a view as possible of the ecotoxicity of Roundup® to a single organism (*C. nilotica*). Once the concentrations at which *C. nilotica* responds under different exposure times and at different biological scales have been established, the study is well placed to compare this extensive single organism knowledge with another commonly used stressor measure, the species sensitivity distribution (SSD). An SSD uses data from multiple species, but fewer response categories, usually lethal (LC50) and sub-lethal (EC_X and NOEC) (USEPA, 2005). This will be explored in Chapter Four of this thesis.

3.2 Materials and methods

3.2.1 Lethal exposure of neonate, juvenile and adult *Caridina nilotica* to Roundup®

Short-term and long-term lethal tests were conducted separately in order to determine the effect of Roundup® herbicide on *C. nilotica*. The experimental method employed for the short-term lethal test was static non-renewal for 48 and 96 hours (Scherman and Palmer, 2000). Based on a range-finding test (Appendix B), the following concentrations and life stages were used in a first definitive test (Experiment 1): 0.0, 1.7, 2.6, 4.1, 6.4 and 8.0 mg/L for neonates (> 7 days post hatching (dph); 10 mg/L was added to this sequence for juveniles (> 7 dph and < 20 dph); and the range 0.0, 5.4, 8.4, 13.1, 20.5, 32 and 50 mg/L was used for adults (> 40 dph). Based on the results of this test, a second definitive test (Experiment 2) was conducted using lower and more refined concentrations: 0.0, 1.3, 2.1, 3.3, 5.1 and 8 mg/L for neonates; this sequence as well as 10 mg/L for juveniles; and 0.0, 4.3, 6.7, 10.5, 16.4, 25.6 and 40 mg/L for adults. Animals were not fed during the experimental period.

A static renewal experimental method was separately conducted to determine the effect of Roundup® herbicide on adult shrimps in a 21-day long-term lethal test. Concentrations used were 0.0, 2.2, 2.8, 3.4, 4.3 and 5.4 mg/L. Test solutions were changed every other day and shrimps were fed TetraMin Tropical Flake to satiation daily and prior to changing the solution. All uneaten food was removed before the next feeding.

In all the experiments (both short-term and long-term tests), each concentration contained 10 shrimps and was replicated three times. Dead shrimps (i.e. immobile, decolourised and degenerated) were recorded twice daily and removed from experimental vessels. The recorded cumulative mortality at the end of 48-hour, 96-hour and 21-day lethal tests was used to estimate LC50, ECx, LOEC and NOEC values of Roundup® for *C. nilotica*. Water physicochemical variables, including pH, dissolved oxygen (DO), electrical conductivity (EC), and temperature were recorded at the beginning and end of the experiment, in all concentrations and control for short-term tests. For long-term tests, these parameters were measured at the beginning of the test and just before changing the solutions.

3.2.2 Sub-lethal growth responses of *Caridina nilotica* to Roundup® exposure

Length and weight

In order to determine *C. nilotica*'s growth responses to Roundup®, 40 dph shrimps were exposed to the herbicide for 25 days (i.e. from 40 to 65 dph). Concentrations used were 0.0, 2.2, 2.8, 3.4, 4.3 and 5.4 mg/L. Each concentration was replicated three times and each replicate contained 10 shrimps, giving a total of 30 shrimps per concentration. A static renewal experimental method was used whereby test solutions were changed every other day. Shrimps were fed TetraMin Tropical Flake to satiation daily and prior to changing of test solutions. All uneaten food was removed before the next feeding. Water physicochemical variables, including pH, DO, EC, and temperature were measured at the beginning of the test and just before solutions were changed.

Shrimp total lengths (TLs) (i.e. the lateral distance from the tip of the rostrum to the posterior-most end of the uropod (Okuthe, 2004)) and wet weights were measured at 40, 45, 50, 55, 60 and 65 dph in order to track growth. These intervals were chosen based on the relationship between age, length and gonad development of *C. nilotica* as described by Okuthe (2004) (Table 3.2).

Table 3.2: Phases of gonad differentiation in *C. nilotica* in relation to age (days post hatch (dph)) and length (based on Okuthe, 2004)

Age (dph)	Mean total length (mm)	Phase of gonadogenesis
0-15	2.90-5.15	Undifferentiated phase
20-40	7.60-10.31	Differentiated male phase
45-65	14.14-26.75	Differentiated female phase

Five individual shrimps (or as many as were available if less than five remained) were randomly removed from the experimental tanks and their TLs measured. All individual shrimps from a particular concentration were placed in a Petri-dish with some of the exposure medium. The Petri-dish was placed in a shallow container containing finely crushed ice, and the container placed under a microscope fitted with a micrometer. As the cold temperature reduced movement, TLs were quickly measured and the shrimps returned to their tanks.

Shrimp weights were measured using an electronic weight balance (ADAM®, PW 124, maximum capacity: 120 g) to 0.000 precision (i.e. three decimal places). Individual shrimps were randomly removed from test solutions and gently placed on the weighing pan in a thin film of water. The water was carefully removed with absorbent paper. Weight was quickly recorded and the shrimp transferred back to a container of water to recover. An average of 10 seconds elapsed from the time the film of water surrounding the shrimp was absorbed to the time it was transferred back to the recovery container. Five individual shrimps (or as many as were available if less than five remained) were weighed and the average weight was taken as the weight of an individual in an experimental tank. These methods of measurement are non-destructive and non-intrusive, as indicated by low mortalities within the control group.

The data obtained from length and weight measurements were used to calculate growth indices including percent weight gain (PWG), percent length gain (PLG), specific growth rate (SGR), condition factor (CF), feed intake (FI), feed conversion ratio (FCR) and feed conversion efficiency (FCE). The formulae used to calculate these growth indices, which were based on Enin, 1994; Araneda *et al*. 2008; and Deekae and Abowei, 2010, are presented in Table 3.3.

Table 3.3: Formulae used to calculate the difference growth indices

Growth index	Formula
Percent weight gain (PWG)	100 (weight gain / initial weight)
Percent length gain (PLG)	100 (length gain / initial length)
Specific growth rate (SGR)	100 ($InW_2 - InW_1 / T_2 - T_1$)
Condition factor (CF)	100 (W / L^3)
Total feed consumed per day (daily feed ration)	Average shrimp size (weight) x feed rate (%) x total number of shrimp in the tank (feed rate is the amount recommended as a percentage of the shrimp's average weight at that time)
Food intake (FI)	Total feed consumed per day / total weight
Food conversion ratio (FCR)	Feed intake / weight gain
Food conversion efficiency (FCE)	Weight gain / feed intake

Note: InW_1 and InW_2 are shrimp wet weights at times T_1 (beginning of test) and T_2 (end of test), respectively; L = shrimp length.

Moulting

Moulting was observed for 14 days; from day-8 to day-21. This sampling period was chosen to ensure that moulting is really due to long-term effect and not just a sudden effect of the herbicide. The daily moult rate for each concentration was calculated as the pooled number of exuviae (shed exoskeleton) recorded for the three replicates of each concentration divided by the pooled number of live shrimps. The cumulative number of moults for each concentration was also calculated as the total of exuviae recorded over the 14 days.

3.2.3 Sub-lethal reproductive responses of *Caridina nilotica* to Roundup® exposure

Gonad histopathology

The same static renewal experimental method as described in section 3.2.3 of this thesis was used to investigate gonadal development of *C. nilotica* in response to Roundup® exposure. Three shrimps were sampled from each concentration during the 25 days long-term sub-lethal exposure (section 3.2.3) at 40, 45, 50, 55, 60 and 65 dph based on Table 3.2. The samples were treated for histopathological analysis following the procedure in Appendix C. A summary of the histology procedure is given below.

Sample fixation and tissue sectioning: The samples were fixed overnight in Davidson's fixative. After fixation, the specimens were dehydrated in graded series of ethanol, cleared in xylene and then embedded in Paraplast. Paraplast blocks were trimmed to the tissue surface and placed in warm water for 20 minutes, after which 5-µm sections were cut, using a microtome. In order to prevent folds of the sections, a flexible paintbrush was used to pick them up from the microtome knife and the sections were floated in a water bath at 48º C. The flattened sections were then placed on glass slides pre-treated with 2 % aminopropyl triethoxy-saline (TESPA) in acetone to avoid loss of sections during the subsequent washing procedure, after which they were air-dried.

Staining, microscopy and photography: The specimens were deparaffinised by placing the sections in xylene (five minutes each) three times, twice in absolute ethanol (two minutes each), twice in 95 % ethanol (two minutes each), and rehydrated in distilled water (one minute). Sections were then stained in Mayer's haematoxylin solution for five minutes and placed in 70 % ethanol. Specimens were then stained and counter-stained in Eosin solution for 30 seconds, placed twice in 95 % ethanol (two minutes each), and twice in absolute ethanol (two minutes each). The sections were cleared three times in xylene and mounted in TESPA-coated slides. Five slides (mounted with sections) were randomly selected from each concentration, examined under the microscope, and photographed using an Olympus 4040 zoom digital camera (Olympus American Inc., USA) with a focus on gonad morphological histopathology.

Embryotoxicity

Embryos were removed from females that had been gravid for less than 24 hours, and exposed to five different glyphosate concentrations and a control, with three replicates per treatment, in incubation chambers. The concentrations of Roundup® used were 0.0, 0.625, 1.25, 2.5, 5 and 10 mg/L. Each chamber contained a minimum of 16 embryos, giving a total of 48 embryos per treatment. Specific developmental ages as identified and described by Ketse (2006) (Table 3.4) were measured in each concentration until hatching. Three developing embryos were randomly selected at each developmental age, placed under a dissecting microscope fitted with a digital camera and photographed. Endpoints for the embryotoxicity test were developmental stage deformity and percentage hatch.

Table 3.4: Ages with corresponding developmental features of *C. nilotica* embryo used as endpoint measures in embryotoxicity tests (after Ketse, 2006)

Age (days)	Developmental feature
1	Egg and yolk
3	Antennae, antennules and optic rudiment
6	Caudal papilla
9	Eye and carapace
12	Heartbeat and pleopods
14	Development of all features
17	Hatching

3.2.4 Sub-lethal biochemical responses of *Caridina nilotica* to Roundup® exposure

Measurement of acetylcholinesterase (AChE) activity

Adult shrimps were randomly sampled from each test vessel at the end of 96-hour short-term and 21-day long-term sub-lethal tests (experiments described earlier in sections 3.2.1 and 3.2.2 respectively). Samples were immediately frozen in liquid nitrogen and stored at -80º C until the AChE activity was measured. Measurement of whole shrimp body AChE activity was based on the method described by McLoughlin *et al.* (2000) (Appendix D). Whole shrimp body lengths were measured, homogenised by maceration in Eppendorf tubes, using Teflon micropestles with 30 µL 1 % Triton-X-100 in 0.02M PBS (pH 8), and diluted with 270 µL 0.02M PBS (pH 8). The homogenate was centrifuged at 14000 g at 4º C for 15 minutes. Clear supernatant was collected, kept at 4º C, and used as an enzyme source. The enzyme activity was determined in quadruplicate for each sample, according to the colorimetric method initially developed by Ellman *et al.* (1961) and adapted to microplates. Briefly, 50 µL of supernatant and 100 µL of the chromogenic agent DTNB (0.008 M, pH 7) were added to a 96-well microtiter plate. Measurement of enzyme activity was initiated by adding 50 µL of acetylthiocholine iodide solution (0.016 M). The final volume was 200 µL. Spontaneous substrate hydrolysis was assessed using two controls: a blank without acetylthiocholine, and a blank without the sample. Absorption of the 2-nitro-5-thiobenzoate anion, formed from the reaction, was recorded at 405 nm every 30 seconds for 10 minutes at 30º C, using a microtiter plate reader (PowerWavex, Bio-Tek Instruments Inc, USA).

Enzyme activity was expressed as micromoles of acetylthiocholine hydrolyzed per minute per milligram of protein. Protein levels for AChE were estimated by the method described by Bradford (1976), using bovine serum albumin (BSA) as standard. Absorption kinetics were calculated in a linear range, and then converted to nanomoles per minute using the equation: *AChE activity rate (mol/min) = Δ abs/min/ε x volume (L),* where $\varepsilon = 1.36 \times 10^4$ L/mol/cm is the molar extinction coefficient of DTNB, *L* is the path length in cm, and *Δ abs* is the change in absorbance per minute (Ellman *et al*., 1961). Standards were prepared using commercial AChE (1 mg/mL) at a concentration range between 0.0 and 0.125 mg/mL to produce a standard curve. Specific standard concentrations were included in subsequent analysis to ensure accurate and reliable results. Each sample and standard was analysed in quadruplicate.

Measurement of lipid peroxidation (LPx)

Whole body samples were assayed following the methods described by Ringwood *et al*. (2003) (Appendix E) and optimised for *C. nilotica*. Adult shrimps were randomly sampled from each test vessel at the end of 96-hour acute and 21-day chronic toxicity tests (experiments described earlier in sections 3.2 and 3.3 respectively), immediately frozen in liquid nitrogen and stored at -80º C until measurement of LPx. The length of two shrimps was measured (previous practice using one shrimp did not yield positive results), added to 100 µL 0.05 M potassium phosphate buffer solution (pH 7, ice cold), homogenised using Teflon Eppendorf micropestles in Eppendorf tubes, and then centrifuged for five minutes at 13000 g at 4º C. Sample supernatant (50 µL) was then added to 700 µL of 10 mM 0.375 % thiobarbituric acid and 7 µL of 2 % butylated hydroxytoluene, and incubated in a heating block at 100º C for 15 minutes. The samples were then centrifuged at 13000 g for five minutes at 22 ± 2º C. A 100 µL sample of supernatant was placed in the well of a 96-well microtiter plate and read at 532 nm using a microtiter plate reader (PowerWavex, Bio-Tek Instruments Inc, USA). A standard curve was prepared using serial dilutions of malondialdehyde (MDA) tetraethylacetal in a concentration range of 6.25 to 3 200 µM MDA. Each sample and standard was analysed in quadruplicate.

Measurement of protein content

The protein content for shrimp whole-body AChE activity and LPx measurements was determined according to the method described by Bradford (1976) (Appendix E). For both AChE activity and LPx, 5 µL of the respective shrimp whole-body sample homogenate was placed in a 96-well microtiter plate. A volume of 250 µL Bradford reagent was added and the plate was allowed to stand for 10 minutes at 22 ± 2º C to incubate. The plate reading was taken at 595 nm using a microtiter plate reader (PowerWavex, Bio-Tek Instruments Inc, USA). Standards were prepared using commercial Bovine Serum Albumin (BSA) ranging between 0.1 and 1.4 mg/mL. The resultant standard curve was used to calculate shrimp whole-body protein content. Final AChE activities (µmol/mL/min) were divided by the sample protein content (mg/mL) and multiplied by 1000 to give AChE activity in nmol/min/mg protein, while final malondialdehyde (MDA) concentrations were expressed as nmol MDA produced/mg protein for LPx. Each sample and standard was analysed in quadruplicate.

3.2.5 Statistical analysis

All data (mortality, growth, reproduction and biochemical) were tested for normality using the Kolmogorov-Smirnov test (Richardson *et al*., 2010). Roundup® 48-hour, 96-hour and 21-day median lethal concentration (LC50) values and the associated 95 % confidence limits were calculated for *C. nilotica*, using USEPA Probit analysis program version 1.5 (USEPA, 1990). The ECx values were calculated using linear regression analysis of transformed herbicide concentration as natural logarithm data against transformed response as percentage inhibition or reduction response measure (Ma *et al*., 2001; Schroer *et al*., 2004). Estimation of ECx values followed the regression model $ECx = a + bc$, where ECx is the percent inhibition or reduction response measure. Step-by-step calculation of ECx values using Microsoft Excel 2010 is presented in Appendix F. Where applicable, effect scores from replicates per treatment were combined in one regression analysis. Dunnett's test was used to estimate the LOEC and NOEC values.

One-way analysis of variance (ANOVA) was performed to test the hypothesis that all mean mortality, growth, reproduction and biochemical values across different concentrations were equal. In the presence of significant observations, Tukey multiple comparison post hoc tests were used to compare the specific difference between any two means. Non-parametric statistics were performed with Kruskal-Wallis ANOVA by ranks to determine *C. nilotica's* differences in growth performances and feed utilisation in the different Roundup® concentrations compared with control shrimps.

A Student's t-test was used to compare mean 48 and 96-hour LC50 values of separate age groups in the definitive short-term lethal tests. The same statistical method was used to compare mean AChE activity or LPx values between 96-hour and 21-day toxicity tests. Pearson's correlation was used to compare the relationship between protein content and AChE activity. A Pearson's correlation value (R^2) of greater than 0.5 implies a strong and positive relationship. Statistics were performed using Statistica Version 9 (StatSoft Inc., 2009) and all statistical decisions were made at alpha = 0.05 *a priori*.

3.3 Results

3.3.1 Lethal exposure of neonate, juvenile and adult *Caridina nilotica* to Roundup®

The mean water quality parameters with standard deviations (± SD) for all three *C. nilotica* life stages exposed to Roundup® in the 48 and 96-hour short-term (Experiments 1 and 2) and 21-day long-term lethal tests are presented in Table 3.5. These ranges of values were all within the acclimated conditions of the culture maintained in the laboratory.

Table 3.5: Mean water quality parameters with standard deviations (± SD) for neonate, juvenile and adult *C. nilotica* exposed to Roundup® in short-term and long-term tests

Shrimp life stage	pH	DO (mg/L)	EC (mS/cm)	Temperature (ºC)
Neonate	8.55 ± 0.03	5.98 ± 0.32	0.92 ± 0.01	24.45 ± 0.34
Juvenile	8.34 ± 0.21	5.99 ± 0.32	0.97 ± 0.05	24.00 ± 0.20
Adult	8.41 ± 0.16	5.89 ± 0.10	0.90 ± 0.01	24.50 ± 0.41

There were no mortalities in the control group for all three shrimp life stages, but there were significant differences between control and treatment groups ($p < 0.05$). Mortalities at 48 and 96 hours were used to estimate the median lethal concentration (LC50) and their 95 % confidence limits of Roundup® for *C. nilotica* different age groups, which are presented in Table 3.6. The lowest observable effect concentration (LOEC) and no observable effect concentration (NOEC) (where the effect is mortality) values were also estimated and are presented in Table 3.6.

Table 3.6: 48-hour, 96-hour and 21-day LC50 values and 95 % confidence limits (CL) with corresponding LOEC and NOEC (all in mg/L) for different ages of *C. nilotica* exposed to Roundup®

Test duration	Life stage	Experiment number	LC50 (95 % CL) (mg/L)	LOEC (mg/L)	NOEC (mg/L)
48-hour	Neonate	1	3.91 (3.30-4.62)	2.6	1.7
		2	5.23 (4.41-6.55)	3.3	2.1
	Juvenile	1	8.90 (8.14-10.16)	8.0	6.4
		2	9.90 (8.89-12.16)	8.0	5.1
	Adult	1	36.81 (27.11-62.60)	13.1	8.4
		2	38.18 (28.58-64.02)	25.6	16.4
96-hour	Neonate	1	2.37 (2.04-2.69)	–	–
		2	2.84 (2.52-3.19)	3.3	2.1
	Juvenile	1	6.77 (6.20-7.32)	6.4	4.1
		2	7.17 (6.45-7.99)	5.1	3.3
	Adult	1	27.79 (19.99-48.02)	13.1	8.4
		2	22.83 (18.69-29.63)	10.5	6.7
21-day	Adult	1	3.12 (2.87-3.43)	2.8	2.2

The ANOVA of mean 48-hour LC50 values across all age groups were found to be significantly different ($p < 0.05$), while the Tukey post hoc test performed to determine specific pairwise differences showed significant difference between all age groups (Figure 3.2). The ANOVA of mean 96-hour LC50 values across all age groups were significantly different ($p < 0.05$), while the Tukey post hoc test revealed that adult LC50 values were significantly different from neonates and juveniles, just as values between neonates and juveniles were significantly different from each other (Figure 3.3).

There was no statistical difference ($p > 0.05$) between mean 48-hour LC50 (4.502 ± 1.172) and mean 96-hour LC50 (2.545 ± 0.139) values for neonates (Figure 3.4). However, there were statistical differences ($p< 0.05$) between mean 48- and 96-hour LC50 values for juvenile and adults, although the mean 48-hour LC50 values for juvenile (9.397 ± 0.706) and adults (37.124 ± 0.44) were significantly higher than the mean 96-hour LC50 values for juveniles (6.967 ± 0.281) and adults (25.305 ± 3.507) respectively (Figures 3.5 and 3.6).

For the 21-day long-term lethal test, percent mortality was compared among different concentrations using one-way ANOVA followed by the Tukey post hoc test. There was no significant difference between control mortality and 2.2 mg/L concentrations, but mortalities in the remaining concentrations were significantly higher. Specific pair-wise differences are shown in Figure 3.7.

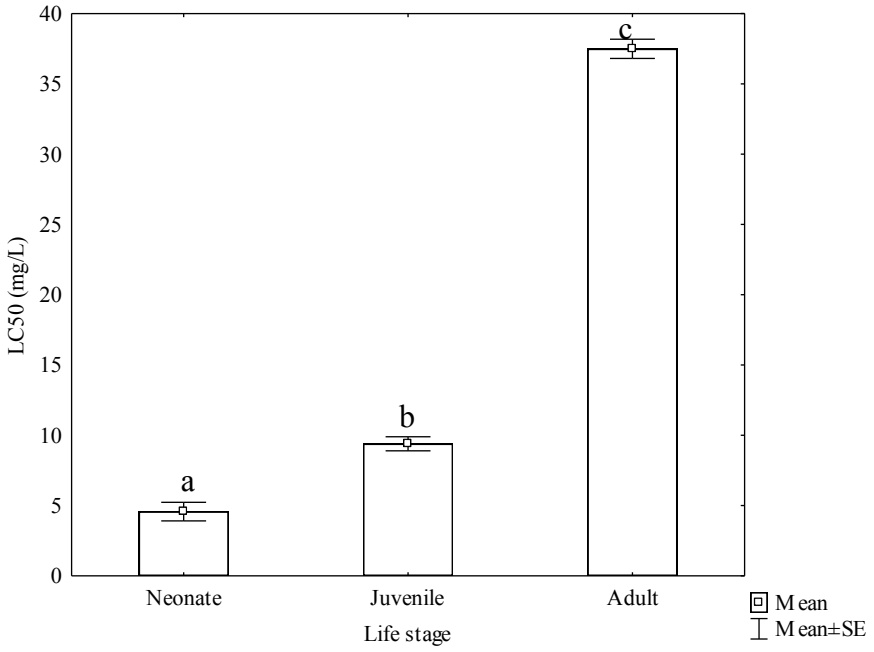


Figure 3.2: Mean 48-hour LC50s (mg/L) of Roundup® for neonate, juvenile and adult *Caridina nilotica*. The means of any two concentrations having the same superscript letter are not significantly different ($p > 0.05$)

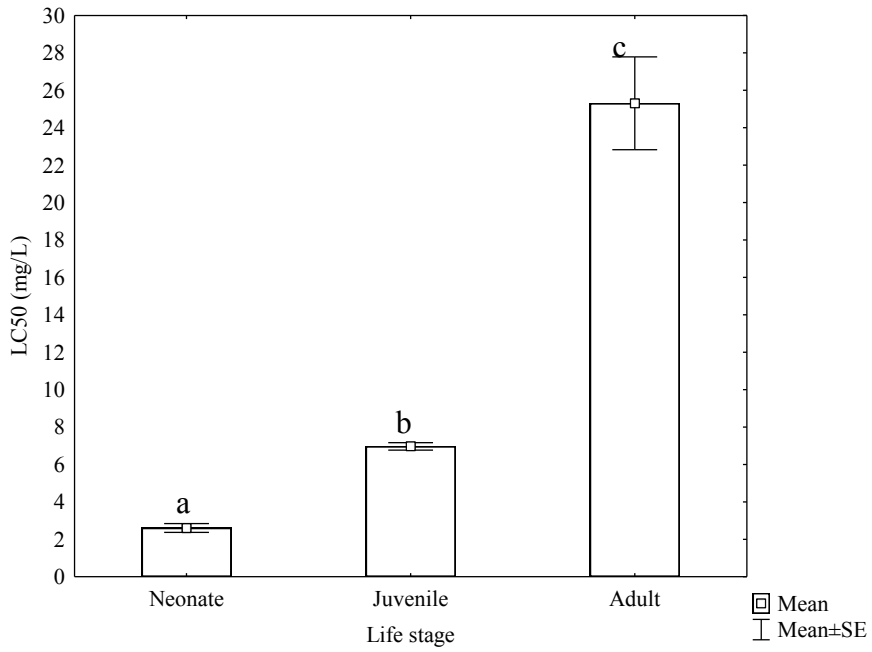


Figure 3.3: Mean 96-hour LC50s (mg/L) of Roundup® for neonate, juvenile and adult *C. nilotica*. The means of any two concentrations having the same superscript letter are not significantly different ($p > 0.05$)

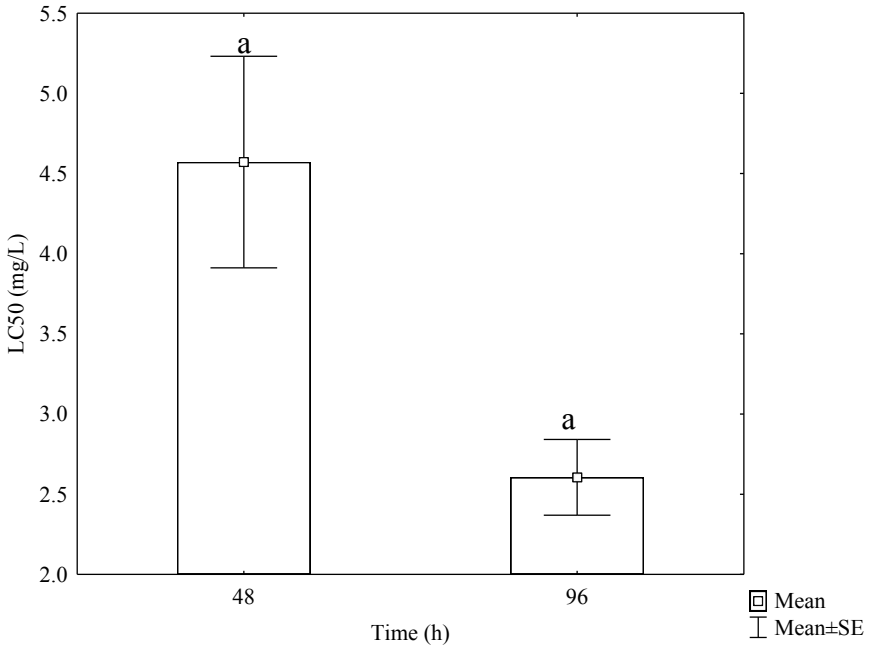


Figure 3.4: Mean 48-hour and 96-hour LC50 (mg/L) of Roundup® for neonate *C. nilotica*. The means of any two concentrations having the same superscript letter are not significantly different ($p > 0.05$)

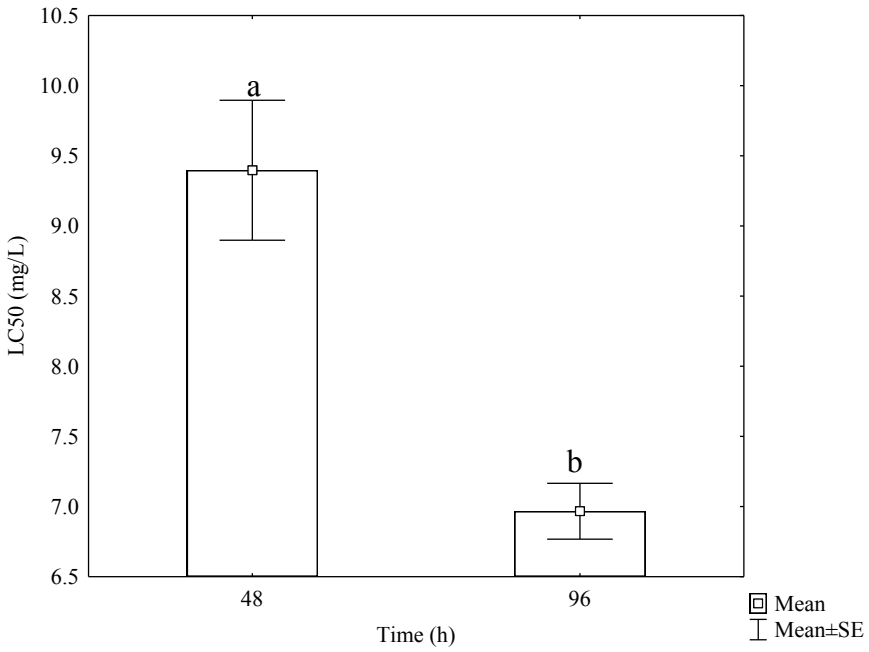


Figure 3.5: Mean 48- and 96-hour LC50 (mg/L) of Roundup® for juvenile *C. nilotica* The means of any two concentrations having the same superscript letter are not significantly different ($p > 0.05$)

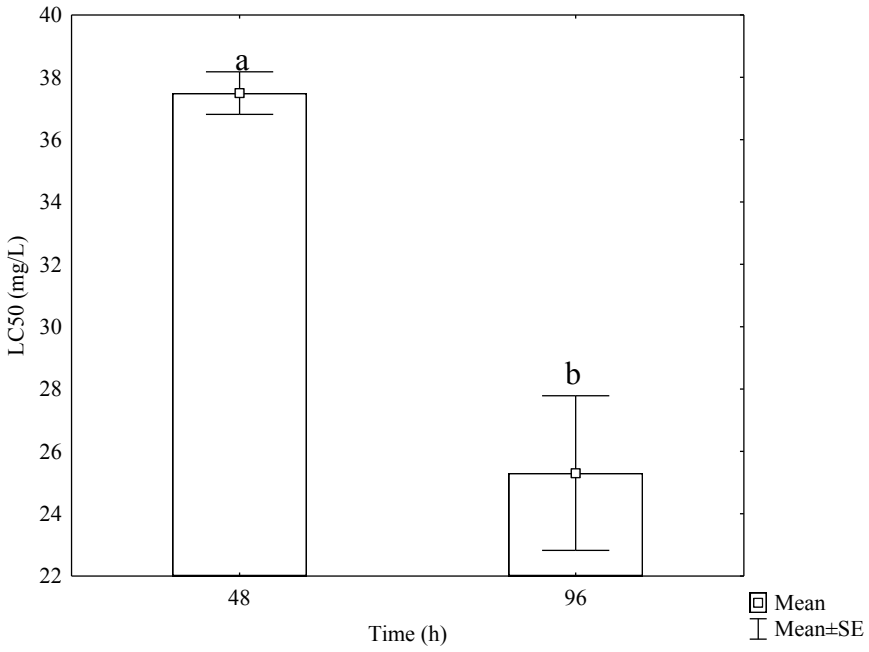


Figure 3.6: Mean 48-hour and 96-hour LC50 (mg/L) of Roundup® for adult *C. nilotica*. The means of any two concentrations having the same superscript letter are not significantly different ($p > 0.05$)

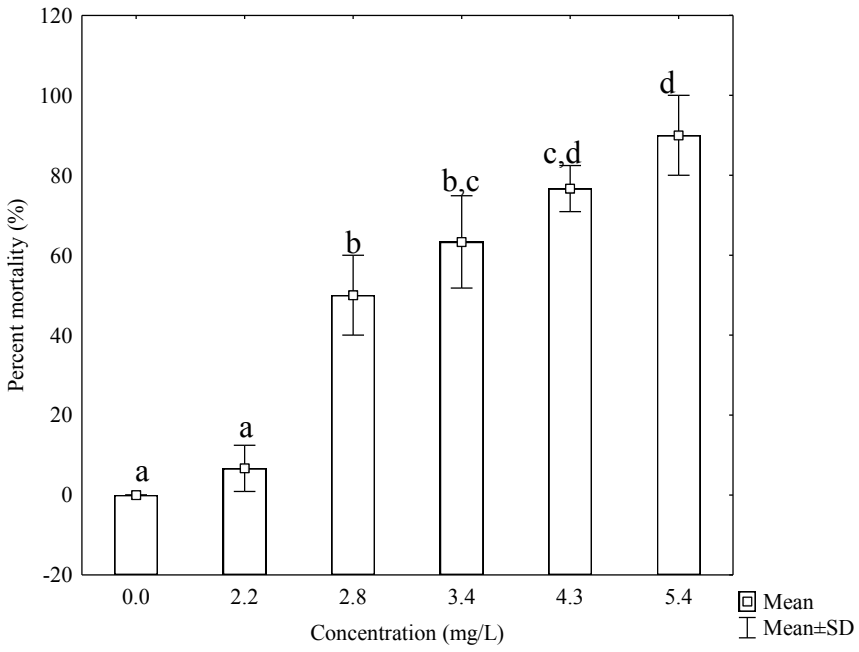


Figure 3.7: *Caridina nilotica* 21-day percent mortality after exposure to different Roundup® concentrations. The means of any two concentrations having the same superscript letter are not significantly different ($p > 0.05$).

3.3.2 Sub-lethal growth responses of *Caridina nilotica* to Roundup® exposure

Length and Weight

Five-day interval changes in shrimp total lengths (TLs) attained in control and the different Roundup® concentrations are presented in Table 3.7. There were general increases in length from 40 dph to 65 dph, but the increase was higher in control shrimp than in the treatment group. One-way ANOVA followed by Tukey post hoc tests were used to identify significant specific pair-wise differences between any two concentrations (Table 3.7). The control group's mean TL was significantly greater than the treatment groups, just as the mean of shrimps exposed to 5.4 was significantly lower than that of all other exposed shrimps and the control (Table 3.7). Bar graphs showing mean lengths of *C. nilotica* exposed to different Roundup® concentrations from 40 to 65 dph are presented in Appendix G. At 65 dph, no mean TL of any two groups was found to be the same.

Table 3.7: Five-day interval changes in total length (in mm) with standard deviations (± SD) of *C. nilotica* exposed to different Roundup® concentrations from 40 to 65 dph. The means of any two concentrations having the same superscript letter are not significantly different (p> 0.05) (n = 30)

Age (dph)	Five-day internal changes in total length of *C. nilotica* (mm) (± SD) in different glyphosate concentrations					
	Control	**2.2 mg/L**	**2.8 mg/L**	**3.4 mg/L**	**4.3 mg/L**	**5.4 mg/L**
40	10.000^{a} ±0.000	10.000^{a} ±0.000	10.000^{a} ±0.000	10.000^{a} ±0.000	10.000^{a} ±0.000	10.000^{a} ±0.000
45	12.500^{a} ±0.300	12.067^{a} ±0.116	12.033^{a} ±0.058	11.933^{b} ±0.116	11.767^{b} ±0.252	11.600^{b} ±0.173
50	15.333^{a} ±0.306	15.200^{a} ±0.200	14.500^{b} ±0.100	14.367^{b} ±0.322	14.167^{b} ±0.289	13.667^{c} ±0.289
55	17.200^{a} ±0.200	16.500^{b} ±0.300	16.033^{c} ±0.252	15.567^{d} ±0.404	15.067^{e} ±0.116	14.800^{e} ±0.200
60	20.667^{a} ±0.577	$20.333^{a,b}$ ±0.289	$19.667^{b,c}$ ±0.577	19.233^{c} ±0.252	19.067^{c} ±0.116	18.167^{d} ±0.289
65	26.833^{a} ±0.289	25.600^{b} ±0.529	24.667^{c} ±0.577	23.667^{d} ±0.577	21.667^{e} ±0.577	20.000^{f} ±0.500

Five-day interval changes in shrimp wet weight attained in control and the different Roundup® concentrations are presented in Table 3.8. There were general increases in weights from 40 dph to 65 dph, but the increase was higher in control shrimp than in the treatment group. One-way ANOVA followed by Tukey post hoc tests were used to identify significant specific pair-wise differences between any two concentrations (Table 3.8). At 65 dph, the mean weight of control group shrimps was significantly greater than that of Roundup® exposed groups. There were significant differences in weight found between control shrimps and Roundup® exposed groups (Table 3.8). Bar graphs showing mean weights of *C. nilotica* exposed to different Roundup® concentrations from 40 to 65 dph are presented in Appendix H.

Table 3.8: Five-day interval changes in wet weight (in mm) with standard deviations (± SD) of *C. nilotica* exposed to different Roundup® concentrations from 40 to 65 dph. The means of any two concentrations having the same superscript letter are not significantly different ($p > 0.05$) ($n = 30$)

Age (dph)	Five-day internal changes in wet weight of *C. nilotica* (g) (± SD) in different glyphosate concentrations					
	Control	**2.2 mg/L**	**2.8 mg/L**	**3.4 mg/L**	**4.3 mg/L**	**5.4 mg/L**
40	0.010^{a} ±0.001	0.010^{a} ±0.001	0.010^{a} ±0.001	0.010^{a} ±0.001	0.010^{a} ±0.001	0.010^{a} ±0.001
45	0.016^{a} ±0.001	0.014^{b} ±0.001	0.013^{b} ±0.001	$0.012^{b,c}$ ±0.002	0.011^{c} ±0.001	0.010^{c} ±0.001
50	0.022^{a} ±0.001	0.019^{b} ±0.001	0.018^{c} ±0.001	0.016^{d} ±0.001	0.014^{e} ±0.001	0.013^{e} ±0.001
55	0.031^{a} ±0.002	0.025^{b} ±0.001	0.024^{b} ±0.001	0.022^{c} ±0.001	0.019^{d} ±0.001	0.018^{e} ±0.001
60	0.050^{a} ±0.002	0.036^{b} ±0.001	0.034^{b} ±0.001	0.032^{c} ±0.001	0.029^{d} ±0.001	0.027^{e} ±0.001
65	0.142^{a} ±0.052	0.072^{b} ±0.004	0.060^{b} ±0.002	0.051^{b} ±0.003	0.047^{b} ±0.002	0.040^{b} ±0.002

Growth performance and food utilisation

Growth performance and feed utilisation of *C. nilotica* in different Roundup® concentrations and control after exposure for 25 days were determined and the results summarised in Table 3.9. Non-parametric statistics were performed with Kruskal-Wallis ANOVA by ranks to determine *C. nilotica's* differences in growth performances and feed utilisation in the different Roundup® concentrations compared with control shrimps. For growth performance, no statistical differences were found between the control group and glyphosate exposed groups in terms of final body weight, percent weight gain (PWG), final body length and percent length gain (PLG)($p > 0.05$). Similarly for food utilisation, no statistical differences were found between the control group and Roundup® exposed groups for food intake, specific growth rate (SGR), condition factor (CF), food conversion ratio (FCR) and food conversion efficiency (FCE) ($p > 0.05$). However, the best apparent growth occurred in control shrimps and generally decreased in exposed groups as Roundup® concentration increased (Table 3.9).

Table 3.9: Mean growth performance and feed utilisation of *C. nilotica* exposed to different Roundup® concentrations from 40 to 65 dph (n = 30)

Growth index	Concentrations (mg/L)					
	Control	**2.2**	**2.8**	**3.4**	**4.3**	**5.4**
Initial body weight (g)	0.010	0.010	0.010	0.010	0.010	0.010
Final body weight (g)	0.142	0.072	0.060	0.051	0.047	0.040
Final weight gain (g)	0.132	0.062	0.050	0.041	0.037	0.030
Percent weight gain (PWG)	1350	620	500	410	370	300
Initial body length (mm)	10.000	10.000	10.000	10.000	10.000	10.000
Final body length (mm)	26.833	25.600	24.667	23.667	21.667	20.000
Final length gain (mm)	16.833	15.600	14.667	13.667	11.667	10.00
Percent length gain (PLG)	168	156	147	137	117	100
Food intake (g/day)	0.014	0.007	0.006	0.005	0.005	0.004
Specific growth rate (SGR)	10.613	7.896	7.167	6.517	6.190	5.545
Condition factor (CF)	0.735	0.429	0.400	0.385	0.462	0.500
Food conversion ratio (FCR)	0.106	0.113	0.120	0.122	0.135	0.133
Food conversion efficiency (FCE)	9.429	8.857	8.333	8.200	7.400	7.500

Moulting

The lowest number of moults was observed in the control group and this generally increased monotonically (Figure 3.8) with increasing exposure concentration. The cumulative number of moults for each treatment group over the 14 days was determined and then transformed to cumulative percent moult (Figure 3.9). One-way analysis ANOVA followed by the Tukey post hoc multiple comparison test revealed that all the mean cumulative percent moults were significantly different from each other (Figures 3.9)

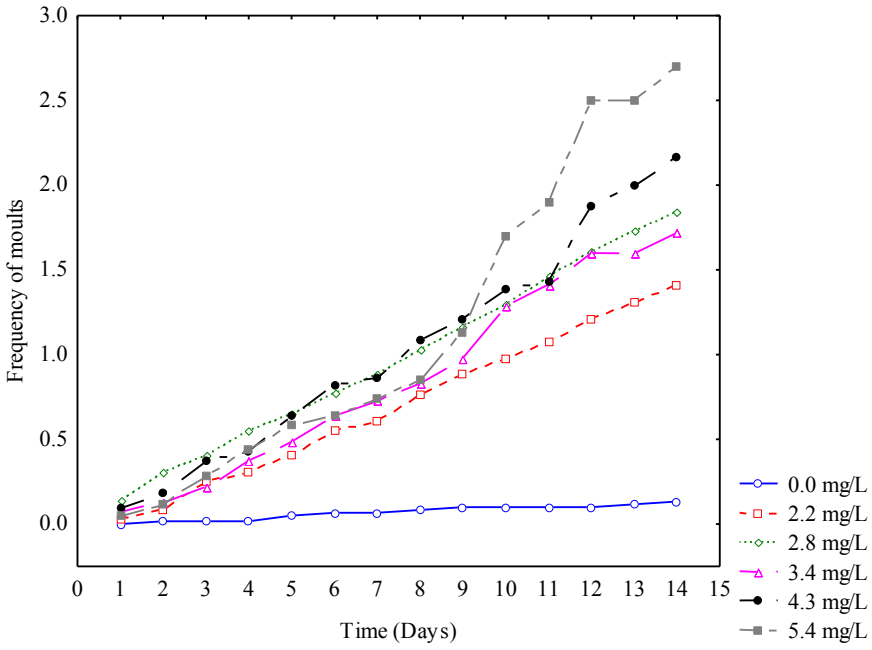


Figure 3.8: *Caridina nilotica* cumulative number of moults per number of shrimps for each Roundup® concentration for 14 days (from day 8 to 21), showing increased moulting with concentration

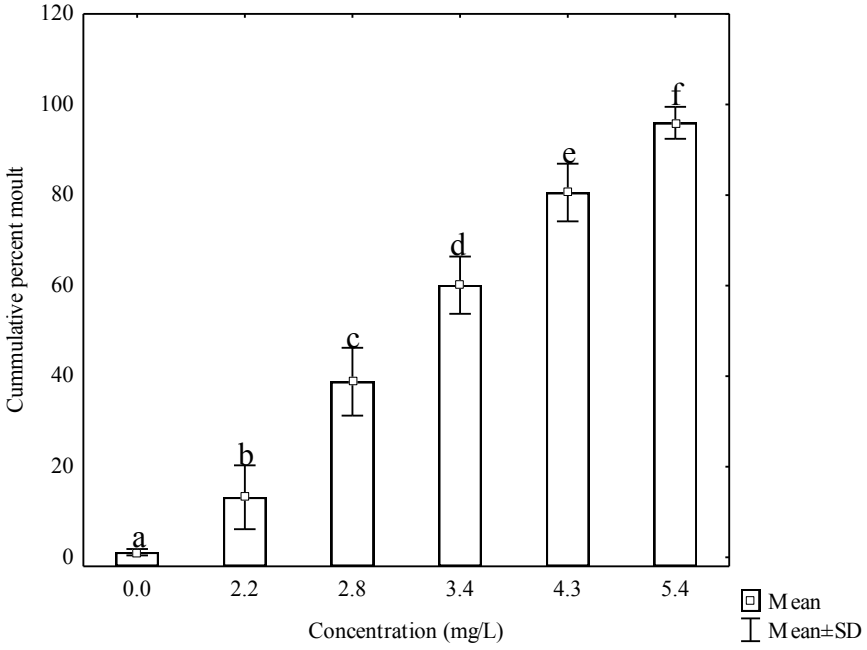


Figure 3.9: *Caridina nilotica* cumulative percent moult per Roundup® concentration for 14 days (from day 8 to 21). The means of any two concentrations having the same superscript letter are not significantly different ($p > 0.05$).

3.3.3 Sub-lethal reproductive responses of *Caridina nilotica* to Roundup® exposure

Embryotoxicity

Embryos hatched in control and all tested concentrations but the hatching success decreased monotonically. The experiment started with 48 embryos per concentration and a total of 21 were sampled for analysis, leaving a total of 27 embryos expected to survive to hatching. The total number of embryos that survived to hatching is presented in Table 3.10. At day-17, embryos hatched in all experimental groups, releasing neonates that were somatically identical to the adult but significantly smaller. Except in the control group neonates, all exposed group neonates had curved bodies (Figure 3.10). A few embryos hatched in lower concentrations (0.625 and 1.25 mg/L) before day-17, but these were not counted. Descriptions of embryonic development in the different Roundup® concentrations until hatching are presented in Appendix I.

Table 3.10: Total number of embryo survivals with corresponding hatching success in different Roundup® concentrations at the end of 17 days experimental period

Concentration (mg/L)	Number survived	Hatching success (%)
0.0	25	93
0.625	21	78
1.25	21	78
2.5	20	70
5.0	18	67
10.0	17	63

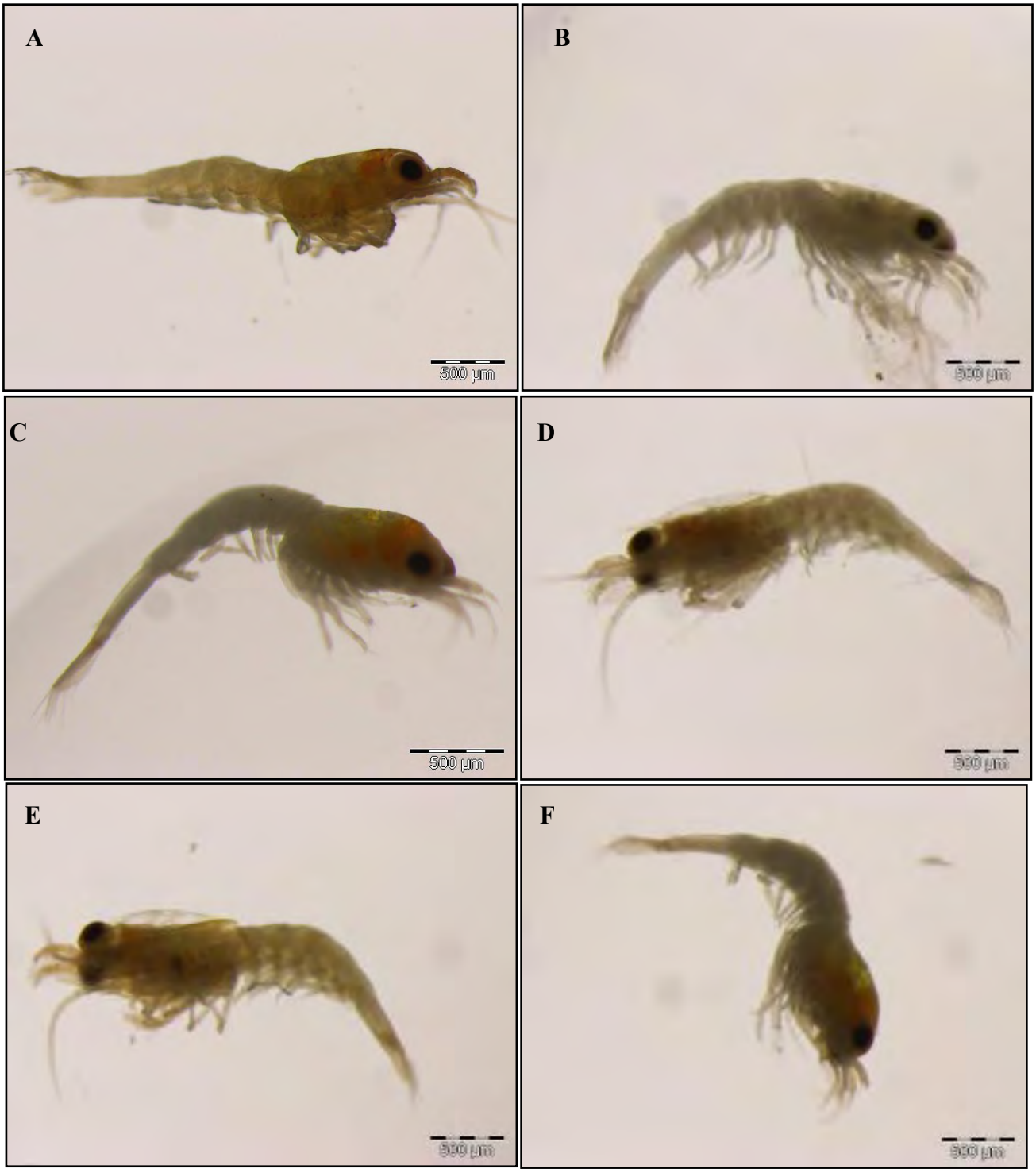


Figure 3.10: Morphological defects in newly hatched embryos after 17 days' exposure to different Roundup® concentrations (A = control; B = 0.625 mg/L; C = 1.25 mg/L; D = 2.5 mg/L; E = 5 mg/L; F = 10 mg/L)

Gonad histopathology

The gonads of shrimps aged from 40 to 55 dph, from controls and those exposed to Roundup®, had ovaries with germ cells, although some male gonads were also noted. However, there were no gonads of either control or exposed shrimps that contained both spermatogonia and ovaries. Some male gonads had spermatogonia with spermatozoa, spermatids and advanced stage secondary spermatocytes. These observations were prevalent in the control rather than in treatment shrimp gonads. Roundup® exposed shrimp stained more darkly with haematoxylin-eosin (H&E) than the control shrimps' gonads (Figure 3.11).

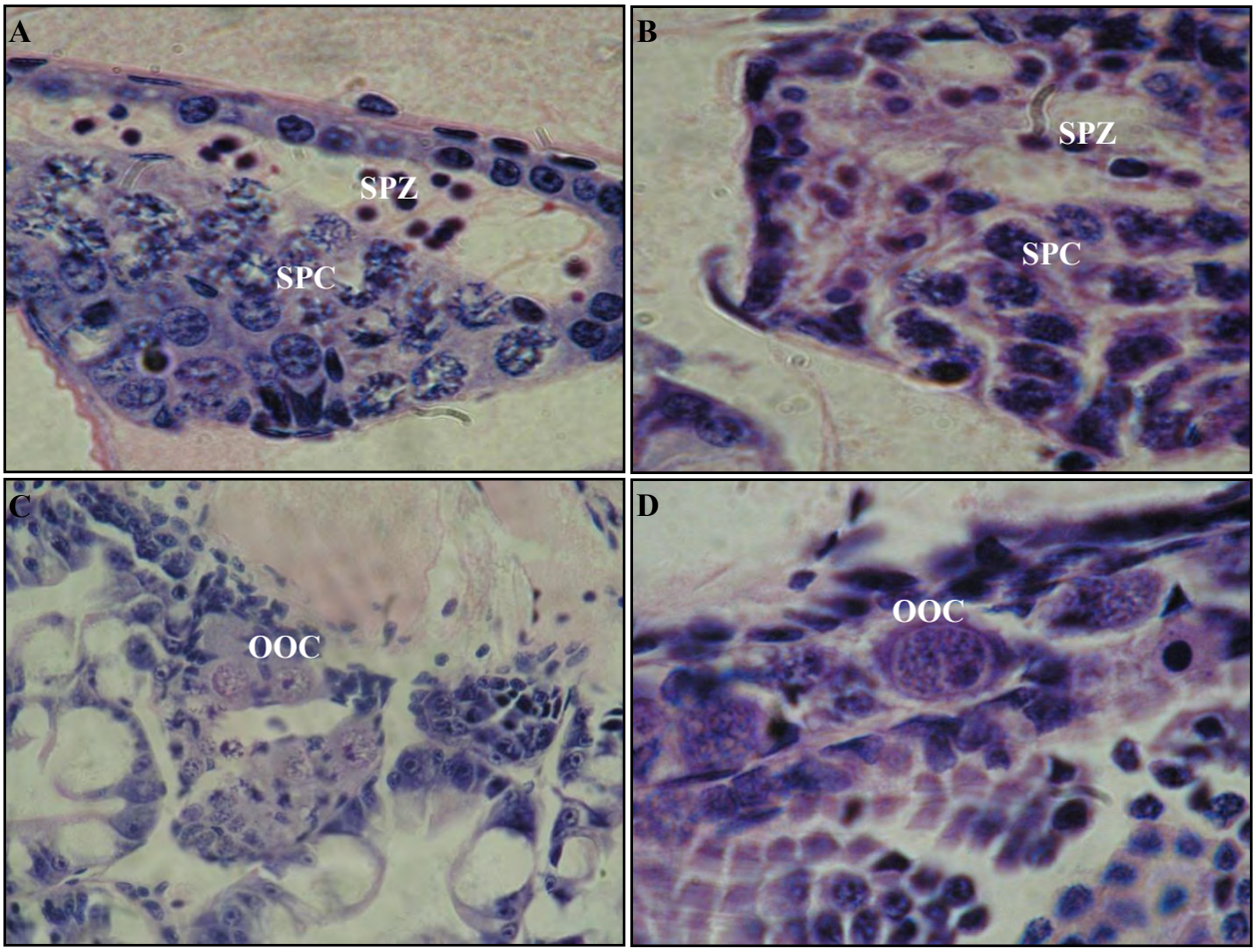


Figure 3.11: Light micrographs of transverse sections of 40-55 dph *C. nilotica* gonads exposed to 2.2-5.4 mg/L Roundup® concentrations: A – control shrimp showing male gonads (H&E; X40); B – Roundup® exposed shrimp showing male gonads (H&E; X40); C – control shrimp showing female gonads (H&E; X40); D – Roundup® exposed shrimp showing female gonads (H&E; X40). (SPZ = spermatozoa; SPC 2 = secondary spermatocytes; OOC 2 = secondary oocytes; H&E = haematoxylin-eosin)

The gonads of shrimps aged from 60 to 65 dph, from both control and treatment groups, had only female gonads with oocytes at different stages of development. The nuclei of both experimental groups were surrounded by a ring of lipid droplets. However, nuclei of Roundup-exposed shrimps stained more darkly with H&E than control shrimps (Figure 3.12).

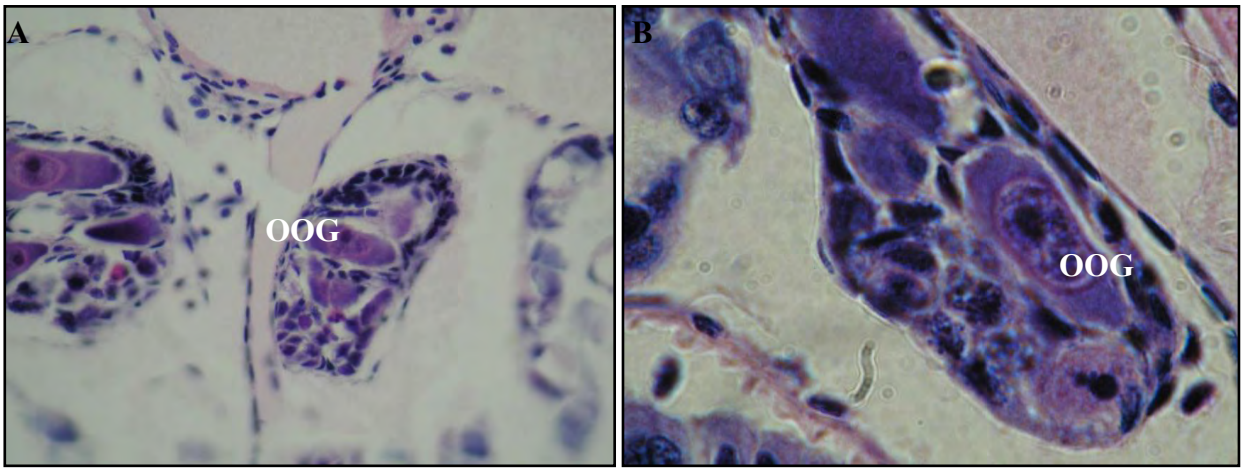


Figure 3.12: Light micrographs of transverse sections of 60-65 dph *C. nilotica* gonads exposed to 2.2-5.4 mg/L Roundup® concentrations: A – control shrimp showing female gonads (H&E; X40); B – Roundup® exposed shrimp showing female gonads (H&E; X40).(OOG = oogonium; H&E = haematoxylin-eosin)

All these results indicate no clear detectable gonadal response to Roundup® exposure.

3.3.4 Sub-lethal biochemical responses of *Caridina nilotica* to Roundup® exposure

Acetylcholinesterase

In order to rectify potential enzyme concentration variations that might result from output differences during the extraction process, AChE activities were subjected to normalisation by dividing with the protein content and were expressed as nmol/min/mg protein. The non-normalised and normalised AChE activity levels for 96-hour short-term and 21-day long-term tests are presented in Table 3.11.

Table 3.11: Non-normalised and normalised AChE values and standard deviations (±SD) of shrimps exposed to different Roundup® concentrations in 96-hour acute and 21-day sub-lethal toxicity tests

Exposure period	Concentration (mg/L)	Non-normalized AChE(±SD) (nmol/min)	Normalized AChE(±SD) (nmol/min/mg protein)
96-hour	0.0	1.17 ±0.40	3.62 ±0.42
	4.3	0.92 ±0.25	3.42 ±1.18
	6.7	0.70 ±0.15	2.53 ±1.40
	10.5	0.88 ±0.52	2.43 ±1.42
	16.4	0.96 ±0.36	2.41 ±1.91
	25.6	1.06 ±0.49	2.00 ±1.11
	40.0	1.20 ±0.33	2.32 ±0.40
21-day	0.0	1.48 ±0.41	3.69 ±0.34
	2.2	1.23 ±0.35	2.85 ±0.71
	2.8	1.12 ±0.22	2.91 ±0.99
	3.4	1.11 ±0.30	2.67 ±0.71
	4.3	1.66 ±0.18	2.30 ±0.45
	5.4	2.17 ±0.27	2.15 ±0.86

Pearson's correlation analysis of the relationship between protein contents and non-normalized AChE activities in the 96-hour short-term lethal test showed no significant linear relationships between AChE activities and protein contents ($p > 0.05$, $R^2 = 0.1128$) (Figure 3.13) but indicated statistically significant linear relationships between protein contents and normalized AChE activities ($p < 0.05$, $R^2 = 0.6047$) (Figure 3.14). For the 21-day test, Pearson's correlation coefficient indicated no significant linear relationships between protein contents and non-normalised AChE activities ($R^2 = 0.0880$, $p = 0.232$) (Figure 3.15) but established statistically significant linear relationship between protein contents and normalized AChE activities ($p < 0.001$, $R^2 = 0.5274$) (Figure 3.16).

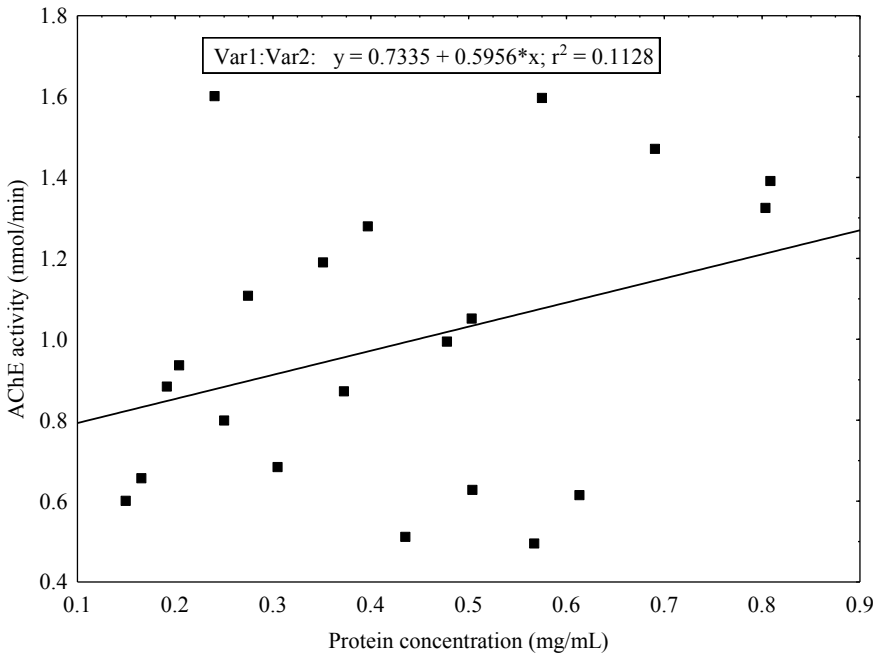


Figure 3.13: Relationship between protein concentration and non-normalized AChE activity measured in whole-body shrimps for 96-hour acute toxicity test

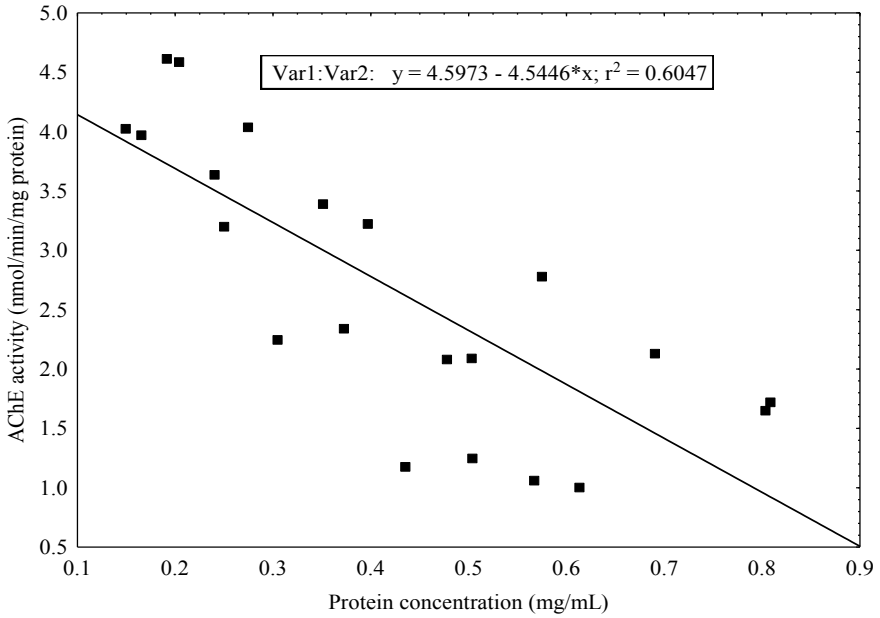


Figure 3.14: Relationship between protein concentration and normalized AChE activity measured in whole-body shrimps for 96-hour acute toxicity test

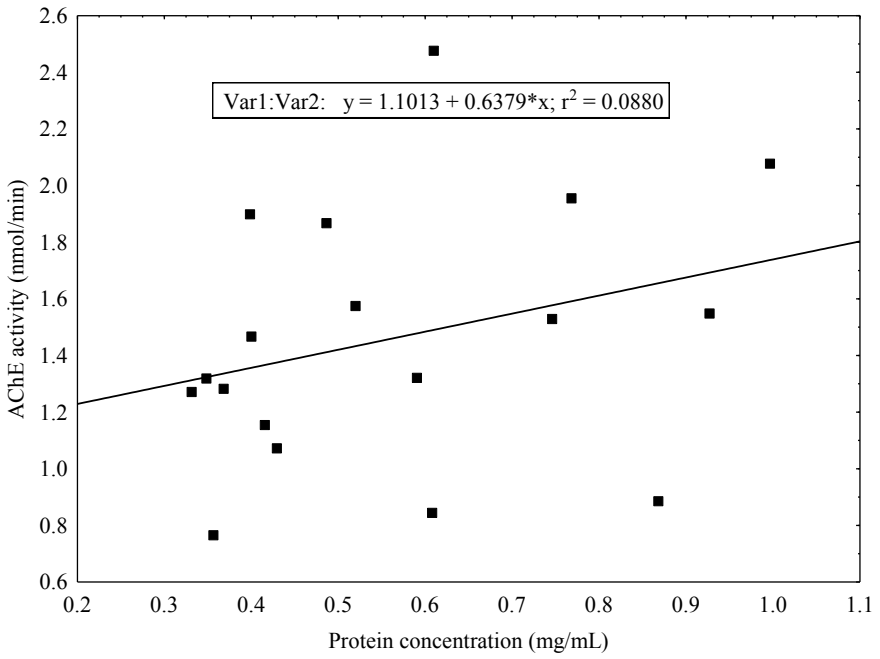


Figure 3.15: Relationship between protein concentration and non-normalized AChE activity measured in whole-body shrimps for 21-day chronic toxicity test

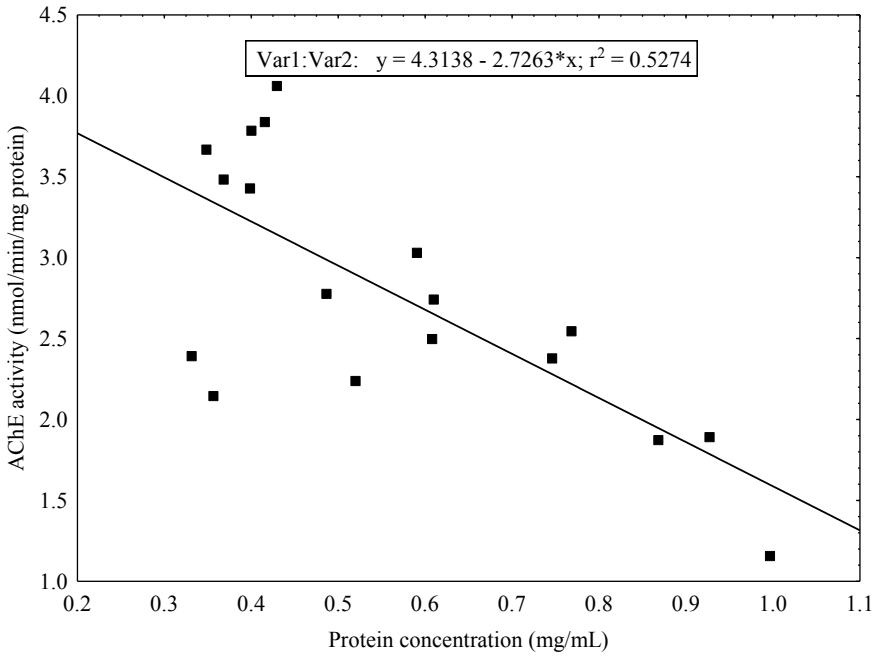


Figure 3.16: Relationship between protein concentration and normalized AChE activity measured in whole-body shrimps for 21-day chronic toxicity test

The normalised AChE activities were tested against the hypothesis that mean values across different concentrations were equal. The null hypothesis was accepted when one-way Analysis of Variance (ANOVA) revealed that the average AChE activities across different Roundup® concentrations were equal for both 96-hour acute toxicity and 21-day chronic toxicity tests ($p > 0.05$) (Figures 3.17 and 3.18). Basal AChE activity was evaluated by Student's t-test analysis of the mean activities between 96-hour and 21-day tests control groups. It was found that the mean AChE activities of the two control groups were not significantly different ($t(4) = 0.44$, $p > 0.05$), and the basal AChE activity was estimated as 3.66 (±0.38) nmol/min/mg protein.

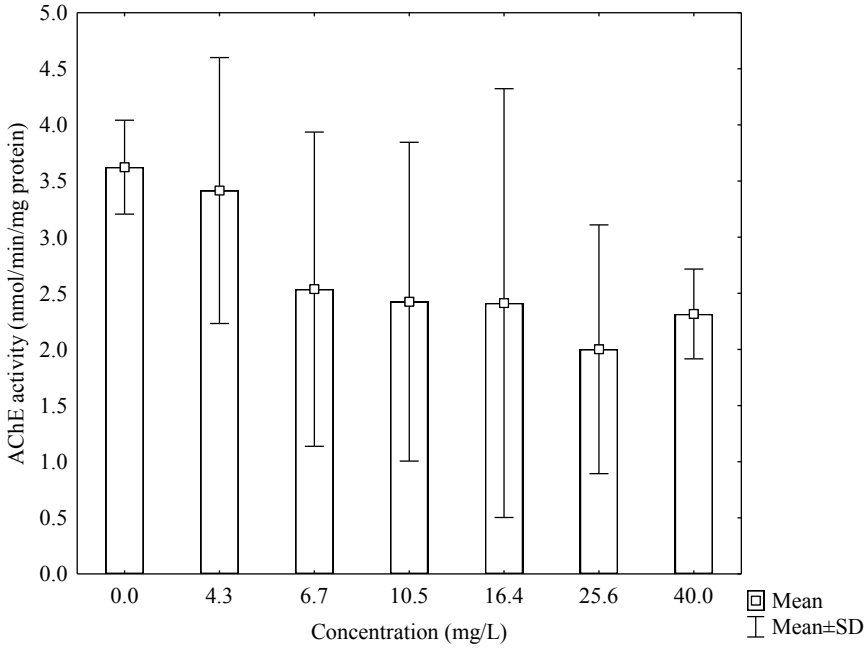


Figure 3.17: Normalised whole-body AChE activity in *C. nilotica* after 96 hours exposure to different Roundup® concentrations

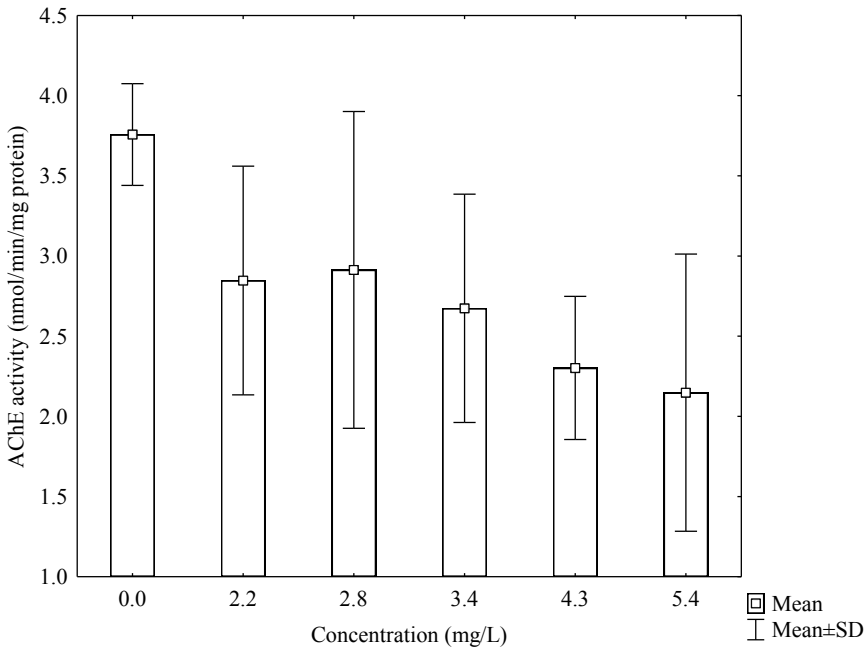


Figure 3.18: Normalised whole-body AChE activity in *C. nilotica* after 21 days exposure to different Roundup® concentrations

Lipid Peroxidation (LPx)

Lipid peroxidation values (in nmol MDA/mg protein) of shrimps exposed to different Roundup® concentrations in 96-hour short-term and 21-day long-term tests are presented in Table 3.12. There were general increases in lipid peroxidation from control to the highest glyphosate concentration. One-way ANOVA followed by Tukey post hoc tests were used to identify significant specific pair-wise differences between any two concentrations (Figures 3.19 and 3.20). Student's t-test found no significant difference between the mean LPx values of 96-hour and 21-day tests ($t(44) = -0.71$, $p > 0.05$).

Table 3.12: Lipid peroxidation values (in nmol MDA/mg protein) of shrimps exposed to different Roundup® concentrations in 96-hour short-term and 21-day long-term tests

Exposure period	Concentration (mg/L)	LPx(±SD) (nmol MDA/mg protein)
96-hour	0.0	0.03(±0.01)
	4.3	0.33(±0.54)
	6.7	0.47(±0.38)
	10.5	0.62(±0.48)
	16.4	0.83(±0.61)
	25.6	1.07(±0.60)
	40.0	2.41(±1.23)
21-day	0.0	0.04(±0.02)
	2.2	0.07(±0.05)
	2.8	0.24(±0.26)
	3.4	0.89(±0.15)
	4.3	1.10(±0.06)
	5.4	1.80(±0.44)

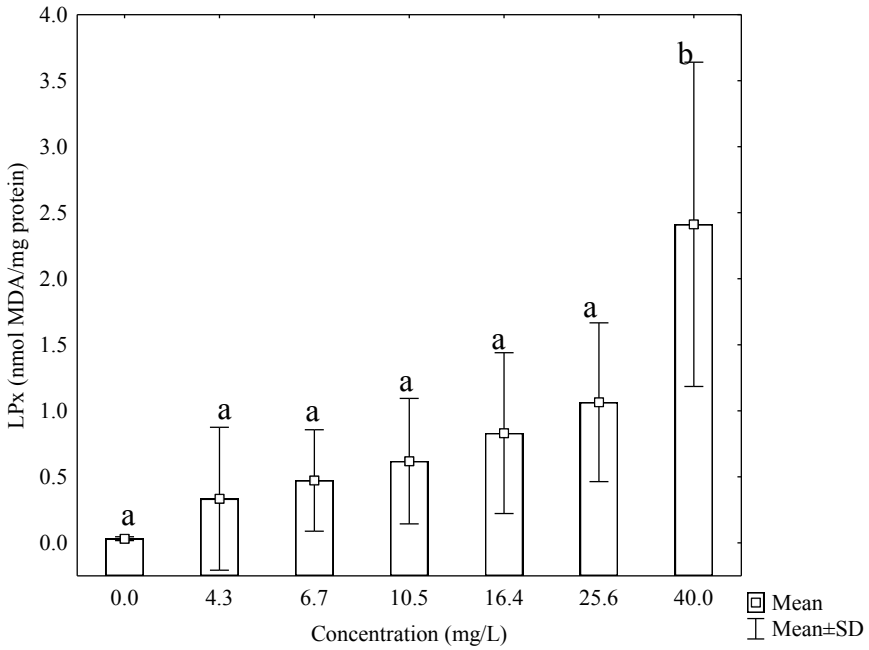


Figure 3.19: Whole-body lipid peroxidation in *C. nilotica* after 96-hour exposure to different Roundup® concentrations. The means of any two concentrations having the same superscript letter are not significantly different ($p > 0.05$)

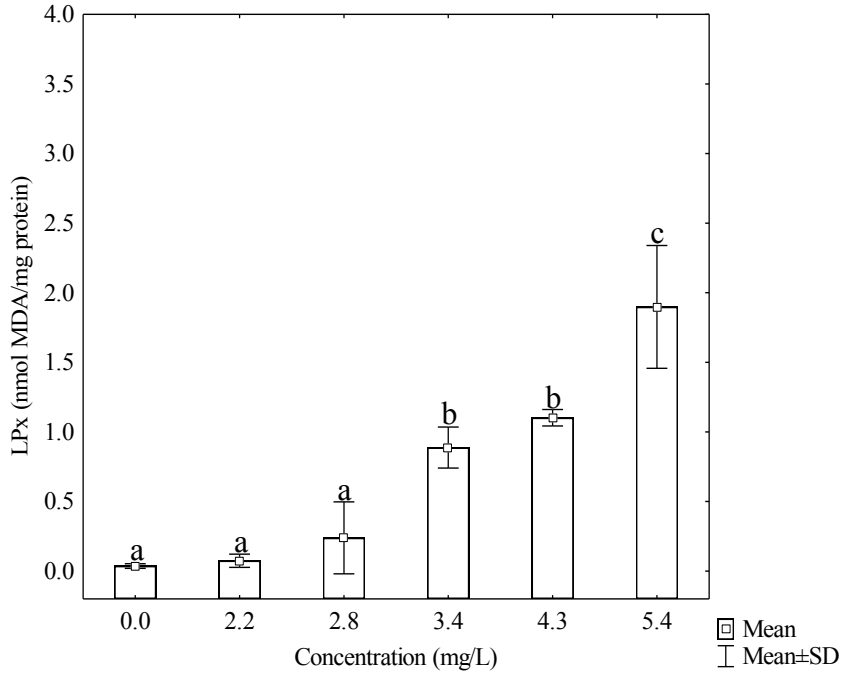


Figure 3.20: Whole-body lipid peroxidation in *C. nilotica* after 21-day exposure to different Roundup® concentrations. The means of any two concentrations having the same superscript letter are not significantly different ($p > 0.05$)

3.4.5 A comprehensive overview of the ecotoxicological effects of Roundup® exposure in *Caridina nilotica*

A comprehensive overview of the ecotoxicological effects of Roundup® to *C. nilotica* under different exposure lengths and at different biological scales is presented (Table 3.13). The concentrations were established at different point estimates including EC10, EC20 and EC50.

Table 3.13: Estimated ECx values for all tested biological systems of *C. nilotica* at different exposure periods and exposure types

Exposure type	**Exposure period (days)**	***C. nilotica* biological system**	**EC10 (mg/L)**	**EC20 (mg/L)**	**EC50 (mg/L)**
Lethal exposure	2	Neonate	1.66	2.14	4.64
		Juvenile	5.50	6.34	9.73
		Adult	7.74	11.77	41.49
	4	Neonate	1.26	1.52	2.67
		Juvenile	4.43	4.94	6.82
		Adult	5.55	8.00	25.05
	21	Adult	2.09	2.35	3.35
Sub-lethal exposure	4	Adult acetylcholinesterase	2.40	5.46	64.84
		Adult lipid peroxidation	5.32	7.21	17.98
	21	Adult acetylcholinesterase	1.48	2.20	7.23
		Adult lipid peroxidation	2.50	2.74	3.64
	25	Adult length	2.51	3.26	7.07
		Adult weight	0.39	0.58	1.82
		Adult moulting	1.80	2.02	2.86
		Embryo	0.02	0.06	1.27
		Adult gonads	-	-	-

3.4 Discussion

The results clearly demonstrate biological stress responses to varying concentrations of the stressor Roundup® at varying biological system scales in the shrimp *C. nilotica*. The data-set provides a detailed inventory of Roundup® as a chemical stressor to the shrimp. Various aspects of the nature of the stress-response relationship will be discussed.

3.4.1 Lethal exposure of neonate, juvenile and adult *Caridina nilotica* to Roundup®

The current study has shown that Roundup® herbicide adversely affects all life history stages of *C. nilotica* as observed in the mortality of shrimps when exposed to the herbicide. Although a few studies exist on the toxicity of Roundup® and other glyphosate-based herbicides to freshwater organisms, especially fishes and crustaceans (Folmar *et al*., 1979; Hartman and Martin, 1984; Alberdi *et al*., 1996; Servizi *et al*., 1987; Tsui and Chu, 2003; Tsui and Chu, 2004), no study has examined the toxicity of Roundup® to *C. nilotica*. It is therefore valuable to compare the LC50 and EC10 concentrations determined in this study to already reported values of other freshwater invertebrates exposed both to Roundup® and other pesticides, bearing in mind that these reported values were mostly from adult organisms.

Folmar *et al*. (1979) reported Roundup® 48-hour LC50s for the scud *Gammarus pseudolimnaeus* as 62 mg/L, while Tsui and Chu (2004) reported it for the daphnid *Ceriodaphnia dubia* as 5.7 mg/L. From the present study, Roundup® 48-hour LC50 for *C. nilotica* ranged from 4.5 to 37.1 mg/L (neonate to adult), which falls within the reported values for other crustaceans as stated above. Folmar *et al*. (1979) reported Roundup® 96-hour LC50 for *G. pseudolimnaeus* as 43 mg/L, while Mayer *et al.* (1986) reported it as 7 mg/L for the crayfish *Orconectes nais*. The Roundup® 96-hour LC50 for *C. nilotica* from the current study ranged from 2.6 to 25.3 mg/L, which falls within that reported above for other crustaceans. Roundup®LC50 values in this study decreased with earlier shrimp life stages, and as the test duration increased from the 48-hour to the 96-hour, which confirms previous studies, including Tsui and Chu (2003, 2004).

The long-term lethal tests have shown that low concentrations (relatively to the application rate) can negatively affect survival of *C. nilotica*. This was demonstrated when 2.8 mg/L Roundup® concentrations caused significant mortality in *C. nilotica.* Therefore, it is necessary to monitor the environmental use of glyphosate since the field application is 2-4 % of the active ingredient, which is about 240 times higher than 2.8 mg/L (Bold, 2007; Jadhav *et al.,* 2008; DWA, 2009). Research has shown that low concentrations of pesticides have a negative impact on survival of arthropods. Duchet *et al.* (2011) exposed *Daphnia pulex* and *Daphnia magna* to lower concentrations of the insecticides spinosad (2, 4 and 8 µg/L) and diflubenzuron (0.2, 0.4 and 0.8 µg/L) for 14 days under laboratory conditions. They reported that both insecticides significantly decreased the number of surviving individuals and production of neonates. Dodson and Hanazato (1995) reported that although low concentrations of carbaryl (1-5 µg/L) did not affect survivorship of *Daphnia* sp., the same concentrations significantly affected other biological factors such as growth rate reduction, reproduction inhibition (and subsequent delay in sexual maturation) and reduced swimming. The results of Dodson and Hanazato (1995) may be explained in the context that survival of organisms is of utmost biological importance. In order to survive, organisms may have to adopt a trade-off strategy that involves compromising and decreasing biological functions such as reduced swimming ability, delayed sexual activity, and even growth.

This study shows that low levels of the herbicide Roundup® may adversely affect *C. nilotica's* health and survival, as was evidenced in some levels of mortality in all three life stages. *C. nilotica* neonates were the most sensitive with a mean 96-hour LC50 of 2.5 mg/L, which is much lower than the application rate (20-30 mg/L), although the application's impact depends on the dilution rate of the applied concentration in the environment**.** Under field conditions, Roundup® is considered to pose less risk due to high adsorption of glyphosate to soil. However, the herbicide should be carefully managed to minimise any negative impact on non-target freshwater organisms. Moreover, it is necessary to include early life stages of toxicity test organisms such as *C. nilotica* in experimental work in order to obtain accurate measures of the potential toxic effects of glyphosate-formulated herbicides. This information would be essential in assessing the ecological risk of using such herbicides.

The long-term lethal response observed in this study suggests that Roundup® adversely affected *C. nilotica's* survival, which may be related to toxicokinetic and toxicodynamic properties of the herbicide. The various estimated values for including LC50 NOEC and LOEC values for all three life stages (neonate, juvenile and adult), can be used to estimate chronic thresholds to protect freshwater ecosystems.

3.4.2 Sub-lethal growth responses of *Caridina nilotica* to Roundup® exposure

Length and weight

The effect of Roundup® on *C. nilotica* growth in this study was seen in the reduction of total body length (TL) and wet weights in all tested concentrations compared to the control shrimp. The decreased growth could be attributed to depletion of energy reserves including glycogen, protein and lipid. This is possible because glycogen is the main food reserve used by crustaceans, and environmental stress, such as exposure to chemicals, may affect its level (Frontera *et al.*, 2011). In the current study, higher levels of lipid peroxidation due to oxidative stress were measured in shrimps exposed to glyphosate concentrations than in unexposed control shrimps. These results are supported by Frontera *et al.* (2011) who, in a separate study, investigated the effects of glyphosate on growth and energy reserves in another freshwater decapod, the crayfish *Cherax quadricarinatus*. They found a significant reduction in the weight of glyphosate-exposed *C. quadricarinatus* compared to control animals. Their report revealed decreased somatic growth in *C. quadricarinatus*, which they attributed to a decrease of both glycogen and lipid reserves.

Reduction in growth could also be attributed to the fact that the shrimp had to use energy to detoxify glyphosate through excretion, thereby altering carbohydrate and protein metabolism, resulting in less available energy from the food for growth. Such a phenomenon would have an adverse effect on food utilisation, and consequently, growth performance. In the current study, food utilisation was poor in treatment shrimps compared to control shrimps. This was evident in the higher percentage gains of body weight and length in control *C. nilotica* compared to Roundup® exposed individuals.

In this study, the effect of Roundup® on growth of *C. nilotica* was manifested in both reduced body weight and length of the shrimp. The decrease in growth of *C. nilotica* in the different Roundup® concentrations was concomitant with growth performance parameters such as percent weight gain (PWG), percent length gain (PLG), specific growth rate (SGR), condition factor (CF), feed conversion ratio (FCR) and feed conversion efficiency (FCE). The results of the current study showed that growth reduction became more pronounced as the concentration of Roundup® increased. The values for FCR for the treatment groups were all higher than the control group. This suggests that there was less wastage of food by the control group than in treatment groups and that the food gets wasted more as the concentration increases. Condition factor indicates the condition or "fatness" of the shrimp and is based on the hypothesis that heavier shrimp are in better condition. Since the value for the control group is higher than the treatment group, it can be said that the control group were in better condition than the treatment groups.

Literature focusing on effects of toxic chemicals on freshwater crustaceans' growth and feed utilisation is scarce. However, a few studies have reported on length-weight relationships and growth conditions of freshwater shrimps without necessarily evaluating the effects of toxic chemicals on these animals (Enin, 1994; Araneda *et al*. 2008; Deekae and Abowei, 2010). This notwithstanding, studies on the toxic effects of chemicals to growth and condition indices of other aquatic organisms, especially fish, have been reported. Kerambrun *et al*. (2012) reported significant decreases in growth rates and condition indices when juvenile turbot *Scophthalmus maximus* (Scophthalmidae) were exposed to metals, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs). Therefore sub-lethal growth responses of *C. nilotica* to Roundup® in this study, which resulted in significant growth impairment, could be attributed to poor growth performance indices (e.g. condition factor), depleted food reserves and decreased GH levels.

Moulting

Studies have found that moulting frequency is affected by environmental toxicants and that crustaceans are more susceptible to environmental stresses during moulting (Mazurová *et al*., 2008). However, the process of moulting in crustaceans is generally controlled by the neuroendocrine system and therefore the effect of toxicants on moulting may either stimulate or inhibit this system (Weis *et al*., 1992).

The results of the current study show that Roundup® affected moulting of *C. nilotica* in a concentration-dependent manner, i.e. the frequency of moulting increased with increasing concentrations, from 0.0 mg/L (control) to 5.4 mg/L (highest concentration). This implies that the herbicide Roundup® has a stimulatory effect on the neuroendocrine system of *C. nilotica*. The basis for hormonal activity of xenobiotics (e.g. endosulfan, an oestrogenic pesticide) mimicking endogenous steroid hormones (e.g. oestrogen) are the structural similarities between the two (Zou and Fingerman, 1997). Thus, it can be postulated that the inhibitory effects of the hormonal activity of xenobiotics on the moulting of crustaceans are due to structural overlaps between the xenobiotics and the endogenous steroid moulting hormones. These similarities may enable these mimics to bind to ecdysteroid receptors, but they then act as antagonists rather than as agonists of the ecdysteroids. As antagonists, these mimics may slow down the moulting process by blocking the receptors for extended periods, preventing endogenous ecdysteroids from binding to the receptor and turning on. In this context, in this study, Roundup® exhibited hormone-like activity since it significantly stimulated moulting in a concentration-dependent manner.

It is possible that Roundup® promoted the moulting process as an agonist by attaching to ecdysteroid receptors as a mimic because there are similarities between it and the endogenous ecdysteroids. Although the present study has shown Roundup® to have a stimulatory effect on the neuroendocrine system of *C. nilotica* by aiding the moulting process, a number of investigations into toxic effects of many xenobiotics on crustacean moulting process reported inhibition of the neuroendocrine system (Lemos *et al*., 2010; Zou and Fingerman, 1997; Mu and Leblanc, 2002; Baldwin *et al*., 1995).

Moulting in *C. nilotica* appears to have potential for development as a bioassay for sub-lethal effects of Roundup® and similar herbicides in the freshwater ecosystem. However, it is possible that the stimulatory effect of Roundup® on the moulting process of *C. nilotica* is a more general response to environmental toxicants and does not directly involve ecdysteroids and their receptors.

3.4.3 Sub-lethal reproductive responses of *Caridina nilotica* to Roundup® exposure

Embryotoxicity

Glyphosate adversely affected hatching of embryos in all tested concentrations compared to the control embryos, which experienced a significant higher hatching success. The rate of hatching success decreased with increasing Roundup® concentrations which is the case in recorded embryotoxicity investigations. Sawasdee and Köhler (2009) performed an embryotoxicity study with the pesticides atrazine and imidacloprid, using the ramshorn snail, *Marisa cornuarietis*. They found that higher concentrations of atrazine significantly increased hatching rate compared to lower concentrations of the pesticide. They also reported that imidacloprid caused a significant decrease in the heartbeat rate of *Marisa cornuarietis* embryos, but they did not observe any effects on other morphological endpoints, such as eyes and tentacles. In contrast to the current study in which glyphosate decreased the hatching rate compared to the control group by 18%, Wirth *et al.* (2001) reported that endosulfan and methoprene respectively increased hatching time by 12% and 10%, when embryos of the grass shrimp *Palaemonetes pugio* were exposed to it. Thus, results of this study suggest that hatching and newly hatched neonates could be used as endpoint measures in embryotoxicity investigations.

Gonad histopathology

In the present study, no adverse morphological or developmental effects were observed in gonads of control shrimps and shrimps exposed to Roundup®. Two possibilities may account for this observation: either the range of glyphosate concentration (2.2-5.4 mg/L) used was too small to cause any serious defects in the gonads, or the shrimp effectively detoxified the herbicide from its system. If the former is the case, then it would be advisable to expose *C. nilotica* to concentrations higher than 5.4 mg/L in short-term or long-term sub-lethal investigations to establish the veracity or otherwise of such a possibility. However, the latter scenario is possible because the growth of Roundup® exposed *C. nilotica* was significantly reduced, suggesting that greater amounts of available energy from food could have been used to detoxify Roundup®, leaving less energy for growth. Thus, it could possibly be a trade-off of growth for reproduction, since procreation will ultimately lead to the survival of the species.

Literature on the histopathological effects of glyphosate-based herbicides on crustaceans is extremely sparse, but a few studies have reported effects of other chemicals. Schirling *et al*. (2006) exposed the amphipod *Gammarus fossarum* to Bisphenol-A (BPA) and evaluated stress response and gonad histology. They found that exposure to BPA accelerated the maturation of oocytes in females and reduced the number and size of early vitellogenic oocytes. Byrne and O'Halloran (2001) used two estuarine bivalve molluscs, *Scrobicularia plana* and *Tapes semidecussatus*, to evaluate the effects of laboratory exposure to sediments collected from estuarine and coastal areas around the Irish and English Coast over a three-year study period. The results of their study revealed that histopathology could produce sensitive endpoints to provide information at the cellular level of biological organisation. In this study, clear histopathological defects could not be detected in either Roundup® exposed gonads or control shrimp gonads. However, the treated gonads were more darkly stained with haematoxylin-eosin (H&E) than the control gonads. Thus, it is possible to speculate that though histopathological defects could not be detected in exposed gonads, the gonads could be used as environmental biomarkers to detect exposure to Roundup®.

3.4.4 Sub-lethal biochemical responses of *Caridina nilotica* to Roundup® exposure

Acetylcholinesterase

AChE activity levels in organisms exposed to toxic chemicals may be concentration-dependent, time-dependent, or both (Xuereb *et al*., 2009a, b). In this study, AChE activity levels decreased as concentrations increased in both 96-hour acute toxicity and 21-day chronic toxicity tests. This suggests that the AChE activity levels in *C. nilotica* are concentration-dependent. However, the results suggest that AChE activity is not time-dependent since there was no significant difference between means in the AChE activity levels between 96-hour acute toxicity and 21-day chronic toxicity tests, levels which ranged between 2.6761 (±1.1772) and 2.7736 (±0.8017) respectively. This is supported by previous studies. Xuereb *et al*. (2009b) exposed *Gammarus fossarum* to the concentrations 0.125, 0.25, 0.5 and 1 µg/L of the organophosphorous pesticide chlorpyrifos (CPE) and 10, 20, 40, 80 and 160 µg/L of the carbamate pesticide methomyl (MT) for 96 hours. They reported a decrease in AChE activity levels in *G. fossarum* for both pesticides.

In a separate study, Beltran and Pocsidio (2010) investigated the effect of AChE levels in the freshwater bivalve mollusk *Corbicula fluminea* when exposed to the pesticide malathion. They found that AChE levels decreased with increasing malathion concentrations. Glusczack *et al*. (2007) reported reduction in AChE activity levels in the brain of the silver catfish *Rhamdia* quelen after exposure to Roundup® of concentrations 0.0 (control), 0.2 and 0.4 mg/L for 96 hours. Their results concur with the results in the current study that Roundup® reduces the AChE activity levels of aquatic animals in a concentration-dependent manner.

In terms of the relationship between AChE activity levels and time-dependency, Xuereb *et al*. (2009b) reported that CPE decreased AChE activity levels over time, contrary to the current study where time did not affect the enzyme's activity in either 96-hour or 21-day toxicity tests. However, their study with MT showed no time-dependent AChE inhibition, which is in agreement with this study. In fact, the decrease in AChE activity levels caused by MT was significant from the lowest tested concentration (10, 20, 40, 80 and 160 µg/L), with mean inhibitions of 13.4±5.7, 21.3±3.6, 38.4±4.3, 53.9±4.4 and 66.2±3.2 % respectively, compared to the control.

The use of biomarkers in aquatic invertebrates as water pollution indicators has been widely recognised in recent years (Parvez and Raisuddin, 2005; Xuereb *et al*., 2009a, b; Richardson *et al*., 2010). Biomarkers are valuable tools for environmental assessment because they make it possible to express chemical stress in biological terms (Xuereb *et al*., 2009a). In order to use an enzyme activity appropriately as a biomarker of contamination requires an extensive knowledge of its variability in terms of intrinsic biotic and environmental factors. It is necessary to establish reference basal enzyme activity levels in the test organism before an enzyme activity is used as a biomarker of pollution. Basal enzyme activity is often derived from the control groups in exposure studies (Xuereb *et al*., 2009a, b). In the current study, the reference basal AChE activity range was established from mean enzyme activities of the control groups, which was found to be 3.69 (±0.34) nmol/min/mg protein. This was used as the reference point at which AChE activity levels in the current study were evaluated. Based on this, AChE activity levels in nmol/min/mg protein in 96-hour acute toxicity test were transformed to percent activity levels to obtain 100, 94, 70, 67, 67, 55 and 64 % for control, 4.3, 6.7, 10.5, 16.4, 25.6 and 40.0 mg/L respectively.

This implies percent activity levels reduced by 0, 6, 30, 33, 33, 45 and 36 % in the respective Roundup® concentrations. Percent AChE activity levels for shrimps in the 21-day chronic toxicity were 100, 76, 78, 71, 61 and 57 % for control, 2.2, 2.8, 3.4, 4.3 and 5.4 mg/L respectively. This means that AChE activity levels diminished by 0, 24, 22, 29, 39 and 43 % in the respective Roundup® concentrations. Many studies have reported AChE activity levels in percentages and have demonstrated that reduction in AChE activity levels is proportional to increased toxicant exposure concentrations. Printes and Callaghan (2004) demonstrated that the organophosphate pesticides chlorpyrifos, malathion and parathion caused a reduction in *Daphnia magna* AChE activity levels up to 50 %, with accompanying adverse effects on mobility, while acephate caused 70 % reduction in *D. magna* AChE activity levels with no adverse effects. Xuereb *et al*. (2009b) reported that chlorpyrifos caused more than 50 % AChE activity inhibition in *Gammarus fossarum*, while the median lethal concentration (LC50) produced 70 % AChE activity inhibition. They also reported that 160 µg/L of methomyl caused 66 % AChE inhibition in *G. fossarum*.

As stated earlier, AChE activities are generally normalised against the protein content in sample extracts and expressed in nanomoles as hydrolysed substrate (nmol/min/ mg protein) (Xuereb *et al*., 2009a, b; Richardson *et al*., 2010). Conversely, the natural variation of structural protein content that is related to physiological changes, such as reproductive status, constitutes a source of variability which may make estimates of the basal level of AChE activity imprecise (Xuereb *et al*., 2009a). The robustness of the basal activity estimated for this study lies in the fact that the shrimps used were laboratory-cultured and may not be affected much by environmental factors because the experiments were conducted under controlled conditions. Furthermore, AChE was extracted from the shrimp's whole body and this implies that the total protein content present in the organism was accessed, which would not be the case if AChE was extracted from specific tissues (Printes and Callaghan, 2003; Richardson *et al*., 2010). Thus, the use of total protein content to normalised AChE activity levels in the current study ensured that the natural variation of structural protein content could have little or no impact on the reported levels of activity.

The ecotoxicological data accrued from this study could be the basis for the possible use of *C. nilotica* AChE activity levels for monitoring the pollution of aquatic ecosystems by Roundup® and other glyphosate-formulated herbicides. The establishment of the basal AChE activity level for 40 dph *C. nilotica* has provided a reference value against which relative AChE activity levels can be measured in a polluted environment. If minimum and maximum reference thresholds are estimated below and above the basal reference values respectively, then any decrease or increase of AChE activity would indicate present or past pollution.

Lipid peroxidation

Lipid peroxidation has been suggested as one of the molecular mechanisms through which certain classes of pesticides induce toxicity in organisms by increasing malondialdehyde (MDA) production (Wu *et al*., 2011). Therefore, MDA formation, usually measured with the thiobarbituric acid reactive species (TBARS) assay, is widely used as an indicative index of LPx (Wu *et al*., 2011). Lipid peroxidation levels in organisms exposed to toxic chemicals may be concentration-dependent, time-dependent, or both, since exposure time and concentration used are important determinants of a chemical's toxicity. The results of this study suggest that LPx is not time-dependent since the Student's t-test revealed no significant difference between the mean LPx values of 96-hour acute toxicity and that of 21-day chronic toxicity tests. Geracitano *et al*. (2002) hypothesised that exposure to an environmental stressor like copper favours reactive oxygen species (ROS) generation, which in turn, induced the enzymatic defense system of animals, preventing lipid peroxidation formation. It can, therefore, be argued that LPx increased in exposed *C. nilotica* in the current study because anti-oxidant enzymes were not induced to counteract damage caused by exposure to Roundup®.

The results of this study revealed that LPx increased with increasing exposure concentrations. This means LPx levels in *C. nilotica* are concentration-dependent. The concentrations used in both acute and chronic toxicity tests caused damage to cells and tissues of the shrimp. This implies that transient and long-term exposure to Roundup® herbicide may cause acute and chronic damage to *C. nilotica*. Like this study, many studies have reported oxidative stress caused by toxic effect of pesticides in different organisms.

In a study to evaluate LPx in the freshwater cladoceran *Daphnia magna* after exposure to sub-lethal levels of menadione, paraquat, endosulfan, cadmium and copper for 48 hours, Barata *et al*. (2005) reported that LPx increased with increasing exposure concentrations for all test chemicals. However, animals exposed to menadione, endosulfan and copper showed more significant increases in LPx than control animals. Wu *et al*. (2011) found that malathion and chlorpyrifos induced MDA production in *Oxya chinensis*, while Damiens *et al*. (2004) reported increased LPx levels after larvae of the oyster *Crassostrea gigas* had been exposed to the pesticides malathion and carbofuran.

Lipid peroxidation is considered an important endpoint measure for biomarker development because the effects of many environmental toxicants are linked to oxidative stress (Barata *et al*., 2005). A sound knowledge of animal oxidative stress variability in terms of intrinsic biotic and environmental factors is a necessary prerequisite for the use of LPx as a biomarker of contamination. Therefore, it is necessary that before LPx is used as a biomarker of water pollution, the reference basal levels in unexposed organisms are ascertained (Xuereb *et al*., 2009). In the current study, the lipid peroxidation reference basal level was estimated from the means of the control groups, which was found to be 0.0338 (±0.0143) nmol MDA/mg protein. This value was used as the reference point at which LPx levels of the current study were evaluated.

The oxidative damage of cells and tissues of animals exposed to pesticides may be the result of insufficient anti-oxidant potential (Banerjee *et al*., 1999). Furthermore, different toxicants may induce different anti-oxidant/pro-oxidant responses in organisms, depending on their ability to produce reactive oxygen species and anti-oxidant enzymes to detoxify them. Results of this study show that Roundup® acted as a pro-oxidant, causing injury to the cells of *C. nilotica* through increasing LPx levels. Barata *et al*. (2005) assessed changes in anti-oxidative processes in juveniles of the freshwater crustacean *Daphnia magna* after exposure to paraquat, endosulfan (pesticides), cadmium, copper (metals), and menadione (quinine) in a 48-hour sub-lethal toxicity test. Lipid peroxidation and activities of key anti-oxidant enzymes, including catalase, superoxide dismutase, glutathione peroxidase, and glutathione *S*-transferases were evaluated. The results showed low responses of anti-oxidant enzyme activities for menadione and endosulfan with increased LPx, but found enhanced levels of anti-oxidant enzyme activities for paraquat with decreased LPx.

Conversely, high levels of both anti-oxidant enzyme activities and LPx were found for copper, whereas cadmium recorded low anti-oxidant enzyme responses with insignificant increases in LPx. Hence, Barata *et al.* (2005) speculated that cadmium's potential for altering the anti-oxidant/pro-oxidant status in *D. magna* was low. They found that endosulfan increased lipid peroxidation in *D. magna*, just as Roundup® did in *C. nilotica* in the current study. In the current study, Roundup® caused oxidative tissue damage in *C. nilotica*. This was determined by LPx and measured as MDA in shrimp tissue. Evidence from this study showed that Roundup® as an environmental contaminant caused significant LPx increase in *C. nilotica*. Therefore, LPx in *C. nilotica* may be used as biomarker of Roundup® pollution of aquatic ecosystems.

3.5 Conclusion

In this study, both lethal and sub-lethal exposure tests (short-term and long-term) have shown that varying concentrations of Roundup® elicit responses in different biological scales in *C. nilotica*. Lethal exposure to Roundup® resulted in some levels of mortality in all three life stages, with neonates being the most sensitive life stage. The responses were both concentration and time-dependent. However, *C. nilotica* generally responded to sub-lethal exposure to Roundup® in a concentration-dependent manner in all tested biological scales including length, weight, moulting (growth), embryos (reproduction) acetylcholinesterase (AChE), lipid peroxidation (LPx) (biochemical), except the gonads. Therefore, all the listed measures could be used as endpoint measures for the detection of Roundup® toxicity to *C. nilotica*. This information can be used to assess the ecological risk of using glyphosate-based herbicides and estimate protective values for the protection of aquatic life.

The aim of this chapter, as stated in the introduction, was to provide a comprehensive perspective on the ecotoxicological effects of Roundup® to a single organism: *C. nilotica*. The overview of this was presented in Table 3.13. This substantive single organism knowledge will be compared with species sensitivity distribution (SSD) using multiple species in the next chapter.

CHAPTER FOUR

DERIVATION OF WATER QUALITY GUIDELINES FOR GLYPHOSATE (USING ROUNDUP®) BASED ON SPECIES SENSITIVITY DISTRIBUTION

4.1 Introduction

In the previous chapter, the response of a single species to a biological stress was investigated using different biological system scales of *Caridina nilotica* exposed to varying Roundup® concentrations. This chapter seeks to evaluate the responses of multiple species exposed to Roundup® (as a biological stress) through species sensitivity distribution (SSD). In this way, the various kinds of investigation of stress-response can be evaluated, and a guideline for Roundup® will be recommended.

4.1.1 Development of glyphosate water quality guideline protocol

The approach used in developing the proposed South African water quality guidelines (WQGs) follows the Australian and New Zealand framework (ANZECC and ARMCANZ, 2000). The ANZECC and ARMCANZ (2000) framework was recommended for the derivation of water quality guidelines for organic chemicals in South Africa because:

- Its aim is to achieve sustainable use of the nations' water resources by protecting and enhancing their quality while maintaining economic and social development. This aim is in line with that of the South African National Water Act (Act 36 of 1998) (see section 2.1 of Chapter One).

- It incorporates the most recent advances in ecotoxicology, ecology, statistics and ecological risk assessment in deriving water quality guidelines (WQG). Although currently under revision, it is widely acknowledged as being the most sophisticated set of water quality guidelines in the world (Warne, 2001).

- It uses the species sensitivity distribution (SSD) method in preference to assessment factors (AF) (Warne, 2001). The SSD is advantageous to AF because it selects the statistical distribution that best fits the toxicity data from a family of distributions rather than trying to apply a single distribution.

- It acknowledges that the national water quality guidelines may not be applied blindly to all freshwater aquatic ecosystems and encourages site-specific assessments in addition to providing a series of risk-based decision trees to support and guide site specific investigations (Warne, 2001).

- It specifically addresses the toxicity of mixtures, which are not included in any other known framework for the development of guidelines (Warne, 2001).

In this study, the Australian and New Zealand framework (ANZECC and ARMCANZ, 2000), together with those of South Africa (Warne *et al*., 2004), United States (USEPA, 2005) and Canada (CCME, 2007) were used as reference documents to develop a water quality derivation protocol for Roundup®. This was used to derive a proposed guideline that could be taken up into the official South African water quality guidelines (SAWQGs) for water resource management.

Water quality guidelines are perceived as environmentally safe levels (ESLs) that would provide adequate protection to aquatic life (Warne, 2001). The ANZECC and ARMCANZ (2000) and Warne *et al*. (2004) frameworks referred to the ESLs as trigger values (TVs), which may be derived using an SSD in preference to AF approach. Therefore, TVs and SSDs are briefly discussed as applied in this study to derive WQGs for Roundup®.

4.1.2 Trigger values

Trigger values (TVs) are protective concentration values used to manage chemical substances in the environment for the protection of aquatic life. Trigger values indicate risk of impact if exceeded and normally result in (i.e. "trigger") some form of management action, which may include further investigation, remediation and/or implementation of strategies (Warne *et al*., 2004).

There are three grades of hierarchical TVs, namely high reliability (HR), moderate reliability (MR) and low reliability (LR). The LR TV is further divided into interim (LR (interim) TV) and environmental concern level (LR (ECL) TV), depending on the quality of data. Warne *et al*. (2004) suggested that derivation of HR TV should always be the target if there is adequate and suitable toxicity data. However, if data to derive HR TV are inadequate, then the hierarchy is descended until the available data meet the minimum requirements for a particular grade of TV (Warne *et al*., 2004).

A TV may be determined using either the assessment factor (AF) approach, which involves dividing the most sensitive toxicity value by an assessment factor (usually 10, 100 or 1000) (Warne *et al*., 2001), or the species sensitivity distribution (SSD) approach, which involves fitting a statistical distribution to toxicity data of a number of species in order to estimate the concentration that should protect any chosen percentage of species (Warne *et al*., 2004). The SSD is the preferred approach, but AF is used where data are constrained (ANZECC and ARMCANZ, 2000; USEPA, 2005; Warne *et al*., 2004; CCME, 2007). The data requirements for using the SSD approach in determining trigger values are presented in Tables 4.1 and 4.2. In the current study, eight South African aquatic organisms belonging to five different taxonomic groups were used to derive a high reliability trigger value for protecting aquatic life from Roundup® exposure.

Table 4.1: Minimum data required by the statistical distribution approach for the three grades of trigger values (after Warne *et al*., 2004)

Level of trigger value	Minimum data requirement
HR	Requires chronic NOEC toxicity data for at least five species that belong to at least four different taxonomic groups
MR	Requires acute toxicity data (i.e. LC50 or EC50) for at least five species that belong to at least four taxonomic groups
LR (interim) for non-polar chemicals only	Requires nineteen estimates of chronic toxicity derived by QSARs

Table 4.2: Types of taxonomically different organisms and major subdivisions to which they belong (after Warne *et al*., 2004)

Major subdivisions of organisms	Types of organisms that are considered as being taxonomically different
Fish	Fish
Invertebrates	Crustaceans, insects, molluscs, annelids, echinoderms, rotifers, hydra
Plants	Green algae, blue algae, red algae, macrophytes
Others	Blue-green algae (cyanobacteria), amphibians, protozoans, coral, fungi and others

4.1.3 Species sensitivity distribution (SSD)

A single-species battery assay is used in aquatic ecotoxicology as a predictive effect assessment tool to assess the effect mechanisms (mode of actions) of toxicants. However, extrapolation of results obtained from single-species test batteries to ecosystems are often over-simplified because important aspects of community ecology are not considered (Schmitt-Jansena *et al.*, 2008). Such over-simplification of the ecosystem may include the following:

- Ecosystem assemblages of species usually cover a wide range of different species sensitivities, but only one or a few aquatic test species are used in ecotoxicological or regulatory testing.

- Different species in a community interact through competition for food and space, but this is not the case in single-species bioassays.

- Important functional groups may be under-represented or completely left out in a single-species test battery assay since such an assessment does not consider the community structure of the ecosystem.

- ❖ Ecosystem functions are not considered in a single-species test batteries assay since assessment strategies are restricted to the species level (Schmitt-Jansena *et al.*, 2008).

Therefore, to achieve a better ecological risk assessment of toxicants, with higher ecological relevance, it is worthwhile integrating theoretical ecology in aquatic ecotoxicology (Schmitt-Jansena, 2008). This can be done by integrating ecological, chemical, analytical and ecotoxicological tools. The role of basic ecology is to derive the mechanistic understanding of ecosystem structure, function and regulation, while aquatic ecotoxicology is used to support regulatory decisions by providing scientifically sound methods to derive environmental quality criteria or to identify and assess relevant contamination in aquatic ecosystems. Therefore, a strong base of theoretical ecology is necessary to develop ecotoxicological measurement tools that are scientifically sound. Such tools will enhance ecological relevance in assessment strategies and reduce uncertainty in the extrapolation process (Schmitt-Jansena *et al.*, 2008). Traditionally, dose-response data from single-species toxicity tests were used in ecological risk assessment processes to protect populations, communities, and ecosystems (Newman *et al*., 2000). This seems to create incongruity, as extrapolations of results to these entities from single-species are often oversimplified (Schmitt-Jansena *et al.*, 2008). Kooijman (1987) proposed an evaluation of species-sensitivity distribution (SSD) to resolve the difference between individual-based data and the complex biological entities addressed in ecological risk assessment. The SSD method is now considered a useful tool in aquatic ecotoxicology for predictive effect assessments of toxicants.

The SSD is based on the principle that living things have inherent biological differences. These differences create diversities in behaviour, geographical distribution, life history, morphology, physiology and taxonomy. This means different species will respond differently to a given toxicant concentration because they have different sensitivities. This variation in species sensitivities can be described by a statistical distribution or empirical function, which gives rise to SSDs (Posthuma *et al*., 2002).

The main hypothesis of the SSD approach states that the sensitivities of a set of species can be described by a "distribution", often a parametric distribution such as normal, triangular or logistic; or nonparametric distribution, such as resampling. Available data from ecotoxicological studies are used as a sample from this "distribution" to estimate the SSD parameters. Specific percentile from the empirical distribution is then used to estimate a safe concentration expected to protect most species of interest. This safe concentration is then used to set an environmental water quality (EWQ) guideline (Posthuma *et al*., 2002). Based on the above treatise, SSD may be defined as the representation of species-sensitivity variation to a toxicant by a statistical or empirical distribution function of responses for a sample of species.

Application of SSD in ecological risk assessment involves combining data from single-species toxicity tests to predict concentrations affecting only a certain percentage of species in a community (Newman *et al*., 2000). For instance, the median lethal concentration (LC50), no observed-effect concentration (NOEC) or lowest observed-effect concentration (LOEC) values from single-species data for many species are separately fitted to a distribution such as the lognormal or log-logistic. A hazardous concentration (HCp), at which a certain percentage (p) of all species is assumed to be affected, is then identified from the resulting distribution of species sensitivities. The most conservative form of this approach uses the lower 95% tolerance limit of the estimated percentage to ensure that the specified level of protection is achieved (Newman *et al*., 2000; Jesenská *et al*., 2011). The SSD or extrapolation methods are not only being incorporated into ecological risk assessments, but also into recommendations for pesticide registration and regulation (Newman *et al*., 2000). Application of the SSD method demands assumptions in order to ensure derivation of reliable water quality criteria. Chapman *et al*. (1996) and Newman *et al*. (2000) stated these assumptions include the following:

- The LC50, EC50 and NOEC derived from single-species toxicity tests have very significant deficiencies as measures of effect on field populations and communities. Thus, the SSD, which is a secondary metric based on such compromised primary metrics, possesses the same deficiencies.

- The extrapolation of the single-species toxicity test measures of effect to field populations and communities may suspiciously result in species loss, which may be acceptable or not. Proponents of the SSD methods are of the view that the loss is acceptable and has no intrinsic value since communities harbour enough redundancy to allow for some loss. Those who oppose the redundant species hypothesis combine the rivet popper hypothesis with the argument that it is better, when faced with uncertainty in ecological risk assessment, to adopt a conservative stance. The rivet popper hypothesis states that "community integrity is reduced by each loss of a species" and contends that ecosystem productivity, stability, sustainability, and nutrient retention decrease as species diversity decreases.

- Species-sensitivity distribution methods could downplay the importance of maintaining dominant and keystone species, as well as influencing species interactions if they are not performed with careful thought.

- Single species toxicity tests are often biased toward mortality data and the use of standard test species that are amenable to laboratory culture and manipulation, even though sub-lethal effects and non-standard test species, respectively, may be at least as important in determining local population extinction. Nevertheless, these deficiencies are shared by other risk assessment applications of ecotoxicological data, including SSD.

- Many data sets plotted alongside values predicted from the assumption of a specific distribution may not be valid, as they show clear deviations from the assumed lognormal distribution. The basic assumption of a uni-modal distribution is even more uncertain since SSD uses pooled data from different taxonomic groups. In order to reduce the size of this problem, specific taxonomic groups were used in the SSD procedure in the present study, but the ambiguity associated with selecting the lognormal distribution still remains.

- ❖ Assessment of questions about adequate sample size and representativeness of the community is difficult in most applications. Most discussions in this regard centre on pragmatic issues of producing an acceptable number of observations for regulatory agencies. Most studies fail either to report confidence limits along with estimates of HCp, to calculate minimal sample sizes, or to discuss the extent of the data set in relation to the community at risk.

Two of the above issues, namely ambiguity in selecting a specific distribution, and estimation of the adequate number of species needed to estimate HCp precisely, are partly answered by bootstrap estimation. Firstly, bootstrap estimation decreases the intricacy of selecting an appropriate distribution since calculations do not require an explicit distribution. The only prerequisite is a random sample of species sensitivities from a set of possible species sensitivities. Secondly, bootstrap methods can be used to assess the number of approximate species needed to minimise variation around the HCp estimate (Newman *et al*., 2000). In this study, the SSD approach was used to derive water quality guidelines (WQGs) for glyphosate. South African WQGs were derived using data from single species toxicity tests conducted with South African aquatic organisms. This was compared with other derived WQGs based on data extracted from the USEPA ECOTOX database, version 4.

Measures for estimating the SSD

The measure normally used to generate SSDs is the no observed effect concentration (NOEC). However, this practice has received wide spread criticism from authors including Chapman *et al*. (1996), Fox (2008), Newman (2008), Warne and van Dam (2008), Landis and Chapman (2011), Jager (2012), and van Dam *et al*. (2012). The authors opined that the NOEC is associated with a number of deficiencies. These deficiencies include the fact that (a) it is one of the test concentrations (b) the procedure by which it is determined "rewards bad experiments" (c) it cannot be determined in some cases (d) its size is a function of the choice of statistical test and level of significance, and therefore (e) definite conclusions cannot be made based on it. In order to eliminate the problems associated with using NOEC data to generate SSDs, other measures such as EC10 or IC10 values are often used as alternatives (Fox, 2008). However, Fox (2008) contended that the generated SSDs (based on EC10 or IC10 values) are problematic and difficult to interpret.

Fox (2008) explained that the problem is related to an SSD based on '*effect*', while the objective is to generate SSDs based on '*no effect*' values. Based on the above discourse, many authors, including van der Hoeven (1997), Kooijman (2006), OECD (2006) and Fox (2008) have proposed deriving SSDs using the NEC. In this study, both NEC and NOEC data were used to generate SSDs for long-term protection of aquatic life. This approach was taken so that the SSDs resulting from NOEC and NEC could be compared and contrasted. However, NEC values were used to derive long-term proposed South African water quality guidelines (WQG), while short-term WQGs were derived using LE(C)50 values.

4.2 Materials and methods

4.2.1 Laboratory single-species toxicity tests

Single-species, short term, lethal, Roundup® exposure tests were conducted with different aquatic organisms belonging to five different South African taxonomic groups. These include insects (*Baetis harrisoni*, *Tanytarsus flumineus*), molluscs (*Burnupia stenochorias*), crustaceans (*Caridina nilotica*, *Daphnia pulex*), fish (*Oreochromis mossambicus*) and green algae (*Chlorella protothecoides*, *Chlorella sorokiniana*). These organisms were collected from either the Swartkops or Balfour Rivers in the Eastern Cape Province, South Africa, or were obtained from laboratory cultures maintained in laboratories of Unilever Centre for Environmental Water Quality (UCEWQ) (Institute for Water Research, Rhodes University, Eastern Cape Province, South Africa). The species, common names, taxonomic group, life stage or age, and collection source were recorded (Table 4.3). All field-collected species were acclimated to laboratory conditions for a minimum of 24 hours, during which time food was provided.

Table 4.3: Characteristics of South African species in laboratory toxicity tests with different glyphosate concentrations

Scientific name	Common name	Taxonomic grouping	Source	Life stage or age	Concentrations used (mg/L)
Baetis harrisoni	Mayfly	Insect	Swartkops River	Larvae	0.5, 1, 2, 4, 8, 10
Burnupia stenochorias	Limpet	Mollusc	Balfour River	Adult	0.5, 1, 2, 4, 8, 10
Caridina nilotica	Shrimp	Crustacean	Laboratory culture	Neonate	1.3, 2.1, 3.3, 5.1, 8
Chlorella protothecoides	Green algae	Plant	Laboratory culture	3-4 days old exponential growth phase	0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25
Chlorella sorokiniana	Green algae	Plant	Laboratory culture	3-4 days old exponential growth phase	0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25
Daphnia pulex	Water flea	Crustacean	Laboratory culture	1-day old	0.02, 0.06, 0.19, 0.65, 2.16, 7.20
Oreochromis mossambicus	Mozambique tilapia	Fish	Hatchery	< 2 weeks fry	1.2, 1.5, 1.9, 2.4, 3.0
Tanytarsus flumineus	Midge	Insect	Swartkops River	Larvae	0.5, 1, 2, 4, 8, 10

Aquatic animal exposure tests

Static non-renewal experimental methods were employed for short-term lethal tests (≤ 96 h), and static renewal for long-term sub-lethal tests (> 96 h ≤ 21 d). Based on the range-finding test, the tested concentrations listed in Table 4.3 were selected and used for both lethal and sub-lethal tests. All exposure tests involved water-only controls. For all animal exposure tests (except *D. pulex*), each concentration contained 10 organisms and was replicated three times. For *D. pulex* toxicity tests, each concentration contained five organisms and was replicated four times. Dead (immobile, decolourised, degenerated) organisms were recorded twice daily and removed from experimental vessels. Animals were not fed during short-term exposure periods, but were fed with TetraMin tropical flakes to satiation twice a day during the long-term experimental period. Experiments were conducted under a 12-hour light: 12-hour dark artificial light regime using Biolux fluorescent tubes in a temperature-controlled room of temperature 25.00º C (± 0.05) in 600-mL glass beakers for the macro-invertebrates and 20-L glass tanks for fish exposure tests.

The experimental chambers were provided with aeration. Water quality parameters, including hydrogen ion concentration (pH), dissolved oxygen (DO), electrical conductivity (EC) and temperature were measured in all concentrations at the beginning and just after change of solution.

Mortality (incorporating immobility) was evaluated in different ways for the different species Generally, animals were deemed dead when they did not respond to any form of repeated tactile stimulation with the aid of a plastic pipette after about 10 seconds under a stereo-microscope (van Wijngaarden *et al*., 2010). The mortality score endpoints were used to calculate the lethal concentrations at which specific percentage of animals died (LCx). Mortality of organisms in controls was less than 10 % for all exposure tests.

Aquatic plant (algae) exposure tests

Two indigenous green algae species *Chlorella protothecoides* and *Chlorella sorokiniana* were obtained from laboratory cultures (Gola, pers. comm., 2012). The test solutions of concentrations 0.0 (control), 0.4, 0.8, 1.6, 3.1, 6.3, 12.5 and 25 mg/L were prepared and dispensed in a predetermined pattern into 24-well microplates. Each concentration and control was replicated six times. Each well received 1.8 mL of test solution, 0.1 mL of algal inoculum and 0.1 mL BG-11 medium as nutrient solution. The size of the inoculum was 300 000 cells/mL. Preparations of algal inoculum and BG-11 medium are presented in Appendix J. The 24-well microplates were incubated at a constant temperature with continuous light for seven days, during which optical density (OD) was read with a microplate-reader every 24 hours. The OD represented the concentration of algae in mg/L (i.e. number of cells per mL). The number of algal cells in the test concentrations was then compared with the number in the control solutions as relative growth rates. Algal exposure test endpoints were calculated from the relative growth rates and percent inhibition. The growth rate was calculated according to the equation $U = (\mathrm{Ln}N_t - \mathrm{Ln}N_0)/(t - t_0)$, where U is the growth rate, N_t the cell number at t time, N_0 the cell number at 0 time, t the sample time for counting cell number, and t_0 is the origin time of the treatment. The effects of Roundup® on the growth rate of algae were calculated as percent inhibition (PI) as follows: $PI = ((U_{ck} - U_{tox}) / U_{ck})\ 100$, where U_{ck} is growth rate in control samples and U_{tox} is growth rate in exposed samples (Li *et al*., 2005).

Statistical analyses

The LCx values for animal exposure tests were calculated using Probit analysis, while ECx values for algae and their confidence intervals were calculated using linear regression analysis of transformed herbicide concentration as natural logarithm data against percentage inhibition (Ma *et al*., 2001; Schroer *et al*., 2004). Thus, estimation of ECx values followed the regression model $ECx = a + bc$, where ECx is the percent inhibition of growth. Where applicable, effect scores from replicates per treatment were combined in one regression analysis. The NOEC (no observed effect concentration) was estimated as the tested concentration which was not statistically different from the control using Statistica version 9 (StatSoft Inc., 2009). NEC (no effect concentration) was estimated using DEBTOX, version 2.0 (Department of Theoretical Biology, Vrije Universiteit, Amsterdam, The Netherlands) computer program. For animal exposure tests, results of the survival data for each test concentration at each time point was entered, and the hazard model was selected. For algal exposure tests, results of the population density in each test concentration at each time point was entered, and the algal growth inhibition model was selected. The statistical analysis performed by the software is based on the maximum log-likelihood theory and provides a 95% confidence interval for estimates of all the parameters of DEBtox.

4.2.2 Extraction of data from USEPA ECOTOX database, version 4

Acute and chronic data were extracted from USEPA ECOTOX database, "cleaned", assessed for quality, and then used to construct international SSDs as described below. Acute data were defined as having an experimental duration between 24 and 96 hours inclusive, while chronic data were obtained from NOEC values for experiments longer than 96 hours (Warne, 2001). In constructing the SSDs, the geometric mean of all values for the same species was used if more than one datum existed for that species (Warne, 2001).

Primary screening (cleaning)

Primary screening involved the "cleaning" of the data by removing data with an inadequate number of treatments, replication and/or controls. Data for which the medium of exposure was seawater were also excluded. In addition, all data in which there were no reports on endpoint, effect measurement, and exposure duration were eliminated. All field-generated data were excluded, and only laboratory generated data used for SSD construction.

Secondary screening (quality rating score)

Both cleaned acute and chronic data were subjected to a secondary screening using a data quality rating score system as described by Warne (2001). This system subjects the data to a series of specific questions and awards marks to each question (Table 4.4). The quality of toxicity data were classified as either complete, moderate or incomplete if the total score was between 85-100, 51-84 or 0-50, respectively (Warne, 2001).

Table 4.4: Questions and corresponding marks used to assess the quality score and quality class of the toxicity data extracted from ECOTOX (Warne *et al*., 2004)

Question	**Possible marks***
Was the duration of the exposure stated?	20 or 0
Were there appropriate controls?	5 or 0
Were the characteristics of the test organism stated?	5 or 0
Were the chemical concentrations measured?	5 or 0
Was the type of exposure (e.g. static, flow through) stated?	5 or 0
Was the test location stated?	4 or 0
Was the grade of purity of the test chemical stated?	4 or 0
Was the type of test media used stated?	4 or 0
Was the hardness measured and stated?	2 or 0
Was the alkalinity measured and stated?	2 or 0
Was the dissolved oxygen content of the test water measured at some stage during or after the test?	2 or 0
Was the temperature measured during the test?	2 or 0
Was the pH of the test water measured at some time during the test?	2 or 0
Was the biological endpoint clearly defined?	20 or 0
Was there a concentration-response relationship either observed or stated?	5 or 0
Was the biological effect quantified i.e. 50% effect, 25% effect?	5 or 0
Was the statistical level of significance for any statistical tests stated (for NOEC data)? Was a valid model used to derive LC50/EC50 values (for LC/EC data)?	4 or 0
Was the stated significance level 0.05 or less (for NOEC data)? Was there an estimate of the variability of the LC50 or EC50 (for LC/EC data)?	4 or 0

* There are only two marks that can be awarded in answering a question: full mark or zero.

4.2.3 Generation of species sensitivity distributions

Species sensitivity distributions (SSDs) were constructed using the *Species Sensitivity Distribution Generator*, a tool used to create custom SSDs (Posthuma *et al*., 2002; USEPA, 2005). The SSD generator fits a commonly applied distribution, the log-probit (i.e., linearized log-normal) to data for concentrations at which different species exhibit a standard response to a stressor (Posthuma *et al*., 2002; USEPA, 2005). Using the SSD Generator to create an SSD involves three fundamental steps:

- Select data for the exposure intensities at which different species exhibit a standard response to the stressor.

- Calculate proportions by first ranking these data from lowest to highest, then converting ranks to proportions: Proportion = (Rank-0.5)/Number of Species.

- Fit a statistical or empirical distribution to Proportion (y-axis) against Stressor Intensity (x-axis) (Posthuma *et al*., 2002; USEPA, 2005).

Step-by-step calculations behind the SSD using the SSD Generator are presented in Appendix K. The SSD Generator was used to construct SSD curves based on the laboratory single-species toxicity tests data and screened data retrieved from ECOTOX database. The 5% hazardous concentration (HC5) of Roundup® (glyphosate) was calculated from the SSDs and used to derive water quality guidelines trigger values (TVs).

4.3 Results

4.3.1 Laboratory single-species toxicity tests

The 48 and 96-hour LE(C)50 values for all tested organisms are presented in Table 4.5. Water flea, *Daphnia pulex,* was the most sensitive among all the animal species tested with the lowest LCx values, while the midge, *Tanytarsus flumineus,* was found to be the most insensitive with the highest LCx values. The 48-hour LC50 of *D. pulex* was about nine times more sensitive than *T. flumineus*.

However, the 96-hour LC50 of *D. pulex* was about 19 times more sensitive than *T. flumineus*. For the algae species evaluated, *Chlorella sorokiniana* 48-hour EC50 was found to be 10 times more sensitive than *C. protothecoides*, while *C. sorokiniana* 96-hour EC50 was 900 times more sensitive than *C. protothecoides*.

The long-term NOEC and NEC results for all organisms tested are presented in Table 4.6. The NEC values were less than their corresponding NOEC values for all animal species tested, except *Daphnia pulex*. In contrast, the NEC values were greater than their corresponding NOEC values for both plant species tested.

Table 4.5: Mean 48-96 h LE(C)50 values and 95% confidence limits (in bracket) for all tested South African aquatic organisms

Scientific name	**Common name**	**Taxonomic grouping**	**48 h LE(C)50 (mg/L)**	**96 h LE(C)50 (mg/L)[b]**
Baetis harrisoni	Mayfly	Insect	8.58 (6.02-15.24)	2.71 (2.14-3.40)
Burnupia stenochorias	Limpet	Mollusc	6.50	4.30 (2.12-7.90)
Caridina nilotica[a]	Shrimp	Crustacean	5.23 (4.41-6.55)	2.842 (2.52-3.19)
Chlorella protothecoides	Green algae	Plant	1.03	0.33
Chlorella sorokiniana	Green algae	Plant	0.27	0.02
Daphnia pulex	Water flea	Crustacean	1.66	0.66 (0.47-0.91)
Oreochromis mossambicus	Mozambique tilapia	Fish	3.72 (3.08-6.36)	3.25 (2.79-4.52)
Tanytarsus flumineus	Midge	Insect	15.55	12.24 (9.45-22.36)

[a] = Toxicity data for *C. nilotica* neonate were used because it was the most sensitive life stage.

[b] = 72 hours EC50s of algae were used instead of 96 hours EC50s.

Table 4.6: NOEC, NEC and EC values calculated for long-term lethal Roundup®-exposed South African aquatic organisms

Scientific name	Common name	Taxonomic grouping	Exposure duration (days)	Long-term NOEC (mg/L)	Long-term NEC (mg/L)[b]	Long-term EC10 (mg/L)
Baetis harrisoni	Mayfly	Insect	10	0.5	0.23 (0.0-0.4)	0.54
Burnupia stenochorias	Limpet	Mollusc	14	0.5	0.06 (0.0-0.3)	0.15
Caridina nilotica[a]	Shrimp	Crustacean	21	2.2	1.13 (0.4-1.6)	2.00
*Chlorella prototheco*ides	Green algae	Plant	4	1.56	0.06 (0.0-2.5)	
Chlorella sorokiniana	Green algae	Plant	4	0.4	0.01 (0.0-3.5)	
Daphnia pulex	Water flea	Crustacean	21	0.056	0.11 (0.0-0.2)	0.14
Oreochromis mossambicus	Mozambique tilapia	Fish	14	1.5	1.40 (1.2-1.5)	1.35
Tanytarsus flumineus	Midge	Insect	10	1.0	0.37 (0.0-0.7)	0.96

[a] = Toxicity data for *C. nilotica* neonate were used because it was the most sensitive life stage.

[b] = 96 hours EC50s of algae were used

4.3.2 Extraction of data from USEPA ECOTOX database, version 4

A total of 1740 data points with glyphosate as the active ingredient were retrieved from the USEPA ECOTOX database, version 4. Primary screening trimmed the retrieved data to 625 (36%) LC50 data points (Table 4.7) and 72 (4%) NOEC data points (Table 4.8). Secondary screenings of the LC50 and NOEC data points scored a complete data quality rating of 90 and 95%, respectively. These data were used to construct the SSDs as suggested by Warne (2001) as follows:

- If there was a single toxicity value for a species, that was adopted as the sensitive value for that species.

- If there were multiple toxicity values for a single endpoint (e.g. lethality) for a species then the geometric mean was determined and used in construction of SSDs.

- If there were multiple toxicity values for multiple endpoints (e.g. lethality, immobilization, reproduction) for a species then the geometric mean for each endpoint was calculated and the lowest geometric mean used as the sensitivity value for that species.

Table 4.7: Summary of acute LC50 data for Glyphosate from ECOTOX database used to derive short term WQG for glyphosate

Scientific name	Common name	Taxonomic grouping	Exposure duration (hour)	LC50 (geometric mean in mg/L)	Number of data points
Anopheles quadrimaculatus	Malaria mosquito	Insect	24	673.43	1
Brachionus calyciflorus	Rotifer	Invertebrate	72	28.00	1
Bufo americanus	American toad	Amphibian	96	69.34	8
Carassius auratus	Goldfish	Fish	48-96	213.83	3
Ceriodaphnia dubia	Water flea	Crustacean	24-48	38.03	12
Chironomus plumosus	Midge	Insect	24-48	258.61	3
Chironomus riparius	Midge	Insect	24-48	1216.00	1
Cnesterodon decemmaculatus	Ten-spotted livebearer	Fish	96	100.00	1
Crinia insignifera	Frog	Amphibian	48-96	39.54	6
Ctenopharyngodon idella	Grass carp	Fish	24-96	60.00	3
Cyprinodon variegatus	Sheepshead minnow	Fish	96	240.00	1
Cyprinus carpio	Common carp	Fish	12-72	5.22	9
Daphnia magna	Water flea	Crustacean	24-96	16.85	9
Gambusia affinis	Mosquito fish	Fish	48	6.20	1
Gambusia yucatana	Yucatan gambusia	Fish	96	17.79	1
Gammarus pseudolimnaeus	Scud (amphipod)	Crustacean	24-96	16.07	7
Heleioporus eyrei	Moaning frog	Amphibian	48	15.60	3
Hyalella azteca	Scud (amphipod)	Crustacean	48-96	73.49	4
Ictalurus punctatus	Channel catfish	Fish	24-96	4.99	22
Lepomis macrochirus	Bluegill	Fish	24-96	18.59	67
Limnodynastes dorsalis	Western Banjo frog	Amphibian	48	0.15	3

Litoria moorei	Western green frog	Amphibian	24-96	13.42	6
Morone saxatilis	Striped bass fish	Fish	24-96	23.50	5
Nephelopsis obscura	Leech	Worm	96	80.67	3
Nitocra spinipes	Harpacticoid copepod	Crustacean	96	22.00	1
Oncorhynchus gorbuscha	Pink salmon	Fish	24-48	48.11	56
Oncorhynchus keta	Chum salmon	Fish	24-96	74.11	44
Oncorhynchus kisutch	Coho salmon	Fish	24-96	46.80	60
Oncorhynchus mykiss	Rainbow trout	Fish	24-96	18.79	147
Oncorhynchus nerka	Sockeye salmon	Fish	24-96	146.97	3
Oncorhynchus tshawytscha	Chinook salmon	Fish	24-96	19.41	59
Orconectes nais	Crayfish	Crustacean	96	7.00	1
Pimephales promelas	Fathead minnow	Fish	24-96	10.81	17
Procambarus clarkii	Swamp crayfish	Crustacean	96	47.31	1
Rana clamitans	Green frog	Amphibian	24-96	11.82	13
Rana pipiens	Leopard frog	Amphibian	96	4.69	8
Rana sylvatica	Wood frog	Amphibian	96	1.84	4
Salmo trutta	Brown trout	Fish	96	3.35	2
Scinax nasicus	Snouted tree frog	Amphibian	24-96	45.65	7
Spirostomum ambiguum	Protozoa	Invertebrate	24	40.70	1
Tetrahymena pyriformis	Ciliate	Invertebrate	24	1750.00	1
Tilapia nilotica	Nile tilapia	Fish	24-96	46.35	3
Utterbackia imbecillis	Paper pondshell	Mollusc	24	18.30	1
Xenopus laevis	African clawed frog	Amphibian	96	0.65	18

Table 4.8: Summary of NOEC data for Glyphosate used to derive SSD_{NOEC} extracted from ECOTOX database

Scientific Name	Common name	Taxonomic grouping	Exposure duration (days)	NOEC (geometric mean in mg/L)	Number of data points
Bufo americanus	American toad	Amphibian	16	1.00	4
Euglena gracilis	Flagellate euglenoid	Protist	7	0.10	4
Hyla versicolor	Gray tree frog	Amphibian	16	1.41	4
Lampsilis siliquoidea	Lamp-mussel	Mollusc	21	0.07	4
Lemna gibba	Inflated duckweed	Plant	7	0.01	2
Myriophyllum sibiricum	Water milfoil	Plant	14	2.99	5
Myriophyllum spicatum	Eurasian water milfoil	Plant	5	1.00	2
Neochetina bruchi	Water hyacinth weevil	Insect	7	1.48	2
Oncorhynchus mykiss	Rainbow trout	Fish	20	0.07	7
Pimephales promelas	Fathead minnow	Fish	6	0.46	3
Pithophora oedogonia	Green algae	Algae	7	15.60	1
Planorbella trivolvis	Marsh rams-horn snail	Mollusc	28	4.93	4
Pomacea lineata	Golden apple snail	Mollusc	5	0.42	3
Pontederia cordata	Pickerelweed	Plant	56	0.00	3
Rana catesbeiana	Bullfrog	Amphibian	16	1.00	4
Rana clamitans	Green frog	Amphibian	59	0.02	11
Rana pipiens	Leopard frog	Amphibian	15	0.31	5
Simocephalus vetulus	Water flea	Crustacean	8	0.01	1
Tilapia nilotica	Nile tilapia	Fish	32	0.02	3

4.3.3 Generation of species sensitivity distribution (SSD) curves

The results from this study (Tables 4.5 and 4.6) and ECOTOX database (Tables 4.7 and 4.8) were used to generate species sensitivity distribution (SSD) curves using The SSD Generator. SSD curves were generated for South African freshwater aquatic organisms based on 48-96 hour short-term LC50 (Figure 4.1), long-term NOEC (Figure 4.2) and long-term NEC (Figure 4.3) values for Roundup®. Similarly, SSD curves were generated for freshwater aquatic organisms from ECOTOX database based on 24-96 hour short-term LC50 (Figure 4.4) and long-term NOEC (Figure 4.5) values for glyphosate. The LC50 values from this study and those estimated from the ECOTOX database were pooled together to generate a single SSD curve in order to compare this with the South African-based and the ECOTOX-based SSDs (Figure 4.6). The model parameters including the goodness of fit in terms of R^2, mean squared error (MSE) and corrected sum of squares (CSSQ) are presented in Appendix L.

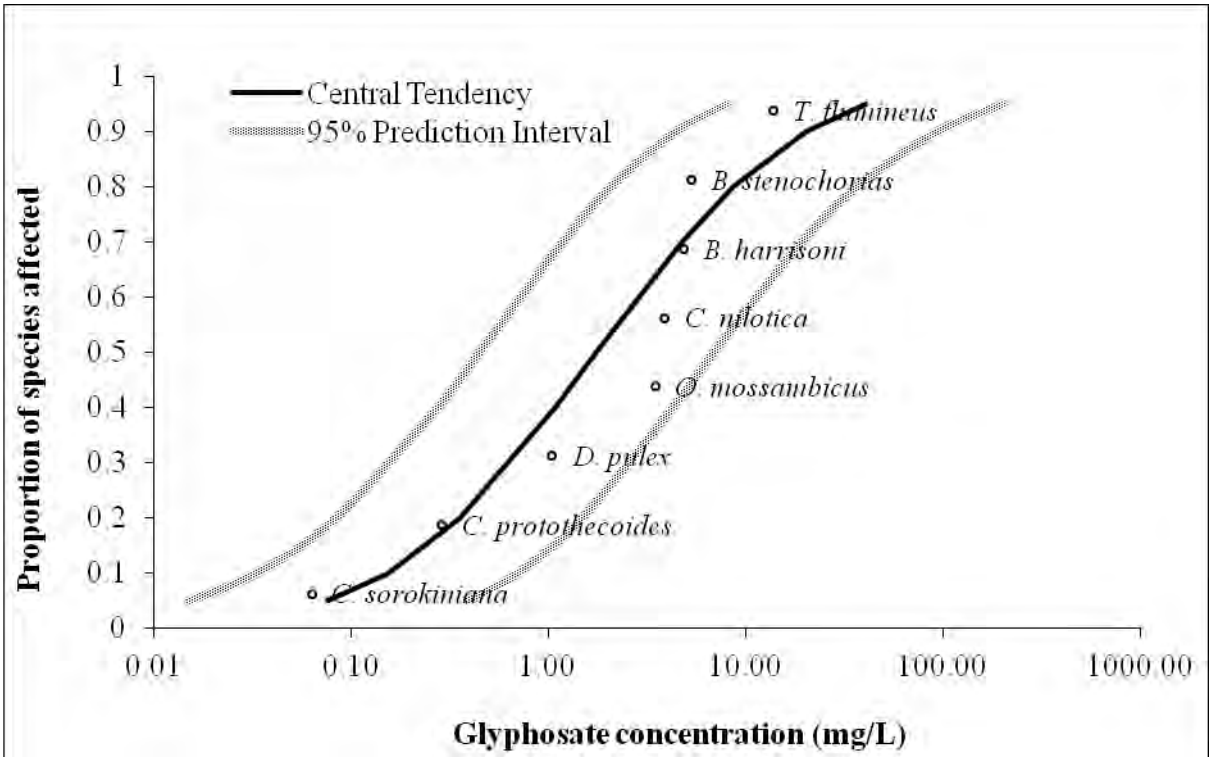


Figure 4.1: Species sensitivity distribution (SSD) curve of South African freshwater aquatic organisms based on short-term 48-96 hour LC50 values for Roundup®

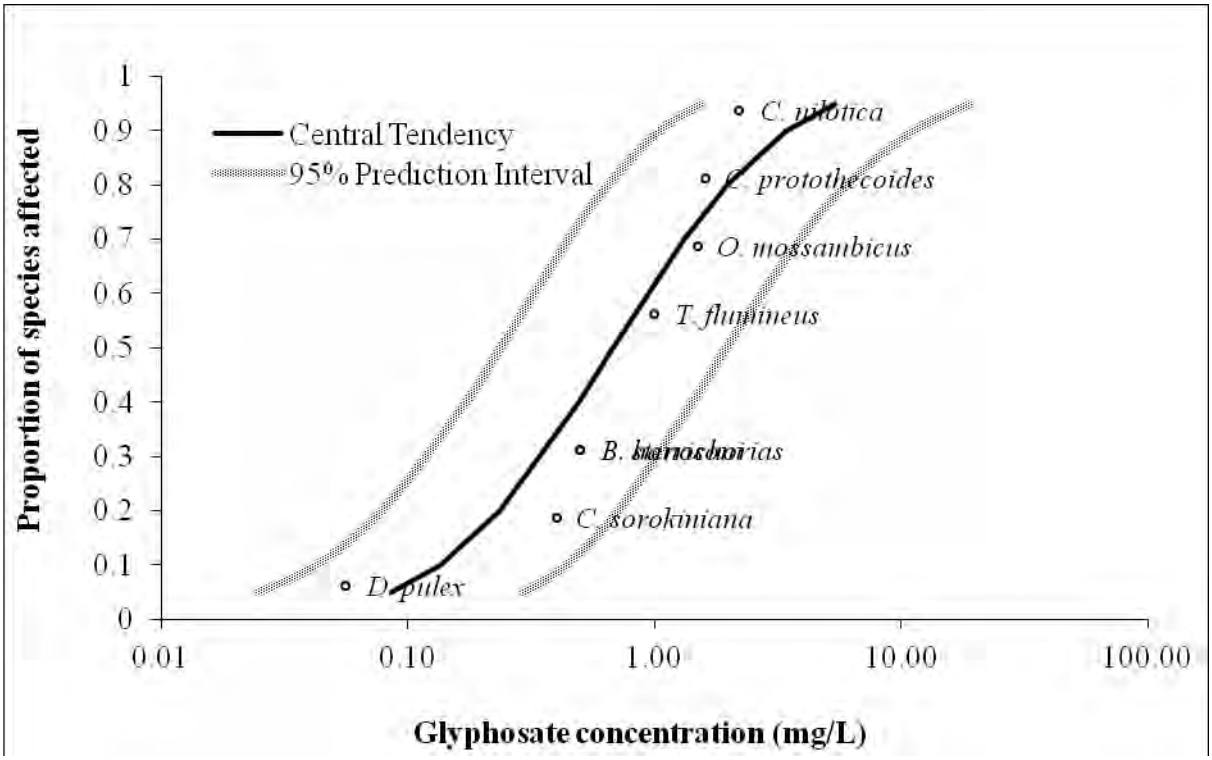


Figure 4.2: Species sensitivity distribution (SSD) curve of South African freshwater aquatic organisms based on long-term NOEC values for Roundup® (*B. harrisoni and B. stenochorias* overlap because their sensitivities lie in the same range)

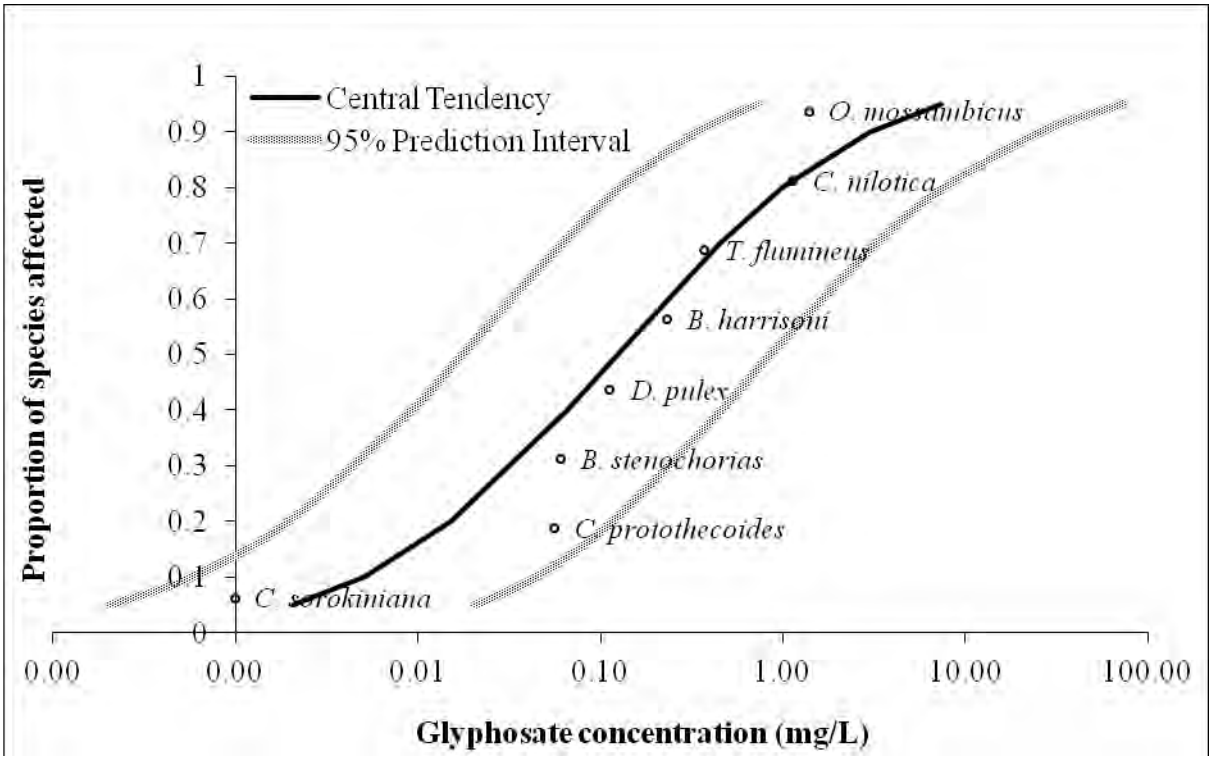


Figure 4.3: Species sensitivity distribution (SSD) curve of South African freshwater aquatic organisms based on long-term NEC values for Roundup®

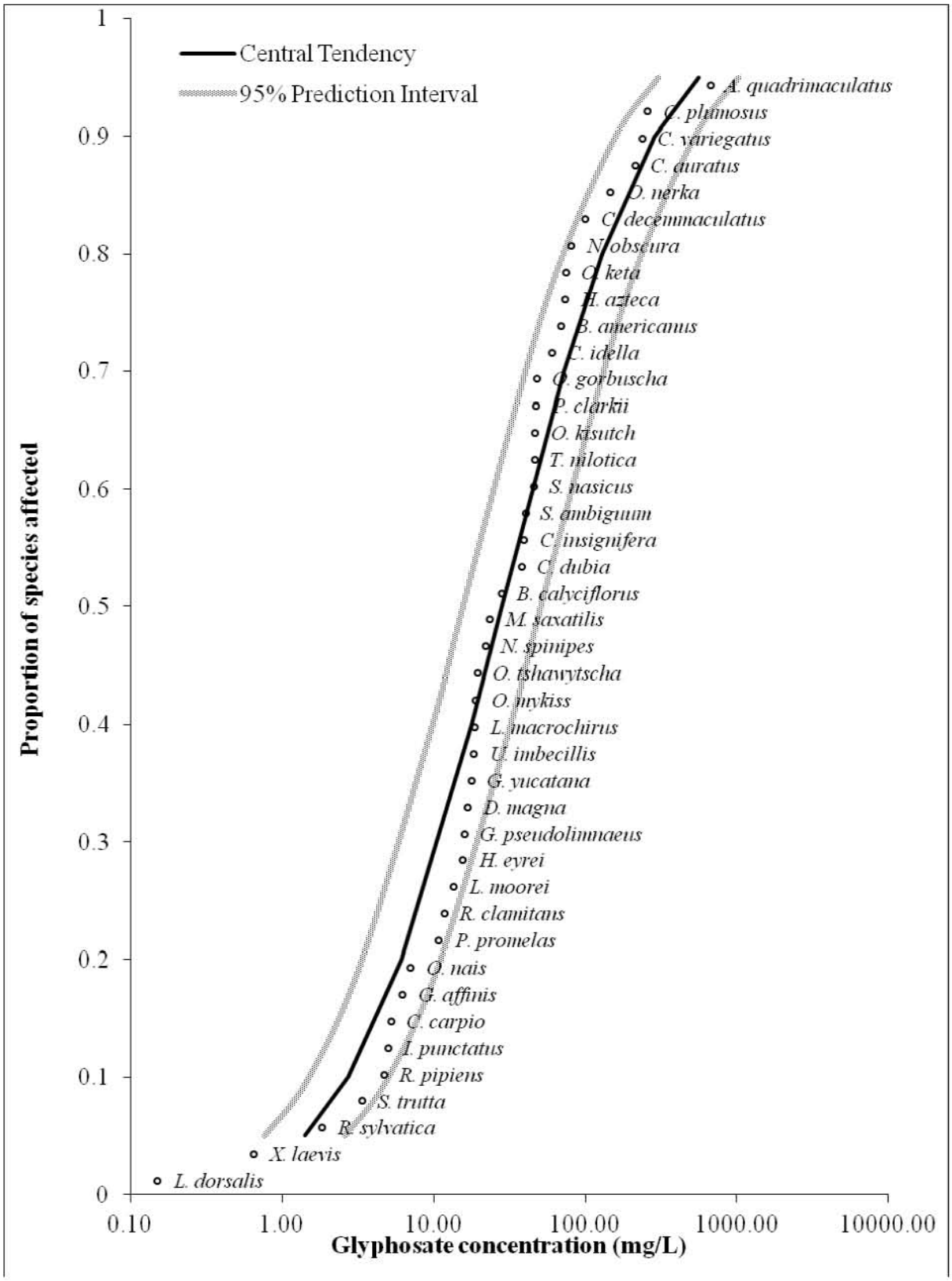


Figure 4.4: Species sensitivity distribution (SSD) curve of freshwater aquatic organisms from ECOTOX database based on short-term 48-96 hour acute toxicity LC50 values for glyphosate

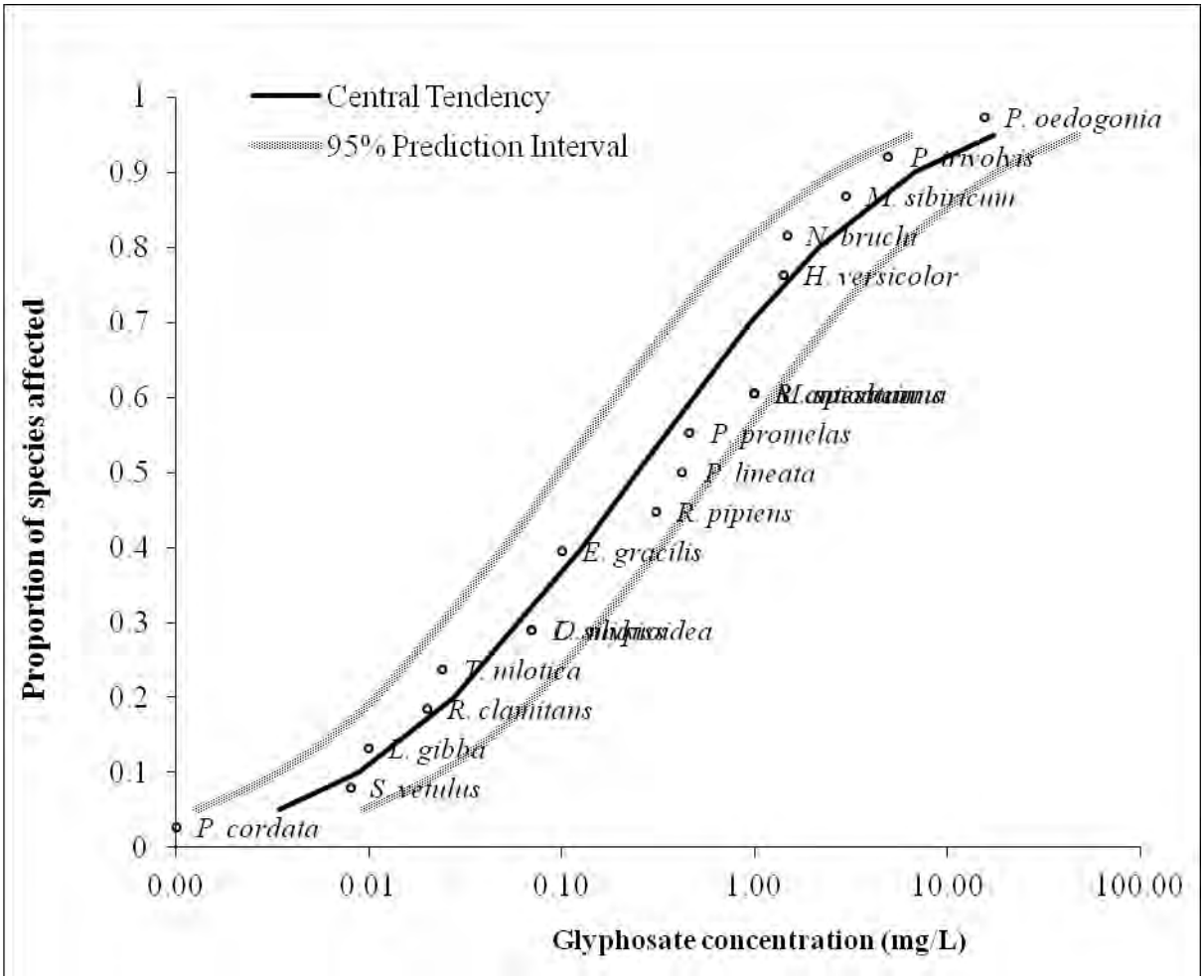


Figure 4.5: Species sensitivity distribution (SSD) curve of freshwater aquatic organisms from ECOTOX database based on long-term NOEC values for glyphosate (the following pair of species overlap because their sensitivities lie in the same range: *B. americanus and M. spicatum*; *O. mykiss and L. siliquoidea*)

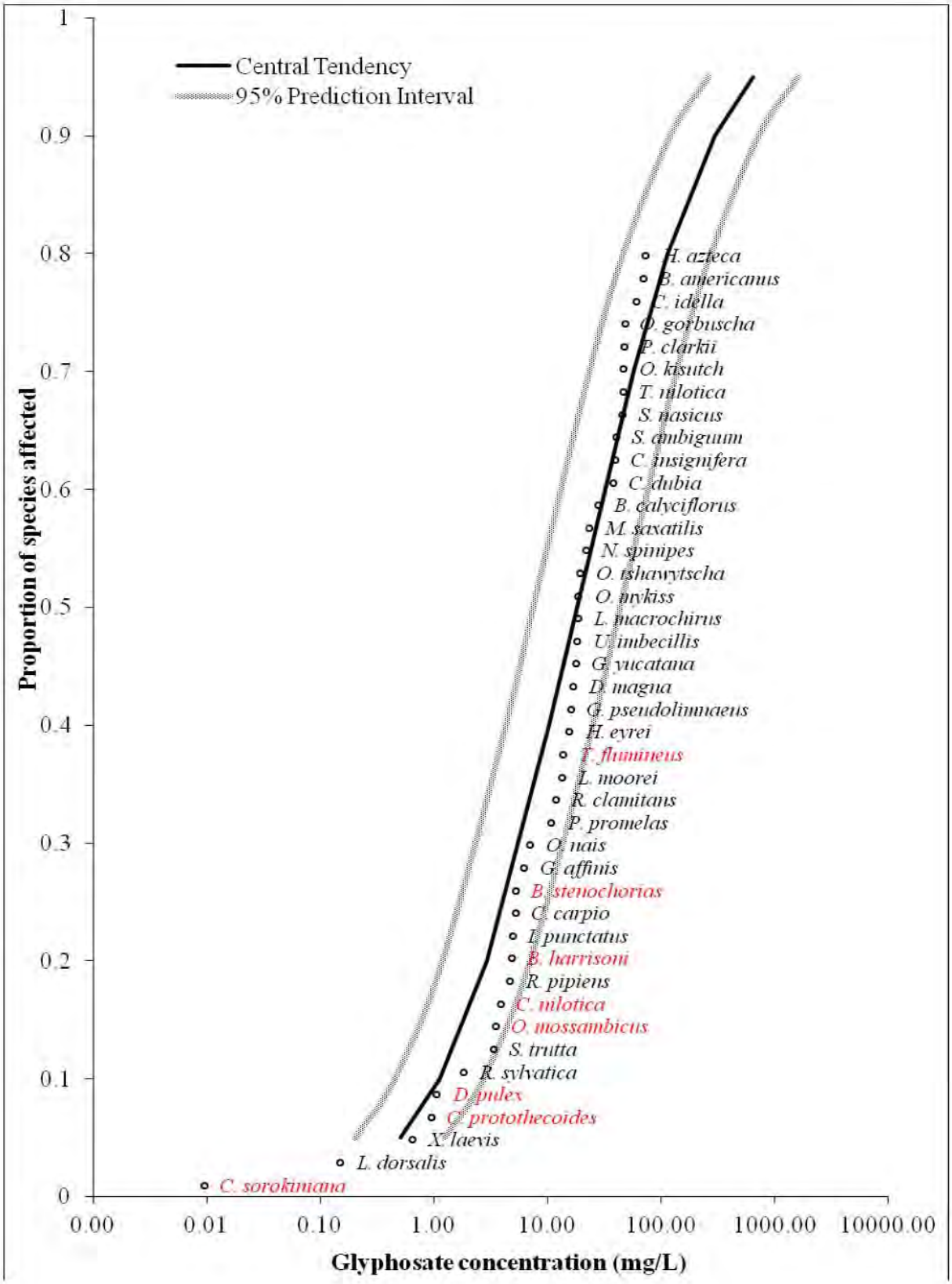


Figure 4.6: Species sensitivity distribution (SSD) curve of freshwater aquatic organisms from a combined South African and ECOTOX database LC50 values for glyphosate (South African species in red)

Based on the generated SSD curves, 95 % protective concentrations (PC95) of Roundup® (glyphosate) were calculated based on LE(C), NOEC and NEC values obtained from the laboratory single-species toxicity tests and the ECOTOX database. These results are presented in Table 4.9.

Table 4.9: WQGs for glyphosate resulting from species sensitivity distribution (SSD) 0.05 proportion (5th percentile) (Central tendency represents the median PC95 value, while predicted interval refers to the boundaries within which the PC95 value is located)

	Central tendency (mg/L)	**Lower predicted interval (mg/L)**	**Upper predicted interval (mg/L)**
From this study			
Short-term LC50	0.076	0.015	0.383
Long-term NOEC	0.085	0.025	0.294
Long-term NEC	0.002	0.000	0.021
From ECOTOX database			
Short-term LC50	1.412	0.771	2.585
Long-term NOEC	0.003	0.001	0.009
South African combined with From ECOTOX database			
Short-term LC50	0.504	0.204	1.245

4.3.4 Generation of biological system scales sensitivity distribution (BS3D) curves

The aim of Chapter Three was to provide a comprehensive perspective on the ecotoxicological effects of Roundup® on a single organism, using *C. nilotica* as a test species. Based on the findings, different effective concentrations (ECx) at which *C. nilotica* responded under different exposure lengths and at different biological system scales were derived and presented in Table 3.13.

The short-term EC50 and long-term EC10 values were used to construct *C. nilotica* biological system scales sensitivity distribution (BS3D) curves (Figures 4.7 and 4.8) using the Species Sensitivity Distribution Generator. The model parameters including the goodness of fit in terms of R^2, mean squared error (MSE) and corrected sum of squares (CSSQ) are presented in Appendix L.

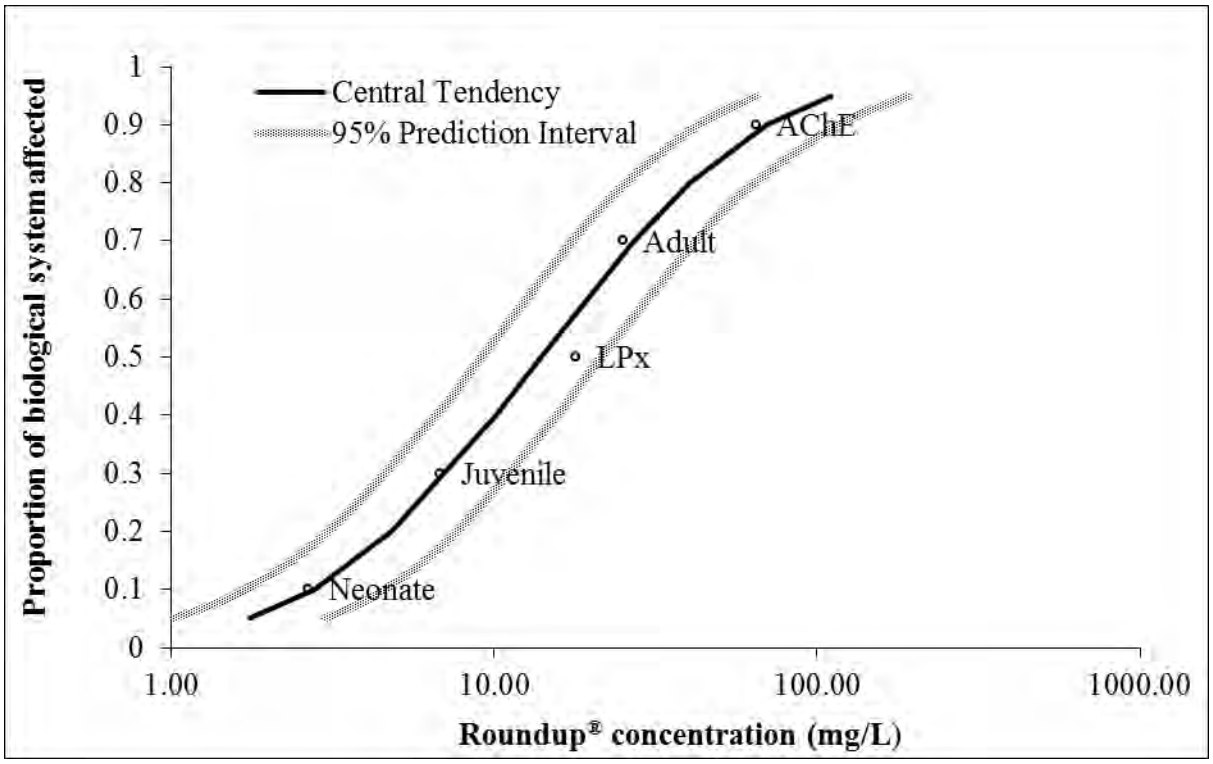


Figure 4.7: *Caridina nilotica* biological system scales sensitivity distribution (BS3D) based on short-term EC50 values (AChE = 4-day adult sub-lethal; Adult = 4-day adult lethal; LPx = 4-day adult sub-lethal; Juvenile = 4-day juvenile lethal; Neonate = 4-day neonate lethal)

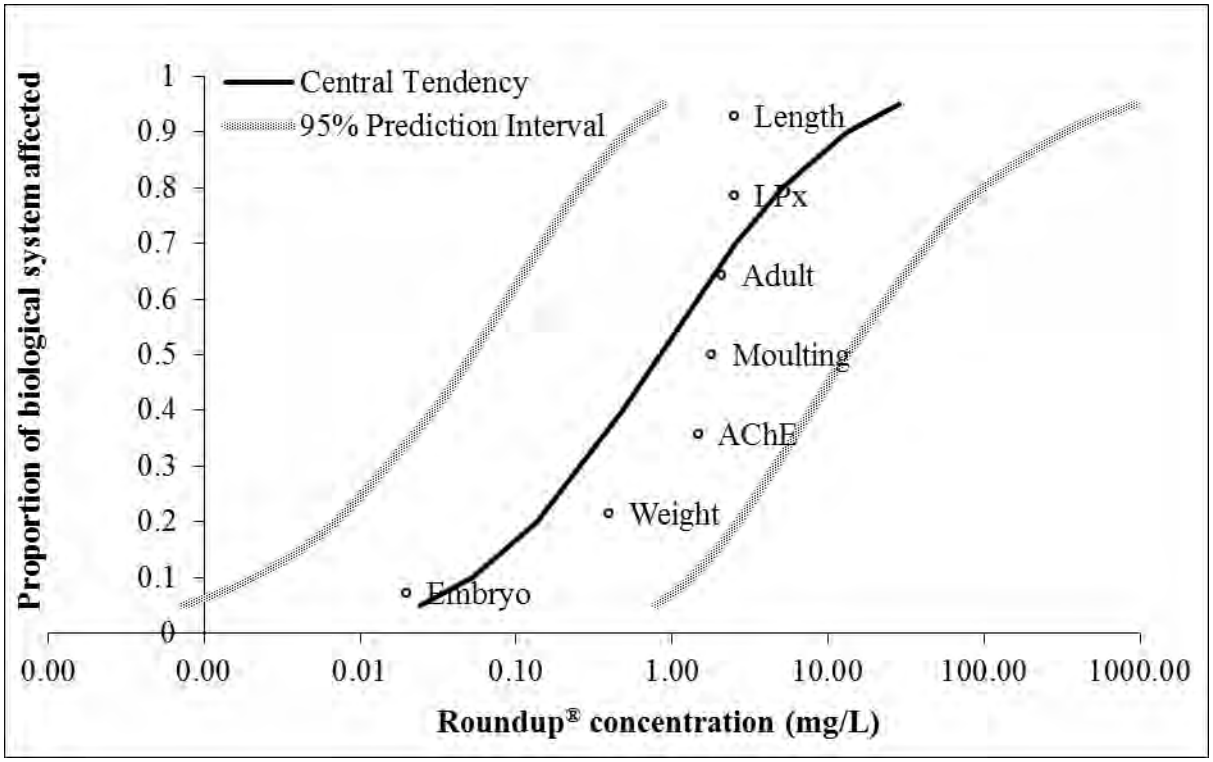


Figure 4.8: *Caridina nilotica* biological system scales sensitivity distribution (BS3D) based on long-term EC10 values (Length = 25-day adult sub-lethal; LPx = 21-day adult sub-lethal; Adult = 21-day adult lethal; Moulting = 25-day adult sub-lethal; AChE = 21-day adult sub-lethal; Weight = 25-day adult sub-lethal; Embryo = 17-day sub-lethal)

Based on the generated BS3D curves, 95% protective concentrations (PC95) of Roundup® were calculated (Table 4.10).The central tendency represents the median PC95 value, while predicted interval refers to the boundaries within which the PC95 value is located. This was then compared with species sensitivity distribution (SSD) using multiple species in Table 4.8.

Table 4.10: WQGs for Roundup® resulting from *C. nilotica* biological system scales sensitivity distribution (BS3D) 0.05 proportion (5^{th} percentile)

	Central tendency (mg/L)	**Lower predicted interval (mg/L)**	**Upper predicted interval (mg/L)**
Short-term EC50	1.76	1.03	3.00
Long-term EC10	0.02	0.00	0.79

4.4 Discussion

4.4.1 Species sensitivity distribution (SSD) curves for South African aquatic species and ECOTOX database

Species sensitivity distribution (SSD) curves provide a relatively quick, qualitative, visual comparison of toxicity data and the hazardous concentration for 5 % of the species (HC5) (i.e. 95 % protective concentration (PC95)) (Hose, 2005). SSDs from this study provided an estimate of species representing five taxonomic groupings affected by glyphosate. Positions of species on different parts of the SSD curves reflect the distribution patterns of the taxonomic groups that these species represent. Based on the 48-96 hour acute toxicity of this study, the algae *C. protothecoides* and *C. sorokiniana* seem very sensitive, appearing at the left tail-end of the curve (Figure 4.1). In contrast, the insects *B. harrisoni* and *T. flumineus,* and the mollusc *B. stenochorias* appeared at the right tail-end of the curve, suggesting their insensitivity to glyphosate (Figure 4.1). The sensitivities of *C. nilotica*, *D. pulex* (crustaceans) and *O. mossambicus* (fish) seem to fall within the same range, as all three species lie in the middle of the curve (Figure 4.1).

Compared to the 48-96 hour acute toxicity results from the ECOTOX database of this study, amphibians (including *Limnodynastes dorsalis*, *Xenopus laevis*, *Rana pipiens*, *R. sylvatica*, *R. clamitans*, *Litoria moorei*, *Heleioporus eyrei*) were among the most sensitive species, appearing at the SSD curve's left tail-end, together with fish (including *Salmo trutta*, *Ictalurus punctatus*, *Cyprinus carpio*, *Gambusia affinis*, *Pimephales promelas*) (Figure 4.4). A mixture of different taxonomic groups, including crustaceans, fish, amphibians and insects, also appeared in the middle of the SSD curve, suggesting that their sensitivities fall within the same range (Figure 4.4). However, at the right tail-end of the curve were mostly insects (including *Anopheles quadrimaculatus*, *Chironomus plumosus*) and fish (*Cyprinodon variegates*, *Carassius auratus*, *Oncorhynchus nerka*, *Cnesterodon decemmaculatus*) (Figure 4.4). This suggests that the sensitivities of these species lie within the same range and that they were among the most insensitive of the tested species. The results from this study (laboratory single-species toxicity tests and the ECOTOX database) suggest that algae were relatively most sensitive to 48-96 hour short-term glyphosate exposure, while crustaceans and molluscs were more sensitive than fish and insects.

Nevertheless, the ranges of sensitivities were not exclusive as there may be overlaps among some or all groups of organisms. Similar observations were made by van Wijngaarden *et al*. (2010) who found fish and some invertebrate species fell within the same range of sensitivities as they appeared together at the SSD curve's middle after exposure to the fungicide fluazinam, while some invertebrate species were found exclusively at the left tail-end, suggesting that they were the most sensitive of the tested species. However, in contrast to the current study, van Wijngaarden *et al*. (2010) reported that algae were insensitive to the toxicity of fluazinam as they appeared at the right tail-end of the SSD curve.

The long-term NOEC results for the present laboratory single-species toxicity tests showed crustaceans (*D. pulex*) to be the most sensitive group, appearing at the SSD curve's left tail-end, while the most insensitive groups included crustaceans (*C. nilotica*), algae (*C. protothecoides*) and fish (*O. mossambicus*), which appeared at the right tail-end of the curve (Figure 4.2). Between these two extremes of sensitivities lie the combined sensitivities of the alga *C. sorokiniana*, insects *B. harrisoni* and *T. flumineus*, and the mollusc *B. stenochorias* (Figure 4.2). These results, like those of 48-96 hour toxicity tests, show that the sensitivities of various taxonomic groups overlap and do not exclude each other.

It is interesting that the sensitivities of insects *B. harrisoni* and *T. flumineus*, and the mollusc *B. stenochorias* are grouped together, just as was observed for the 48-96 hour toxicity tests. However, the positions of these groups of organisms have been pushed down from the right tail-end of the 48-96 hour toxicity tests SSD curve (Figure 4.1) to the middle of the NOEC SSD curve (Figure 4.2). This observation suggests that these groups of organisms become more sensitive to glyphosate toxicity with increasing length of exposure.

The long-term NEC results for the present laboratory single-species toxicity tests showed algae (*C. protothecoides*, *C. sorokiniana*) to be the most insensitive group, appearing at the SSD curve's right tail-end (Figure 4.3). At the left tail-end of the curve lie the mollusc *B. stenochorias* and crustacean *D. pulex* showing that their sensitivities lie within the same range (Figure 4.3). Similarly, the sensitivities of the insects *B. harrisoni* and *T. flumineus*, the crustacean *C. nilotica,* as well as the fish *O. mossambicus* lie are within the same range as they appeared together in the middle of the SSD curve (Figure 4.3).

The long-term NOEC SSD curve from the ECOTOX database of this study, showed the aquatic plants (*Pontederia cordata, Lemna gibba*) and the crustacean *Simocephalus vetulus* at the left tail-end, suggesting that they are the most sensitive taxonomic groups (Figure 4.5). At the right tail-end of the SSD curve lie the alga *Pithophora oedogonia*, the plant *Myriophyllum sibiricum*, the mollusc *Planorbella trivolvis*, the insect *Neochetina bruchi* and the amphibian *Hyla versicolor*, which suggests that all these groups of organisms were insensitive to long-term exposure to glyphosate (Figure 4.5). Similar observations were made in the middle of the SSD curve where different species belonging to different taxonomic groups including plants, insects, amphibians, crustaceans, fish and molluscs were found together (Figure 4.5). These observations for both NOEC and NEC suggest that long-term exposure to glyphosate affects the sensitivity of all groups of organisms involved.

The results also show that the number sample size does not necessarily influence the sensitivity of the SSD, although it gives the curve a robust goodness of fit (high R^2). For example large sample sizes from the ECOTOX database resulted in higher R^2 values for both LC50 and NOEC based SSDs (0.97 and 0.96, respectively) compared to the SSDs derived from LC50 and NOEC (0.89 and 0.86, respectively) values for this study. The SSD_{LC50} TV derived from this study (0.08 mg/L) was more sensitive than the ECOTOX database SSD_{LC50} TV (1.41 mg/L). However, the SSD_{NOEC} TV derived from this study (0.085 mg/L) was less sensitive than the ECOTOX database SSD_{NOEC} TV (0.003 mg/L). These observations show that fitting species sensitivity to a good model to generate an SSD is as good as species sensitivity to the test chemical. These two mutually exclusive situations were achieved in the current study.

The combined SSD_{LC50} data (ECOTOX database plus this study) resulted in providing less adequate protective concentration 0.504 (0.204-1.245) mg/L to South African species compared to South African species alone 0.076 (0.015-0.383) mg/L. Therefore, it was not advisable to derive a water quality guideline based the international alone or the combined data. This will suppress the sensitivity of the South African data. Thus, it was rather necessary to derive the guideline based on the South African data alone.

4.4.2 Derivation of water quality guidelines for glyphosate

In this study, the method used to derive the water quality guidelines (WQGs) for glyphosate follows that of ANZECC and ARMCANZ (2000), Warne *et al*. (2004) and USEPA (2005). However, the type of WQG follows that of CCME (2007), which provides separate guidance for both short-term and long-term exposures. Thus, separate proposed South African water quality guidelines (WQGs) were derived for short-term and long-term exposure to Roundup® (glyphosate), and compared with guidelines based on glyphosate data from the ECOTOX database.

Short-term WQG

The objective of the short-term WQG is to protect most species from death during severe but transient events such as inappropriate application, improper disposal, and spill events. Inappropriate application or improper disposal includes spraying under worst conditions of heavy rainfall, or in severe wind, and not following label instructions. Thus, the short-term WQG is intended to protect most species in the aquatic environment during unfortunate and catastrophic events, but not indefinitely. The short-term proposed South African water quality guidelines (WQG) and ECOTOX WQG are presented in Table 4.9. The ECOTOX WQG was many times (about 19 times) higher than the WQG. This may be attributed to the large difference in the total number of data points used in the SSD; 8 for WQG and 625 for ECOTOX WQG.

Long-term WQG

The objective of long-term WQGs is to protect all aquatic species and life stages against adverse effects during chronic exposure. Chronic exposure of aquatic organisms to a pesticide may result from gradual release from soil or sediment, gradual entry through groundwater or runoff, repeated application within the same localised region, and long range transport events. The long-term WQG and ECOTOX WQG are shown in Tables 4.9. Depending on either the NOEC or NEC data, two different WQG long-term trigger values for the protection of aquatic life could be derived. However, having two different TVs for the same chemical creates confusion in terms of which should be taken as the environmentally safe level. This situation stems from the fact that the scientific community has no official position on using NOEC data to develop SSDs in spite of its limitations, as discussed earlier in this chapter.

Using the NOEC values in deriving the guidelines showed ECOTOX WQG (international) to be about 28 times less than that of this study. This may also be attributed to the large difference in the total number of data points used in the SSD, as explained previously for the short-term WQG. However, only NEC values were used to derive the proposed South African WQG long-term trigger value for the protection of aquatic life.

4.4.3 Comparison of species sensitivity distribution (SSD) and biological system scales sensitivity distribution (BS3D) derived guidelines

The South African-based aquatic species sensitivity distribution derived water quality guidelines (WQG_{SSD}) (Table 4.9) were compared to *C. nilotica* biological system scales sensitivity distribution derived water quality guidelines (WQG_{BS3D}) (Table 4.10). The comparison revealed that short-term LC50 WQG_{SSD} was about 88 times smaller than the magnitude of short-term EC50 WQG_{BS3D}. However, the magnitudes of WQG_{SSD} based on long-term NOEC and NEC were both greater than the long-term EC10 WQG_{BS3D} (about 4 times and 2 times, respectively).

These observations seem to suggest that the different biological system scales of *C. nilotica* are more sensitive to long-term Roundup® exposure than SSD results including the species. This means that, in deriving a long-term water quality guideline, it is probably worth considering the long-term effect on different biological system scales. However, the short-term EC50 WQG_{BS3D} seems to be much less sensitive compared to the LC50 WQG_{SSD}. Thus, it would probably be better to use the short-term WQG_{SSD} rather than the short-termWQG_{BS3D} to derive a guideline.

4.5 Conclusion

The derived WQG short-term trigger value (TV) indicating the potential for severe effects to sensitive freshwater organisms during transient events for glyphosate based on the species sensitivity (SSD) method (i.e. LC50 WQG_{SSD}) is 0.076 (0.015-0.383) mg/L, while the long-term TV for the protection of aquatic life is 0.002 (0.000-0.021) mg/L (i.e. NEC WQG_{SSD}). The short-term and long-term ECOTOX WQGs (international) for glyphosate are 1.412 (0.771-2.585) and 0.003 (0.001-0.009) mg/L, respectively.

The derived WQGs may be applied to other glyphosate-based herbicides in South Africa after evaluation by the relevant body. Clear inconsistencies were found between the derived South African WQGs and the ECOTOX WQGs (international), primarily due to differences in data as described earlier in the previous section. Thus, it is recommended that caution should be taken when data are extracted from the ECOTOX database and used as surrogates to derive local (catchment or national) WQGs.

This study has shown that in-depth study with one species using the BS3D approach can provide ecotoxicological data, which can be used to derive site-specific guidelines to protect sensitive species or taxonomic groups. However, it is not clear whether BS3D should be used as a stand-alone or be incorporated into the more conventional approach of SSD. Thus, further study is needed on the use of the BS3D approach in a guideline derivation programme.

CHAPTER FIVE

MACROINVERTEBRATE-BASED BIOMONITORING IN RELATION TO A GLYPHOSATE-BASED HERBICIDE APPLICATION IN THE SWARTKOPS RIVER

5.1 Introduction

The Swartkops River (also referred to as "the river" in this Chapter) was used as an experimental system to evaluate the effects that field application of glyphosate-based herbicides has on freshwater ecosystems. The South African National Department of Water Affairs, through the Working for Water Programme, frequently used glyphosate-based herbicides to control weeds in this river system (Bold, 2007; DWA, 2009). This chapter briefly describes the physicochemical and biological conditions of the Swartkops River before and after spraying with Kilo Max WSG by the Working for Water Programme. This provided the best possible opportunity to investigate an *in situ* effect of a glyphosate-based herbicide formulation. Kilo Max WSG is glyphosate-formulated herbicide and is referred to as "glyphosate" hereafter in this chapter. The study areas, including geology, climate, ecology, biological and socio-cultural importance and factors that pose threats to the river's catchment are briefly discussed. Details of the study sites, data collection and analyses methods, results, discussions and conclusion are also presented.

5.1.1 Description of study area

The Swartkops River is located in the Eastern Cape Province of South Africa and stems from the confluence of Kwa-Zunga and the Elands Rivers. These two rivers originate 155 km away in the Groot Winterhoek Mountains in the Western Cape but merge just above Uitenhage to form the Swartkops River, which discharges into the Indian Ocean at Algoa Bay in Port Elizabeth (E-FA, 2009; Odume, 2011) (Figure 5.1).

The Kwa-Zunga (north of Swartkops) has multiple, narrow, and well-watered ravines, whereas the Elands (southwest of Swartkops) is relatively drier (DWAF, 1996b). The Swartkops River catchment size is about 1555 km^2 and falls within the South Eastern Coastal Belt (lower zone) ecoregion (Dallas, 2007). The river drains the M10 catchment, which is divided into four main quaternary catchments. These are M10A (Kwa-Zunga River), M10B (Elands River), M10C (Swartkops River: Elands Confluence), and M10D (Swartkops River: Despatch-River Mouth) quaternary catchments (Haigh, 2002). The sampling sites for this study fall within the M10C and M10D quaternary catchments (Figure 5.1).

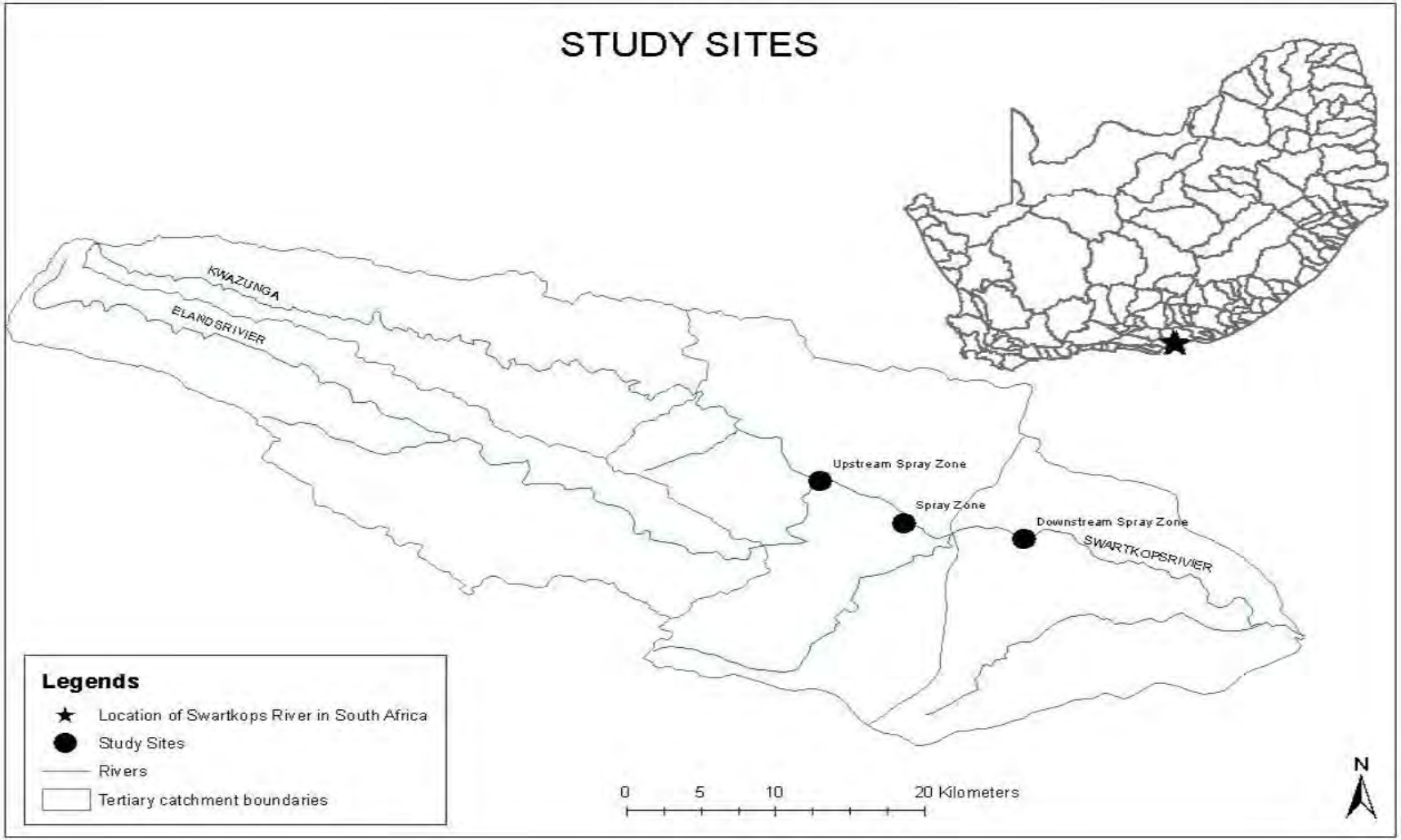


Figure 5.1: A map of the Swartkops River showing the Kwa-Zunga and Elands Rivers that join to form it, and the three sampling sites

The Swartkops River's catchment geology differs from most catchments in the southern Cape primarily in that it has large areas of marine and estuarine origin on the floodplain. The upper reaches of the river flow over the quartzites of the Table Mountain Group, while the downstream flows over weakly consolidated shales of the Uitenhage Group (Baird *et al.* 1986). The Uitenhage group, which underlies the alluvial deposits in the area, is fairly porous and permeable. The underlying geology forms an easily erodible trough, upon which silts, clay, sand and gravel have been deposited in tertiary to recent times. The trough comprises layers of coarse conglomerates inter-bedded with sandstone and mudstone of the Enon formation (marine and fluvial origin), greenish-grey slate, siltstones and sandstones of the Kirkwood formation (fluvial origin) as well as thinly bedded grayish-green mudstones and siltstones of the Sundays formation (marine origin) (Baird *et al.* 1986). The Elands sub-catchment has a higher proportion of Bokkeveld shales, but the geology on the coastal plain consists mainly of stabilised sand dunes without good water retention ability (Haigh, 2002). Three main soil types occur in the catchment (DWAF, 1996b). Soils in the upper catchment are derived from Table Mountain Sandstone (TMS), while the soils in the middle areas are of deep alluvial nature, and the areas closer to the sea are predominantly sand. Soils in the lower Kwa-Zunga, lower Elands, upper Swartkops, Brak and Chatty sub-catchments are suitable for agriculture (DWAF, 1996b). The sedimentary deposit of the river makes it erode easily, allowing the extensive meandering flow path of the river system on the floodplain (DWAF, 1996b).

The river's catchment receives rain throughout the year with mean annual rainfall between 655 mm to 750 mm, and a monthly average of about 55 mm (Haigh, 2002). Frequent flooding occurs when monthly rainfall spans a period of a few days. The mean annual runoff (MAR) is about 84.2 x 10^6 m^3 and the Kwa-Zunga/Swartkops draining system contributes about 0.3 % to the total MAR in South Africa (Haigh, 2002).

The climate is generally warm and temperate with large fluctuations in daily and seasonal temperatures. The mean daily maximum temperature in the low-lying areas is approximately 32° C in January and 18° C in July, while the mean daily minimum temperature is 15° C in January and 5° C in July (Haigh, 2002).

The prevailing winds in summer are south-westerly, occasionally interchanged by south-easterly winds, but warm, dry, north-westerly and north-easterly winds prevail in winter. The wind plays a big role in the temperature regime by reducing temperatures as well as humidity in summer (Haigh, 2002).

5.1.2 Threats to the Swartkops River

The lower catchment of the Swartkops River is subjected to different levels of pollution, especially sites downstream of Uitenhage, but the upper catchment lies within a pristine inaccessible area of the Groot Winterhoek Mountains (Odume, 2011). The entire Swartkops catchment is invaded by alien plants species including *Opuntia ficus-indica* (prickly pear), *Opuntia aurantiaca* (jointed cactus), *Acacia mearnsii* (black wattle), *Acacia longifolia* (longleaf wattle) and *Pinus pinaster* (cluster pine) (Haigh, 2002). These invasive alien plants pose a significant threat to the indigenous vegetation and the integrity of the catchment as they tend to encourage channel band instability and sediment production. These species also reduce stream flow and impact negatively on in-stream habitat. The presence of *Acacia mearnsii* is currently recognised as the biggest threat because it evapo-transpires large volumes of water. However, it is often harvested by local people for fuel. Alien fish species, such as largemouth bass (*Micropterus salmoides*) found in the Swartkops River constitute a threat to the survival of indigenous fish species, preying on the latter (Haigh, 2002).

Industrial activities are major anthropogenic sources of water quality impacts in the river and they include the Uitenhage and Despatch Wastewater Treatment Works, a stone quarry, a power station, the motor industry, wool industry, tanneries and aquaculture, with only limited agriculture taking place (Odume, 2011). Runoff from industrial or informal residential areas has resulted in high levels of

metals entering the river system (Taljaard *et al.,* 1998). There has been a pronounced increase in phosphate concentrations in the Swartkops River in the recent past with phosphate concentration doubling in less than five years (de Villiers and Thiart, 2007). Seasonal nutrient profiles show that nitrate levels are increasing, with concentration exceeding 400 µg/L for at least five months in a year, which favours conditions for eutrophication for most of the year in the river catchment (E-FA, 2009).

5.1.3 Sampling sites

Three sampling sites, namely spray zone (SZ), upstream of spray zone (USZ) and downstream of spray zone (DSZ), were selected in the Swartkops River and they all lie within the same ecoregion level II (i.e. South Eastern Coastal Belt, lower zone). The following considerations were given in choosing the sampling sites:

- One of the sites (i.e. SZ) was sprayed with glyphosate to control invasive alien aquatic plants as part of the Department of Water Affairs' Working for Water programme. Two sites were selected upstream and downstream of the SZ.

- All the sites fall within the same ecoregion. Since, within an ecoregion, there are relative similarities in both biotic and abiotic components of the ecosystem, macroinvertebrates sampled at sites within the same ecoregion should be similar (Kleynhans *et al*., 2005).

- All SASS5 biotopes, including stones (in and out of current), vegetation (marginal and aquatic) and gravel, sand and mud (GSM) to provide enough habitats for macroinvertebrates are well represented at all the sites.

- A year-long macroinvertebrate-based biomonitoring study was done at these sites by Odume (2011) and therefore provides baseline information for the current study.

Spray zone (SZ)

The spray zone (SZ) (S33º 46'53.5" E 25º 23'36.6") (Figure 5.2) is located in the industrial city of Uitenhage. The site is severely impacted with domestic, agricultural, wastewater and industrial effluents. The wastewater treatment works near Nivens Bridge is located close to the site, and livestock farming and other agricultural practices are evident around it. There is extensive growth of water hyacinth and other aquatic weeds at the site. Although there is evidence of habitat degradation at this site, diversity of the sampling biotope is generally good (Odume, 2011). Spraying with glyphosate to control water hyacinth was done at this site for a week by Working for Water (WfW).

Figure 5.2: Spray zone (SZ) showing water hyacinth before spray and habitat degradation

Upstream of spray zone (USZ)

Upstream of spray zone (USZ) (S 33º 45' 08.4" E 25º 20' 32.6") (Figure 5.3) is located upstream of Uitenhage and about 16.4 kilometres downstream of the Groendal dam. The impoundment due to the dam could negatively impact on macroinvertebrate composition of the USZ. However, USZ represents the best available condition within the accessible areas in the Swartkops River compared to SZ and DSZ. Furthermore, water quality and diversity of the biotope at this site is generally good with little habitat degradation (Odume, 2011). The USZ served as a positive control due to its relatively pristine nature.

Figure 5.3: Upstream of spray zone (USZ) showing the biotopes sampled

Downstream of spray zone (DSZ)

Downstream of spray zone (DSZ) (S 33º 47' 34.0" E 25º 27' 58.7") (Figure 5.4) is located at Despatch close to a quarry. The DSZ is impacted with agricultural and municipal runoff as well as by effluents from the Despatch wastewater treatment works. These have consequently resulted in a thick growth of aquatic weeds and water looking greenish at the site. There is a small culvert at the site, which modifies the flow and has degraded the habitat. Although biotope diversity at this site is generally good, GSM and stones are not extensively represented. The DSZ served as a negative control because its physicochemical and biological conditions are relatively worse than SZ and DSZ (Odume, 2011).

Figure 5.4: Downstream of spray zone (DSZ) showing the biotopes sampled

5.2 Materials and methods

5.2.1 Aquatic macroinvertebrates sampling before and after glyphosate spray

Macroinvertebrate samples were collected at three sites before and after spraying with glyphosate. The before-spray data were obtained from the raw data collected by Odume (2011) as a separate study, in which samples were collected seasonally over a period of one year in spring-summer, 2009, and autumn-winter, 2010. The after-spray data were collected after the water hyacinth in the SZ had been sprayed with glyphosate by WfW in summer 2011. Weekly samplings were conducted in all three sites for four weeks after spraying.

Samples of macroinvertebrates were collected, following the South African Scoring System version 5 (SASS5) protocol, which requires the collection of macroinvertebrates from three distinct biotopes, namely stones (stones in- and out-of current), vegetation (marginal and aquatic vegetation) and sediment (gravel, sand and mud (GSM)) (Dickens and Graham, 2002). A SASS5 net (30 x 30 cm frame with mesh size 1000 μm) was used for the sampling. During each sampling event, three replicates were taken from each of the three biotopes (stones, vegetation and GSM) making a total of nine samples from each site per sampling event. Collected macroinvertebrates were emptied into a white SASS5 tray that was half-filled with river water. Families of macroinvertebrates present were identified, recorded on a SASS5 sheet, preserved in 70 % ethanol and transported to the laboratory. In the laboratory, samples were sorted, and abundance count and family identifications were checked to ascertain field accuracy. Three replicate samples were taken to ascertain the representativeness of a single sample and to provide robust statistical analysis of the biotic indices. Nonetheless, only the first sample collected from each biotope was used to calculate SASS5 scores and average score per taxon (ASPT) since the SASS5 protocol requires the collection of only one sample from each biotope.

5.2.2 Biological metrics and environmental water quality relations before and after glyphosate spray

Biological metrics are measurable units or processes of a biological system that are perceived to change in value with the level of pollution in aquatic ecosystems (Ofenböck *et al.,* 2004). The following macroinvertebrate metrics (Table 5.1) together with SASS5 scores, ASPT and number of taxa (biotic indices) were used as biological metrics to determine the relationship between macroinvertebrate assemblage and water physicochemical variables before and after glyphosate spray. The selected biological metrics were reported to be sensitive to pollution in the Swartkops River (Odume, 2011; Odume *et al*., 2012).

Table 5.1: Macroinvertebrate metrics of community composition, richness and diversity applied for bioassessment in the sampling sites before and after glyphosate spray (after Odume, 2011)

Metric	Definition
Composition (Relative abundance)	
% Chironomidae + Oligochaeta	Percentage of individuals in Chironomidae and Oligochaete taxa relative to entire sample
% ETOC	Percentage of individuals in Ephemeroptera, Trichoptera and Odonata relative to entire sample
Richness	
ETOC richness	Absolute number of taxa in Ephemeroptera, Trichoptera, Odonata and Coleoptera
Margalef's family richness index	Accounts for both number of taxa and individuals and is independent of sample size
Diversity	
Shannon diversity index	Information statistic index which takes account of the contribution of individual taxa to the diversity while assigning greater weight to most dominant taxa
Equitability (or Evenness)	Measures the relative even distribution of abundance of taxa within a sample

5.2.3 River health assessment of sampling sites before and after glyphosate spray

The South African River Health Programme (RHP) incorporates several components of aquatic biota, including macroinvertebrates, fish and riparian vegetation. The macroinvertebrate component uses SASS scores and ASPT values to describe the health status of a river (Dallas, 2000; Dickens and Graham, 2002). Thus, in order to determine if glyphosate spray affects river health at the spray zone (SZ), environmental water quality of each sampling site was assessed using SASS5 Scores and ASPT values before and after glyphosate spray. Biological bands and ecological categories for the South Eastern Coastal Belt (lower zone) ecoregion according to Dallas (2007), which were used for interpretation of results, are presented in Table 5.2.

Table 5.2: Biological bands and ecological categories for South Eastern coastal belt (lower zone) ecoregion (extracted from Dallas, 2007)

Biological band	Ecological category name	Description	SASS5 score	ASPT value
A	Natural	Unmodified natural	149-180	7.1-8.0
B	Good	Largely natural with few modifications	100-148	6.0-7.0
C	Fair	Moderately modified	83-99	5.4-5.9
D	Poor	Largely modified	63-82	5.1-5.3
E/F	Seriously/critically modified	Seriously/critically modified	< 62	< 5

5.2.4 Measurements of physicochemical water quality variables before and after glyphosate spray

Water physicochemical variables, including dissolved oxygen (DO), pH, temperature, electrical conductivity (EC) and turbidity, were measured on site using Cyberscan DO300, Cyberscan pH 300, mercury-in-glass thermometer, Cyberscan Con300 and Orbeco-Hellige 966 meters, respectively. Water samples for BOD_5 *analysis w*ere collected in sterilised 500-mL glass bottles, while samples for nitrate-nitrogen (NO_3-N), nitrite-nitrogen (NO_2-N), ammonium-nitrogen (NH_4-N) and orthophosphate-phosphorus (PO_4-P) were collected in 250-mL plastic bottles, and transported to the laboratory in a cooler box filled with ice cubes. NO_3-N and NO_2-N were analysed according to Velghe and Claeys (1983), while NH_4-N and PO_4-P were analysed using Spectroquant® ammonium and phosphate concentration test kits. Readings were done spectrophotometrically using a Bio-Tek micro-plate reader. Each sample was analysed in quadruplicate and averaged, since average readings were considered more representative with reduced variability. BOD_5 was analysed according to APHA (1992), while chlorophyll *a* concentrations for periphyton and phytoplankton were analysed fluorometrically with a Turner Design 10-AU digital fluorometer according to Arar and Collins (1997).

5.2.5 Statistical analysis

One-way analyses of variance (ANOVA) followed by Tukey post hoc multiple comparison tests were carried out to test the hypothesis that there were no significant difference of water quality variables measured between sampling sites, with alpha set at $p > 0.05$, using Statistica (StatSoft Inc., 2009). The same statistical methods were applied to evaluate significant differences between sampling sites for biological metrics. Principal Component Analysis (PCA) was used for the ordination of sampling sites and periods (seasons for before glyphosate spray; weeks for after glyphosate spray) based on the measured physicochemical water quality variables. Canonical Correspondence Analysis (CCA) was used to explain the relationship between biological metrics and physicochemical water quality variables in order to determine which variables influenced the observed spatio-temporal distribution of selected biological metrics. Before CCA was performed, data for physicochemical water quality and biological metric were log (x+1) transformed in order to down weight large values and reduce their influence on the analysis. Multi-collinearity analyses of the data were performed after CCA in order to remove biological and physicochemical variables that were redundant. A variable was rendered redundant if Pearson's correlation (i.e. R^2) was found to be greater than 0.8. Physicochemical water quality and biological metric data were separately subjected to paired t-tests so as to evaluate the observed differences between before and after glyphosate spray.

5.3 Results

5.3.1 Physicochemical water quality variables and biological metrics conditions before glyphosate spray

Physicochemical water quality evaluation before glyphosate spray

Means, standard deviations and ranges of water quality variables measured before glyphosate spray at the three sampling sites, i.e. spray zone (SZ), upstream of spray zone (USZ) and downstream of spray zone (DSZ) are presented in Table 5.3. One-way analysis of variance (ANOVA) followed by a Tukey post hoc test revealed no statistical significant differences between the three sampling sites for pH, temperature, turbidity and five days biochemical oxygen demand (BOD_5).

However, although dissolved oxygen (DO) did not differ significantly between SZ and USZ ($p > 0.05$), DSZ differed significantly from the other two sites ($p < 0.05$). Analyses for electrical conductivity (EC), nitrate-nitrogen (NO_3-N), nitrite-nitrogen (NO_2-N) and ammonium-nitrogen (NH_4-N) revealed similar observations as DO, where no significant differences were found between SZ and USZ, but DSZ was significantly different from the other two sites. Orthophosphate-phosphorus (PO_4-P) was significantly different among all three sites, with USZ having the lowest concentrations, followed by SZ and DSZ recording the highest concentrations. Phytoplankton and periphyton chlorophyll *a* were significantly lower in USZ than in SZ and DSZ, but no significant difference was found in SZ and DSZ.

Table 5.3: Means, standard deviations and ranges (in brackets) of measured physicochemical water quality variables (n = 4) among the three sampling sites before glyphosate spray. The mean values of any two sites having the same superscript letter are not significantly different ($p > 0.05$)

WQ Variable	Sampling sites			
	SZ	USZ	DSZ	p-value
DO (mg/L)	6.99 ± 1.84^{a} (5.53-9.48)	6.40 ± 1.26^{a} (4.73-7.47)	1.78 ± 0.79^{b} (0.90-2.62)	0.00
pH	7.12 ± 1.08 (5.69-7.00)	6.6 0 ± 1.17 (5.13-7.75)	7.24 ± 0.57 (6.65-8.01)	0.63
Temperature (ºC)	17.71 ± 7.72 (9.80-27.3)	17.78 ± 4.60 (12.10-22.00)	19.08 ± 4.66 (14.30-24.00)	0.93
Electrical Conductivity (mS/m)	40.75 ± 8.45^{a} (30.00-48.80)	30.22 ± 1.96^{a} (28.10-32.80)	255.50 ± 15.33^{b} (234.00-270.00)	0.00
Turbidity (NTU)	6.15 ± 1.47 (4.70-8.00)	7.78 ± 2.78 (5.00-10.25)	9.58 ± 11.03 (3.10-26.00)	0.77
BOD_5 (mg/L)	8.66 ± 5.43 (4.78-16.68)	4.75 ± 1.44 (3.62-6.86)	12.76 ± 6.98 (7.06-22.94)	0.15
Nitrate-nitrogen (NO_3-N) (mg/L)	0.92 ± 1.36^{a} (0.03-2.93)	0.12 ± 0.07^{a} (0.06-0.21)	2.01 ± 0.55^{b} (1.19-2.36)	0.04
Nitrite-nitrogen (NO_2-N) (mg/L)	0.07 ± 0.09^{a} (0.01-0.21)	0.02 ± 0.01^{a} (0.01-0.07)	0.17 ± 0.04^{b} (0.12-0.21)	0.01
Ammonium - nitrogen (NH_4-N)(mg/L)	0.57 ± 0.81^{a} (0.07--0.32)	0.21 ± 0.09^{a} (0.15-0.34)	3.25 ± 2.30^{b} (1.05-5.24)	0.03
Orthophosphate-phosphorus (PO_4-P) (mg/L)	1.11 ± 0.56^{a} (0.46-1.65)	0.01 ± 0.00^{b} (0.00-0.01)	6.60 ± 0.77^{c} (5.46-7.20)	0.00
Phytoplankton chlorophyll *a* (µg/L)	3.84 ± 2.88^{a} (1.60-7.96)	0.45 ± 0.11^{b} (0.34-0.60)	1.68 ± 0.10^{a} (1.56-1.79)	0.05
Periphyton chlorophyll *a* (µg/cm^2)	386.16 ± 200.31^{a} (145.87-628.90)	28.54 ± 6.44^{b} (20.29-35.88)	608.62 ± 160.06^{a} (485.73-633.96)	0.00

Principal Component Analysis (PCA) before glyphosate spray classified the sites into three distinct groups based on the measured physical and chemical water quality variables (Figure 5.5). The PCA axes 1, 2 and 3 with eigenvalues 5.21, 2.01, and 1.25 accounted for approximately 47.40, 18.25 and 11.41 % of the total variance, respectively. Thus, the first three eigenvalues accounted for approximately 77.06 % of total variation among measured water quality variables, indicating good ordination (Table 5.4).

The PCA results revealed an apparent classification of the sites into predominantly USZ (to the left) and predominantly DSZ (to the right). The SZ is loosely classified between these two zones (Figure 5.5). The separation of the DSZ from site USZ is an indication of poor water quality at DSZ (Figure 5.5), which may be attributed to organic loads, industrial effluents and other anthropogenic activities. The loose grouping of SZ between USZ and DSZ shows that water quality at this site was better than DSZ but poorer than USZ. The poorest water quality situation at the SZ was recorded during winter (i.e. SZ4), which is when it clusters with DSZ (Figure 5.5).

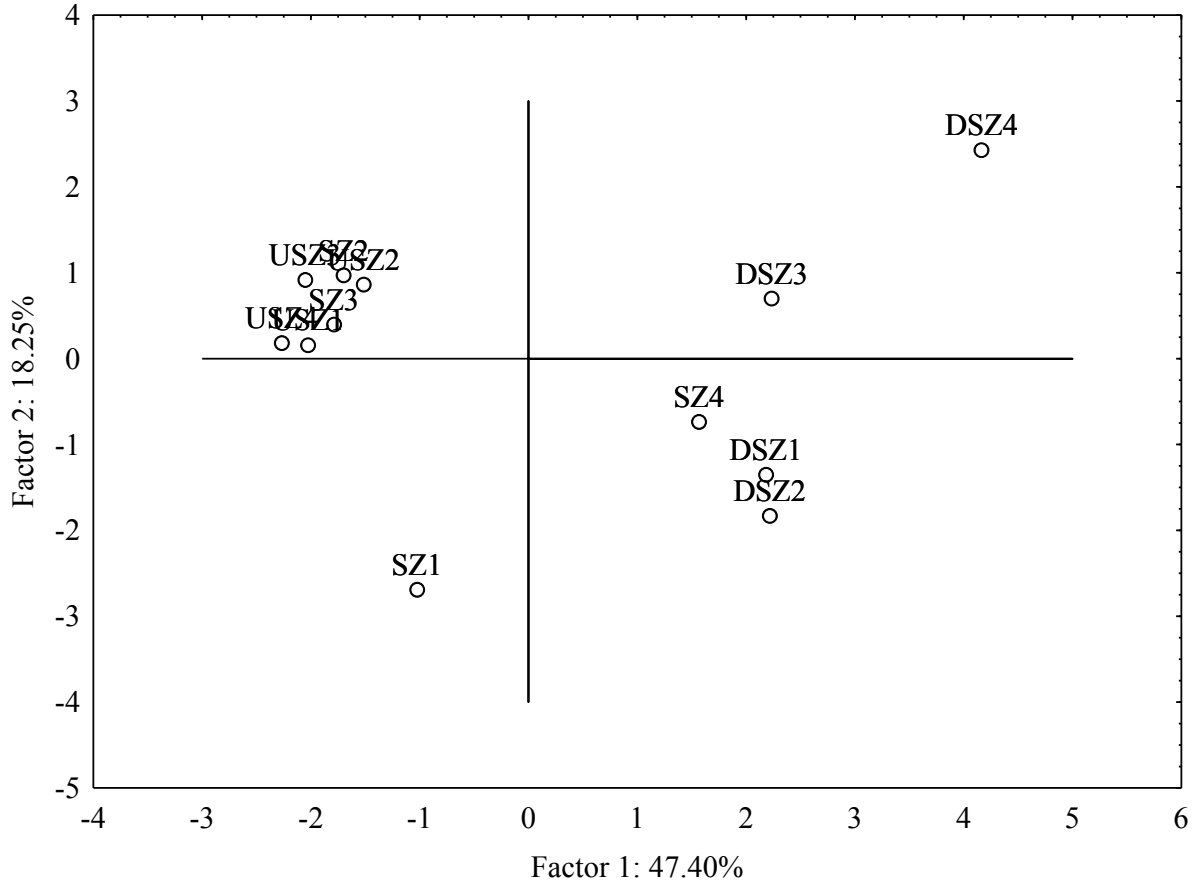


Figure 5.5: Before glyphosate spray PCA ordination bi-plots for physicochemical water quality variables at all three sampling sites in the Swartkops River for each sampling season. Letters represent sites (SZ = spray zone; USZ = upstream spray zone; DSZ = downstream spray zone) and numbers attached to letters represent different sampling seasons (1 = Autumn; 2 = Winter; 3 = Spring; 4 = Summer). (The 6 points clustered in the top left quadrant are USZ1, USZ2, USZ3, USZ4, SZ2 and SZ3)

Table 5.4: Eigenvalues of correlation matrix used in PCA ordination bi-plots for physicochemical water quality variables at all three sampling sites before glyphosate spray. Only first three eigenvalues greater than 1 (shaded) were used in the analysis

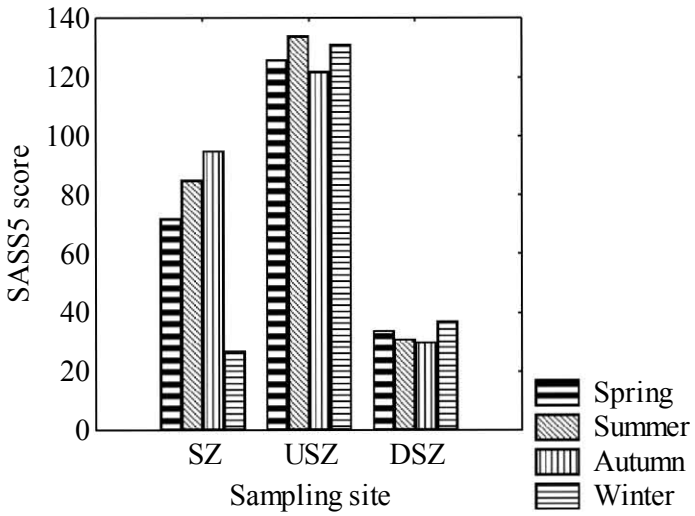
Eigenvalue number	Eigenvalue	Total variance (%)	Cumulative Eigenvalue	Cumulative total variance (%)
1	5.21	47.40	5.21	47.40
2	2.01	18.25	7.22	65.65
3	1.26	11.41	8.48	77.06
4	0.99	8.98	9.46	86.04
5	0.80	7.27	10.26	93.31
6	0.39	3.55	10.65	96.86
7	0.23	2.08	10.88	98.94
8	0.06	0.53	10.94	99.47
9	0.05	0.48	10.99	99.95
10	0.01	0.05	11.00	100.00
11	0.00	0.00	11.00	100.00

Biological metrics evaluation before glyphosate spray

Means, standard deviations and ranges of biological metrics measured before glyphosate spray of the three sampling sites (i.e. spray zone (SZ), upstream of spray zone (USZ) and downstream of spray zone (DSZ)) for all four seasons are presented in Table 5.5. There were statistically significant differences in SASS5 scores, number of taxa, % Chironomidae + Oligochaeta, % ETOC, ETOC richness, Margalef's family richness, Shannon-diversity index and equitability among all three sampling sites (Table 5.5). In all cases, USZ recorded the highest scores, followed by SZ and DSZ, respectively. However, ASPT analysis revealed no significant difference between SZ and DSZ, but USZ was significantly higher than the other two sites, with lower ASPT values (Table 5.5). Seasonal performances of biological metrics as observed in the three sampling sites before glyphosate spray are presented in Figure 5.6.

Table 5.5: Means, standard deviations and ranges (in brackets) of measured biological metrics (n = 4) between the three sampling sites before glyphosate spray. The mean values of any two sites having the same superscript letter are not significantly different ($p > 0.05$)

Biological metrics	Sampling sites			
	SZ	USZ	DSZ	p-value
SASS5 score	69.75 ± 30.02^{a} (27.00-95.00)	128.25 ± 5.32^{b} (122.00-134.00)	33.00 ± 3.16^{c} (31.00-37.00)	< 0.01
Number of taxa	16.50 ± 5.57^{a} (9.00-22)	23.75 ± 0.50^{b} (23.00-24.00)	9.50 ± 1.29^{c} (8.00-11.00)	< 0.01
ASPT	4.07 ± 0.72^{a} (3.00-4.47)	5.40 ± 0.28^{b} (5.08-5.69)	3.49 ± 0.18^{a} (3.36-3.75)	< 0.01
% Chironomidae + Oligochaeta	54.76 ± 1.70^{a} (32.21-70.61)	43.53 ± 3.16^{b} (30.56-58.00)	78.33 ± 4.67^{c} (63.48-97.54)	< 0.01
% ETOC	16.37 ± 4.01^{a} (0-47.30)	28.21 ± 3.37^{b} (14.84-42.20)	1.03 ± 0.46^{c} (0-2.83)	< 0.01
ETOC richness	4.67 ± 0.58^{a} (0-6)	8.33 ± 1.53^{b} (7-11)	1.67 ± 0.58^{c} (0-3)	< 0.01
Margalef's family richness index	2.15 ± 0.25^{a} (1.02-2.76)	3.18 ± 0.18^{b} (2.32-3.80)	1.14 ± 0.10^{c} (0.69-1.45)	< 0.01
Shannon index	1.59 ± 0.12^{a} (0.98-1.81)	1.98 ± 0.05^{b} (1.35-2.49)	0.89 ± 0.06^{c} (0.60-1.12)	< 0.01
Equitability	0.63 ± 0.02^{a} (0.56-0.80)	0.69 ± 0.04^{b} (0.51-0.86)	0.46 ± 0.01^{c} (0.33-0.56)	< 0.01


SASS5 score
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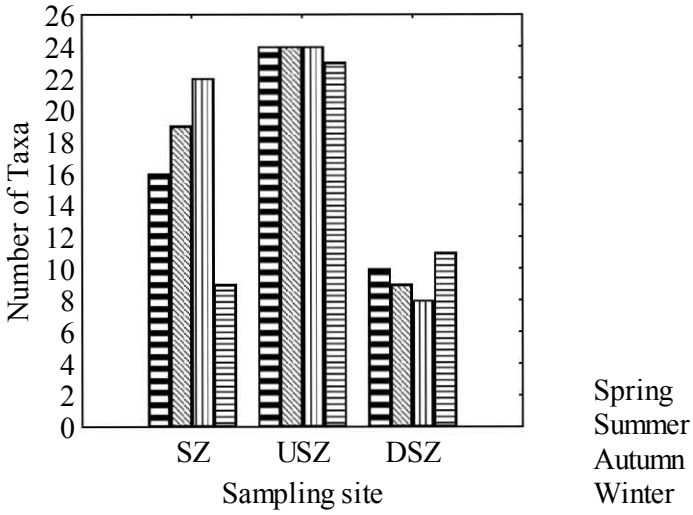
120
100
80
60
40
20
0
SZ
USZ
DSZ
Sampling site
Spring
Summer
Autumn
Winter


Number of Taxa
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22
20
18
16
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12
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8
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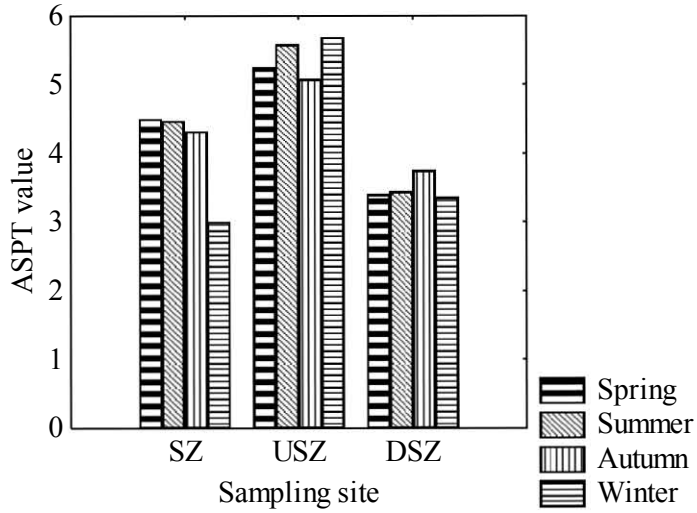
2
0
SZ
USZ
DSZ
Sampling site
Spring
Summer
Autumn
Winter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2
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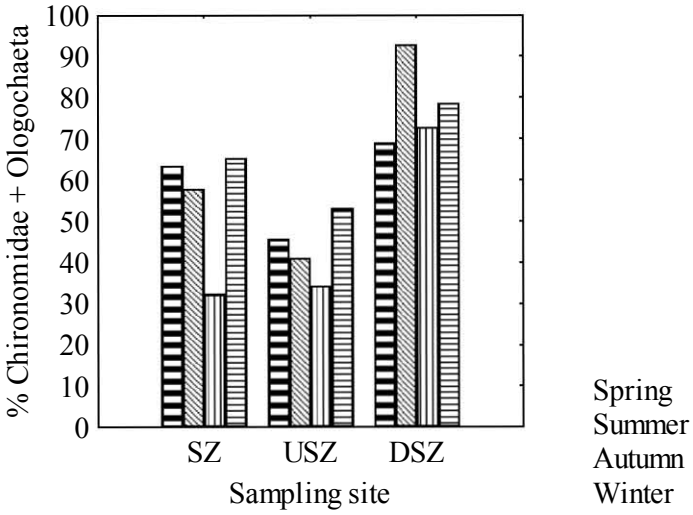
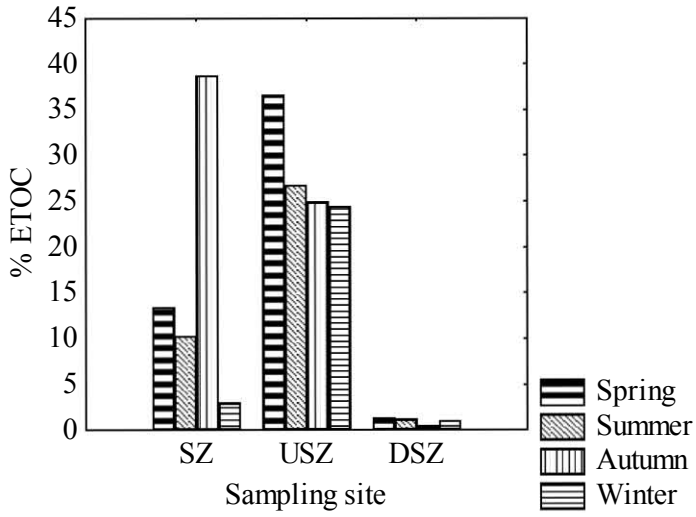
0
SZ
USZ
DSZ
Sampling site
Spring
Summer
Autumn
Winter


% Chironomidae + Ologochaeta
100
90
80
70
60
50
40
30
20
10
0
SZ
USZ
DSZ
Sampling site
Spring
Summer
Autumn
Winter


% ETOC
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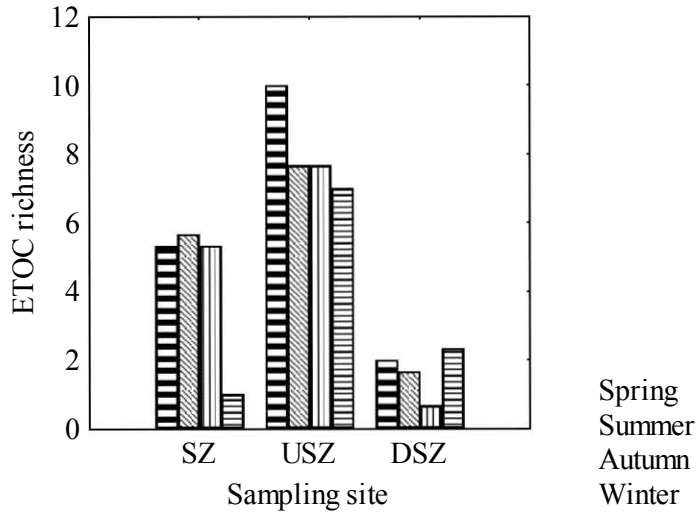
40
35
30
25
20
15
10
5
0
SZ
USZ
DSZ
Sampling site
Spring
Summer
Autumn
Winter


ETOC richness
12
10
8
6
4
2
0
SZ
USZ
DSZ
Sampling site
Spring
Summer
Autumn
Winter

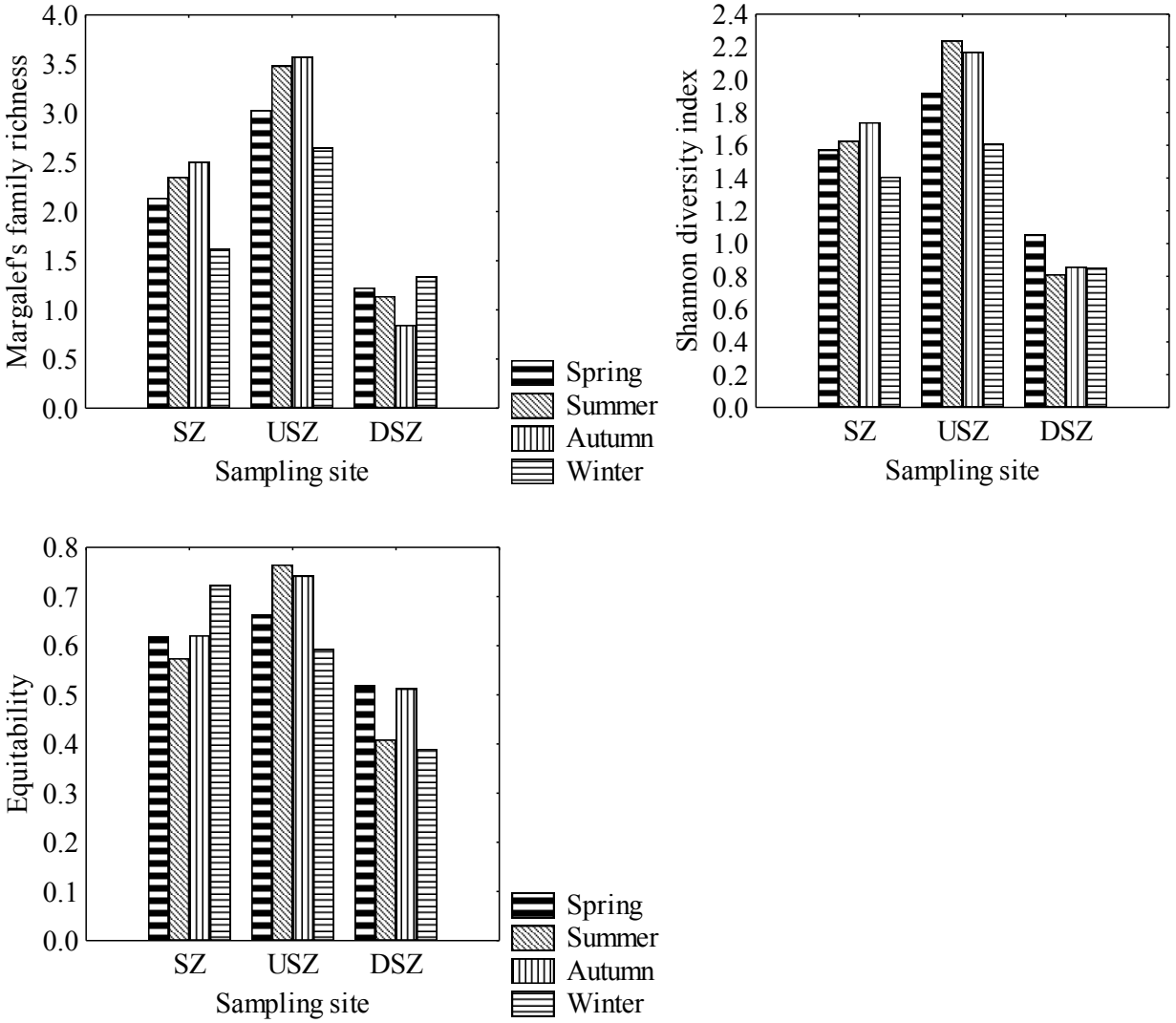


Figure 5.6: Seasonal variation of each biological metric at the three sampling sites in the Swartkops River before glyphosate spray

5.3.2 Physicochemical water quality variables and biological metrics conditions after glyphosate spray

Physicochemical water quality evaluation after glyphosate spray

Means, standard deviations and ranges of water quality variables measured after glyphosate spray at the three sampling sites, i.e. spray zone (SZ), upstream of spray zone (USZ) and downstream of spray zone (DSZ) are presented in Table 5.6. One-way analysis of variance (ANOVA) followed by Tukey post hoc tests were used to evaluate differences in water quality variables between sites. No significant difference between dissolved oxygen (DO), pH, temperature and turbidity for any of the three sampling sites was observed.

However, electrical conductivity (EC) was not significantly different between SZ and DSZ, but USZ, with a lower EC concentration, differed significantly from SZ and DSZ. Five days biochemical oxygen demand (BOD_5) was significantly different between all three sampling sites with the lowest concentration recorded for USZ, followed by SZ, and the DSZ recording the highest value. Analysis of nitrate-nitrogen (NO_3-N) and nitrite-nitrogen (NO_2-N) revealed similar observations of no significant difference between SZ and DSZ, but a significantly lower value was recorded in USZ. No significant differences were found between SZ and USZ for ammonium-nitrogen (NH_4-N) and orthophosphate-phosphorus (PO_4-P), but DSZ values were significantly higher than the other two sampling sites (SZ and USZ). Chlorophyll *a* phytoplankton was not significantly different at the three sampling sites, but significant differences were found for periphyton chlorophyll *a* at all sites.

Table 5.6: Means, standard deviations and ranges (in brackets) of measured physicochemical water quality variables (n = 4) among all sampling sites after glyphosate spray. Mean values of any two sites having the same superscript letter are not significantly different ($p > 0.05$)

WQ Variable	Sampling sites			
	SZ	USZ	DSZ	p-value
DO (mg/L)	6.49± 1.38 (5.21-8.54)	6.51 ± 0.40 (5.94-6.80)	5.28 ± 1.12 (3.79-6.51)	0.22
pH	7.16± 0.48 (6.64-7.80)	7.26 ± 0.85 (6.24-8.30)	8.36± 0.81 (7.65-9.21)	0.08
Temperature (ºC)	22.13± 2.02 (20.00-24.50)	19.12 ± 4.87 (12.5-24.00)	21.30 ± 2.31 (19.50-24.00)	0.45
Electrical Conductivity(mS/m)	393.00 ± 148.66^{a} (261-561)	18.07 ± 14.13^{b} (8.23-39.00)	339.00 ± 13.34^{a} (322.00-354.00)	< 0.01
Turbidity (NTU)	5.60± 1.74 (3.00-6.70)	3.31± 0.36 (3.00-3.68)	7.84 ± 3.85 (2.20-10.60)	0.08
BOD_5 (mg/L)	7.88 ± 2.08^{a} (6.56-10.96)	3.90 ± 1.54^{b} (2.16-5.88)	12.63 ± 1.00^{c} (11.64-13.98)	< 0.01
Nitrate-nitrogen (NO_3-N) (mg/L)	1.61 ± 0.74^{a} (0.97-2.67)	0.10 ± 0.07^{b} (0.03-0.19)	2.11 ± 0.49^{a} (1.57-2.68)	< 0.01
Nitrite-nitrogen (NO_2-N) (mg/L)	0.16 ± 0.10^{a} (0.01-0.23)	0.01 ± 0.00^{b} (0.00-0.01)	0.18 ± 0.07^{a} (0.09-0.24)	0.01
Ammonium - nitrogen (NH_4-N)(mg/L)	1.28 ± 0.68^{a} (0.43-2.10)	0.04 ± 0.02^{a} (0.00-0.05)	3.05 ± 1.18^{b} (1.62-4.35)	< 0.01
Orthophosphate-phosphorus (PO_4-P) (mg/L)	1.06 ± 0.99^{a} (0.40-2.50)	0.01 ± 0.00^{a} (0.00-0.01)	6.35 ± 0.61^{b} (5.72-7.04)	< 0.01
Phytoplankton chlorophyll *a* (µg/L)	3.44± 3.39 (0.46-7.17)	1.30 ± 0.61 (0.52-1.93)	5.30 ± 2.56 (2.74-8.27)	0.13
Periphyton chlorophyll *a* (µg/cm^2)	266.06 ± 31.59^{a} (239.23-306.09)	145.03 ± 96.96^{b} (48.96-262.19)	384.98 ± 121.53^{c} (244.81-518.40)	0.02

Based on the measured physicochemical water quality variables, Principal Component Analysis (PCA) classified the sites after glyphosate spray into three distinct groups (Figure 5.7). The PCA axes 1, 2 and 3 corresponding to eigenvalues 5.74, 1.86, and 1.29 accounted for approximately 52.19, 16.94 and 11.74 % of the total variance, respectively. Thus, the first three eigenvalues accounted for approximately 80.87 % of the total variation among measured water quality variables, indicating good ordination (Table 5.7). The PCA results revealed an apparent classification of the sites into predominantly USZ to the right, predominantly DSZ to the left, and SZ sandwiched between these two zones (Figure 5.7).

Unlike before glyphosate spray classification of the sampling sites, there was clear separation of SZ from USZ and DSZ. However, the classification of SZ is more towards DSZ than USZ, indicating that water quality at this site is better than DSZ but poorer than USZ (Figure 5.7).

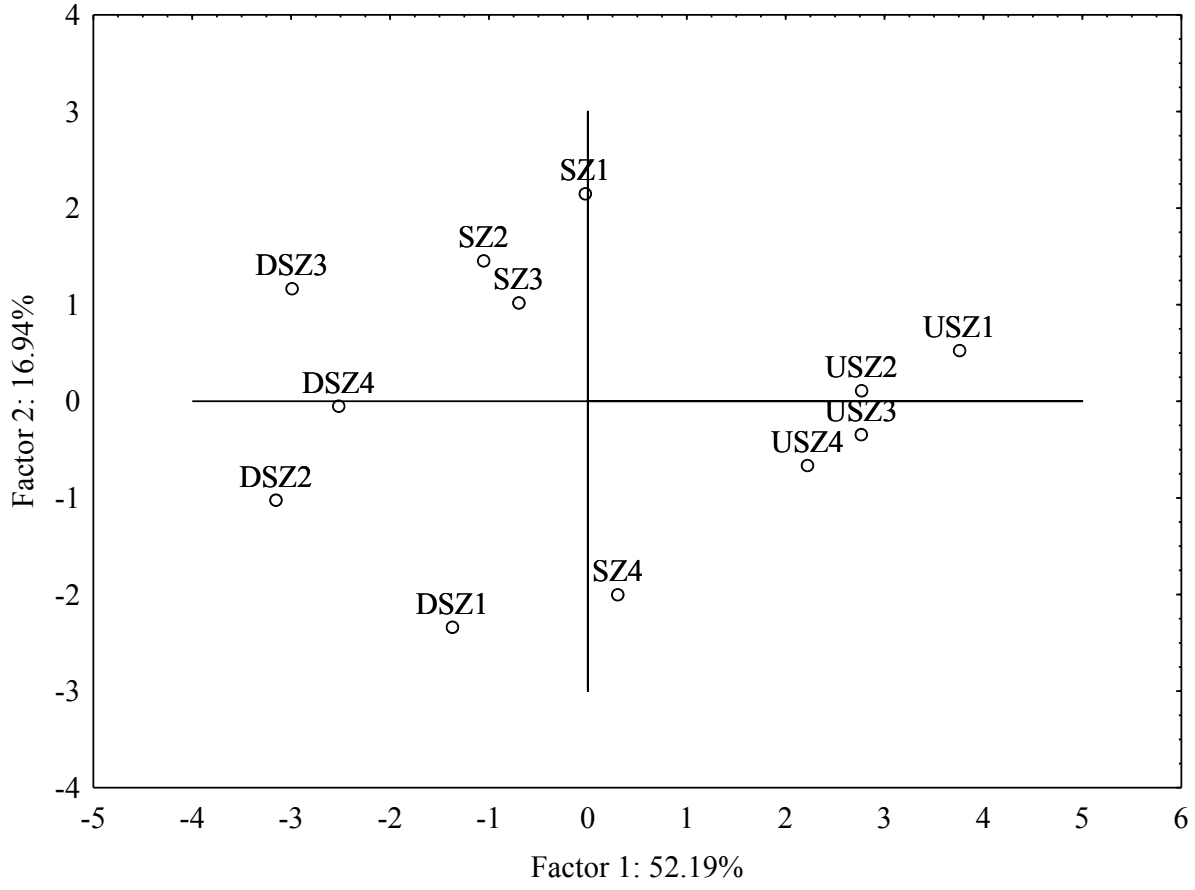


Figure 5.7: After glyphosate spray PCA ordination bi-plots for physicochemical water quality variables at all three sampling sites in the Swartkops River for each sampling season. Letters represent sites (SZ = spray zone; USZ = upstream spray zone; DSZ = downstream spray zone) and numbers attached to letters represent different sampling seasons (1 = Week 1; 2 = Week 2; 3 = Week 3; 4 = Week 4)

Table 5.7: Eigenvalues of correlation matrix used in PCA ordination bi-plots for physicochemical water quality variables at all three sampling sites after glyphosate spray. Only first three eigenvalues greater than 1 (shaded) were used in the analysis

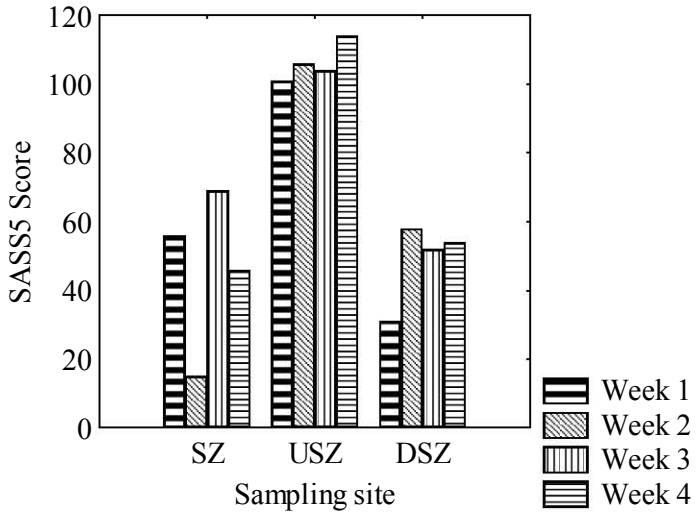
Eigenvalue number	Eigenvalue	Total variance (%)	Cumulative Eigenvalue	Cumulative total variance (%)
1	5.74	52.19	5.74	52.19
2	1.86	16.94	7.60	69.13
3	1.29	11.74	8.90	80.87
4	0.87	7.91	9.77	88.78
5	0.76	6.92	10.53	95.71
6	0.20	1.84	10.73	97.55
7	0.15	1.32	10.88	98.87
8	0.07	0.65	10.95	99.51
9	0.05	0.48	11.00	99.99
10	0.00	0.01	11.00	100.00
11	0.00	0.00	11.00	100.00

Biological metrics evaluation after glyphosate spray

Means, standard deviations and ranges of macroinvertebrate metrics measured after glyphosate spray at the three sampling sites (i.e. spray zone (SZ), upstream of spray zone (USZ) and downstream of spray zone (DSZ)) are presented in Table 5.8. One-way analysis of variance (ANOVA) followed by Tukey post hoc tests revealed significant differences in SASS5 scores, number of taxa, % Chironomidae + Oligochaeta and Shannon-diversity index between all three sampling sites (Table 5.8). However, ANOVA revealed no significant difference for ASPT values between SZ and DSZ, but USZ was significantly higher than the other two sites with lower ASPT values (Table 5.8). The metrics ASPT, % ETOC, ETOC richness and Margalef's family richness were significantly higher at USZ than at SZ and DSZ, which were not significantly different from each other (Table 5.8). ANOVA evaluation of Equitability revealed no significant difference between SZ and USZ, but DSZ values were significantly lower than the other two sampling sites (Table 5.8). Weekly variation of each biological metric as observed in the three sampling sites after glyphosate spray are presented in Figure 5.8.

Table 5.8: Means, standard deviations and ranges (in brackets) of measured biological metrics (n = 4) among all sampling sites after glyphosate spray. Mean values of any two sites having the same superscript letter are not significantly different

Biological metrics	Sampling sites			
	SZ	USZ	DSZ	p-value
SASS5 score	46.50± 23.01[a] (15.00-69.00)	106.25± 5.56[b] (101-114)	48.75± 12.09[a] (31.00-58.00)	0.00
Number of taxa	13.00 ± 5.48[a] (5.00-17.00)	19.50 ± 1.73[b] (17.00-21.00)	14.50 ± 0.16[a] (10.00-17.00)	0.00
ASPT	3.46 ± 0.45 (3.00-4.06)	5.47 ± 0.42 (5.05-5.94)	3.34± 0.16 (3.10-3.47)	0.09
% Chironomidae + Oligochaeta	38.18 ± 3.70[a] (20.50-61.55)	24.48 ± 2.84[b] (6.94-38.89)	48.26 ± 5.70[c] (11.68-70.05)	0.00
% ETOC	3.99 ± 0.30[a] (1.13-6.60)	48.79 ± 5.45[b] (36-50-63.47)	11.13 ± 5.14[a] (3.37-24.71)	0.00
ETOC richness	3.33 ± 0.58[a] (1-5)	11.33 ± 0.58[b] (7-15)	4.33 ± 0.58[a] (3-6)	0.00
Margalef's family richness index	1.75 ± 0.08[a] (1.46-2.10)	3.49 ± 0.26[b] (2.20-4.49)	2.01 ± 0.10[a] (1.40-2.48)	0.00
Shannon index	1.80 ± 0.04[a] (1.55-1.97)	2.26 ± 0.03[b] (1.74-2.67)	1.52 ± 0.18[c] (0.97-2.05)	0.00
Equitability	0.72 ± 0.02[a] (0.59-0.82)	0.74 ± 0.01[a] (0.66-0.86)	0.58 ± 0.05[b] (0.37-0.76)	0.00


SASS5 Score
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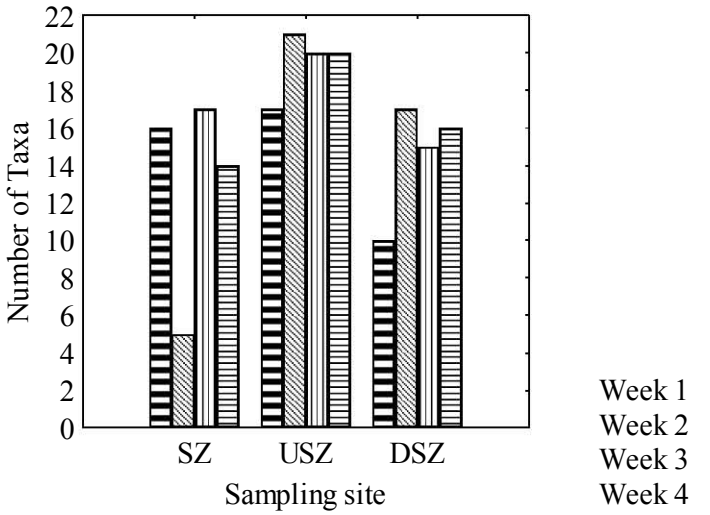
100
80
60
40
20
0
SZ
USZ
DSZ
Sampling site
Week 1
Week 2
Week 3
Week 4


Number of Taxa
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2
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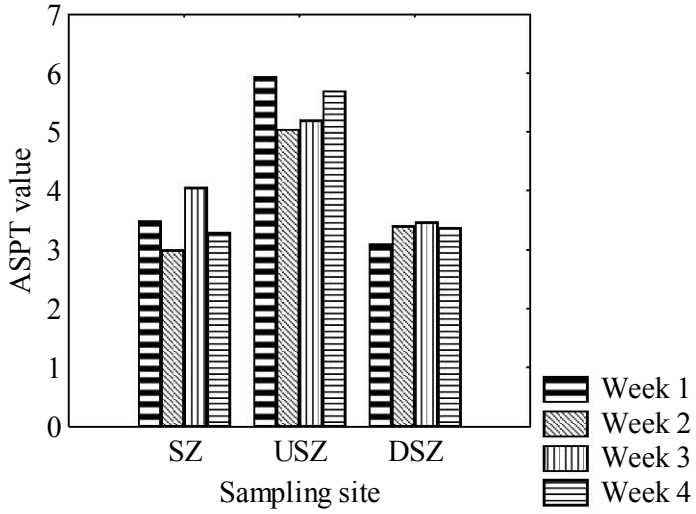
USZ
DSZ
Sampling site
Week 1
Week 2
Week 3
Week 4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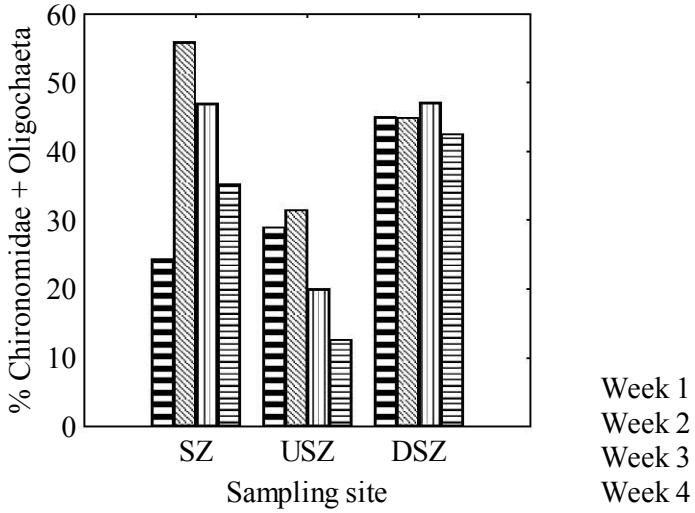
SZ
USZ
DSZ
Sampling site
Week 1
Week 2
Week 3
Week 4


% Chironomidae + Oligochaeta
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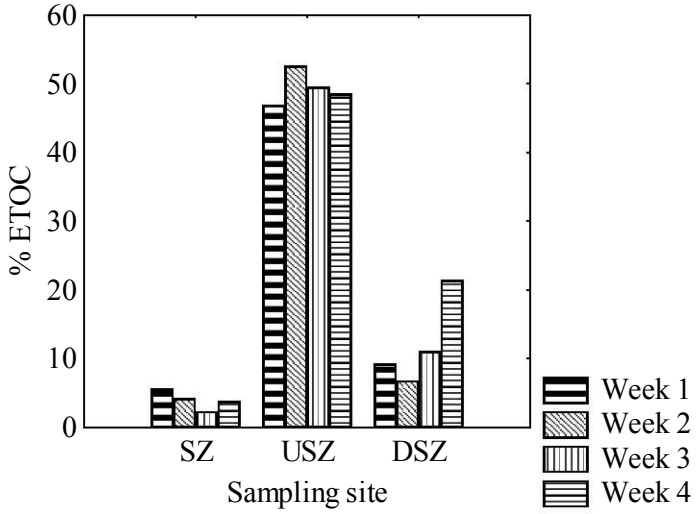
50
40
30
20
10
0
SZ
USZ
DSZ
Sampling site
Week 1
Week 2
Week 3
Week 4


% ETOC
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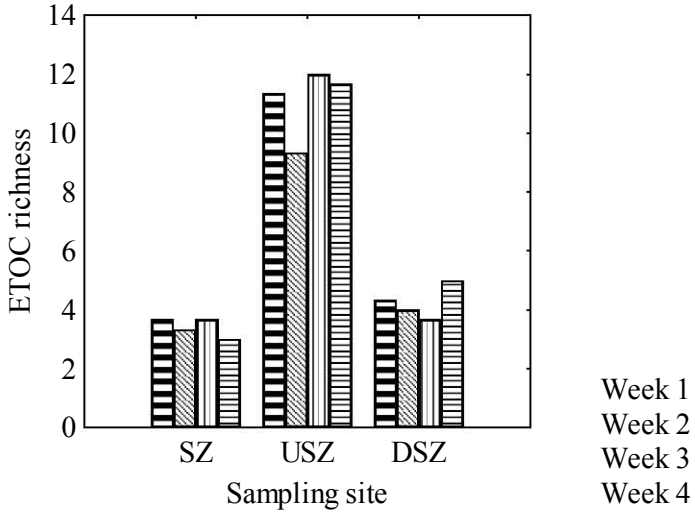
40
30
20
10
0
SZ
USZ
DSZ
Sampling site
Week 1
Week 2
Week 3
Week 4


ETOC richness
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8
6
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0
SZ
USZ
DSZ
Sampling site
Week 1
Week 2
Week 3
Week 4

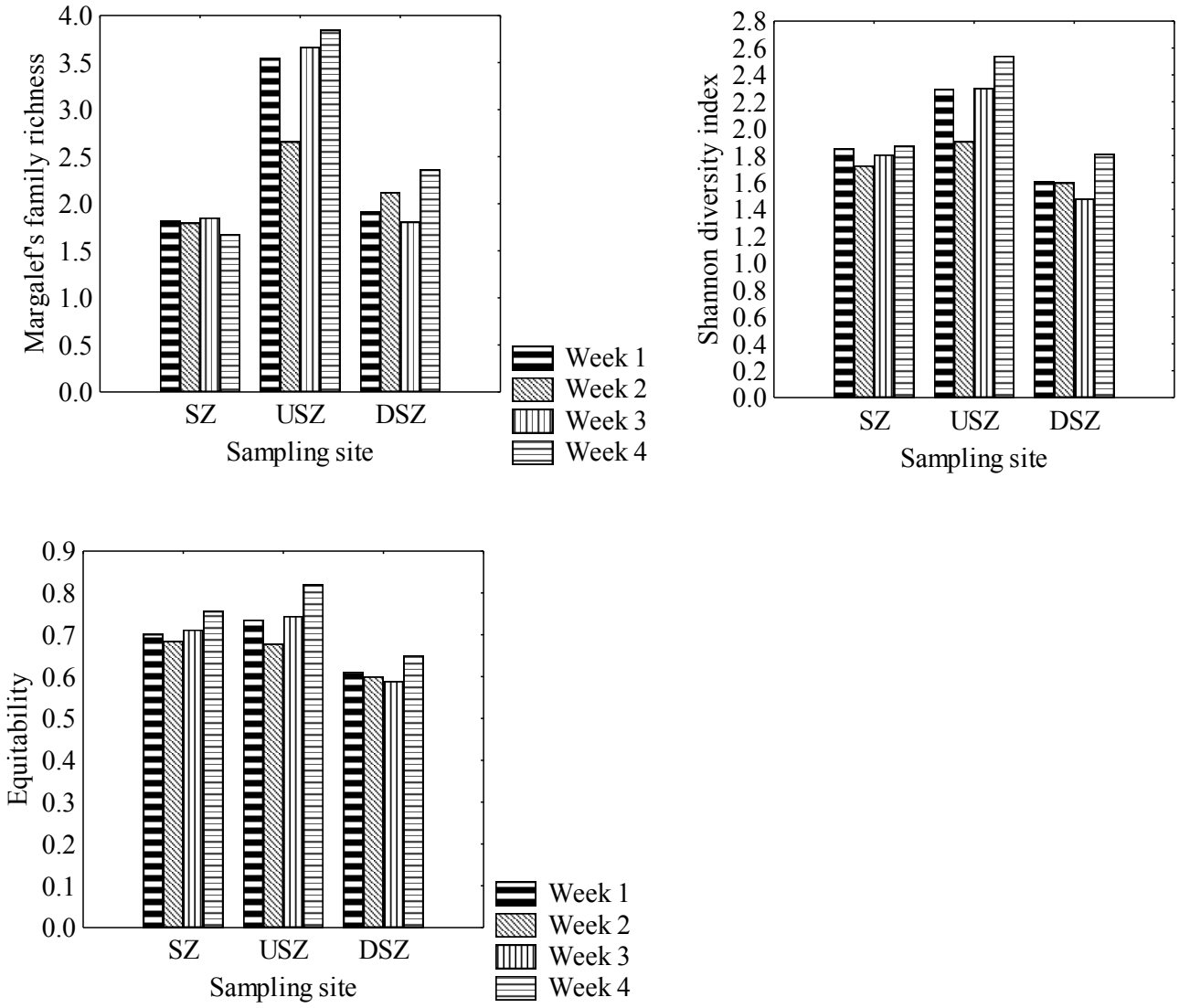

Figure 5.8: Weekly performance of each biological metric at the three sampling sites in the Swartkops River after glyphosate spray

5.3.3 Comparisons between physicochemical water quality variables and biological metrics before and after glyphosate spray

Variations in physicochemical water quality variables before and after glyphosate spray

In order to determine if there was a significant difference between a physicochemical water quality variable measured before and after glyphosate spray, a paired t-test was separately performed on the difference for each variable for all three sampling sites, i.e. spray zone (SZ), upstream of spray zone (USZ) and downstream of spray zone (DSZ). The calculated t-values and p-values (two-tails) are presented in Table 5.9.

Water physicochemical analysis at DSZ for dissolved oxygen (DO) revealed that the mean difference between before and after spray values was statistically different ($p < 0.05$), but not significantly different in SZ and USZ. A paired t-test for turbidity showed that the mean difference was statistically significant in USZ ($p < 0.05$) but not significantly different in SZ and DSZ. Although electrical conductivity (EC) analyses revealed significant mean differences in SZ and DSZ ($p < 0.05$), there was no evidence that the mean difference was statistically different in USZ ($p > 0.05$). Paired t-tests conducted to test the mean differences for pH, temperature and five days biochemical oxygen demand (BOD_5) showed no statistically significant mean differences between the three sampling sites ($p > 0.05$). Nutrient analysis for ammonium-nitrogen (NH_4-N) provided evidence that the mean difference was statistically different in SZ and USZ ($p < 0.05$) but not significantly different in DSZ ($p > 0.05$). However, paired t-tests performed to test the mean differences for nitrate-nitrogen (NO_3-N), nitrite-nitrogen (NO_2-N), orthophosphate-phosphorus (PO_4-P), phytoplankton chlorophyll *a* and periphyton chlorophyll *a* did not reveal any statistically significant differences ($p > 0.05$) for the three sampling sites.

Table 5.9: Mean differences, standard deviations, paired t-test and p-values calculated for each physicochemical variable before and after glyphosate spray events at the three sampling sites in the Swartkops River

WQ variables	Sampling sites		
	SZ	**USZ**	**DSZ**
DO (mg/L)	0.51 ± 2.58 (t = 0.39, p = 0.72)	-0.11 ± 1.47 (t = 0.15, p = 0.89)	-3.49 ± 1.67 (t = 4.17, p = 0.03)
pH	-0.04 ± 1.27 (t =0.06, p = 0.96)	-0.66 ± 1.26 (t =1.05, p = 0.37)	-1.13 ± 0.98 (t =2.31, p = 0.10)
Temperature (ºC)	-4.41 ± 8.15 (t =1.08, p = 0.36)	-1.35 ± 1.40 (t =1.93, p = 0.15)	-2.23 ± 4.19 (t =1.06, p = 0.37)
Electrical Conductivity(mS/m)	-352.25 ± 146.57 (t = 4.81, p = 0.02)	12.15 ± 14.50 (t =1.68, p = 0.19)	-83.50 ± 22.13 (t = 7.55, p = 0.01)
Turbidity (NTU)	0.55 ± 1.92 (t =0.57, p = 0.61)	4.48 ± 2.43 (t = 3.69, p = 0.04)	1.74± 9.91 (t = 0.35, p = 0.75)
BOD_5 (mg/L)	0.79 ± 3.51 (t =0.45, p = 0.69)	0.85 ± 2.34 (t =0.73, p = 0.52)	0.13± 6.92 (t =0.04, p = 0.97)
Nitrate-nitrogen (NO_3-N) (mg/L)	-0.69 ± 1.81 (t =0.77, p = 0.50)	0.02± 0.13 (t =0.27, p = 0.80)	-0.11 ± 0.96 (t =0.22, p = 0.84)
Nitrite-nitrogen (NO_2-N) (mg/L)	-0.09 ± 0.20 (t =0.94, p = 0.42)	0.02 ± 0.01 (t =3.00, p = 0.06)	-0.01 ± 0.09 (t =0.11, p = 0.92)
Ammonium - nitrogen (NH_4-N)(mg/L)	-0.71 ± 0.43 (t = 3.28, p = 0.05)	0.18 ± 0.08 (t = 4.45, p = 0.02)	0.20 ± 3.38 (t =0.12, p = 0.91)
Orthophosphate-phosphorus (PO_4-P) (mg/L)	0.047 ± 0.86 (t =0.11, p = 0.92)	-0.00 ± 0.01 (t =0.52, p = 0.64)	0.25± 1.17 (t =0.43, p = 0.70)
Phytoplankton chlorophyll *a* (µg/L)	0.40 ± 5.46 (t =0.15, p = 0.89)	-0.84 ± 0.67 (t =2.47, p = 0.09)	-3.62 ± 2.59 (t =2.80, p = 0.07)
Periphyton chlorophyll *a* (µg/cm^2)	120.10 ± 212.90 (t =1.13, p = 0.34)	-116.49 ± 102.44 (t =2.27, p = 0.11)	223.63 ± 154.55 (t =2.89, p = 0.06)

Variations in biological metrics before and after glyphosate spray

A paired t-test was performed for each biological metric for all three sampling sites (i.e. spray zone (SZ), upstream of spray zone (USZ) and downstream of spray zone (DSZ)) in order to determine if there was a significant difference between a measured biological metric before and after glyphosate spray. The calculated t-values and p-values (two-sided) are presented in Table 5.10. Analysis of SASS5 scores and % Chironomidae + Oligochaeta revealed that the mean differences between before and after spray values were statistically different in all three sampling sites ($p < 0.05$) (Table 5.10).

Evaluation of the Number of Taxa showed that mean difference between before and after spray values was statistically different in SZ and DSZ ($p < 0.05$) but not USZ. In contrast, the mean difference between before and after spray ASPT values in USZ was significantly different ($p < 0.05$) but those of SZ and DSZ were not significantly different ($p > 0.05$). The mean difference between before and after spray ETOC richness values was statistically not different in SZ and USZ ($p < 0.05$) but statistically different in DSZ ($p < 0.05$). Similar observations (as in ETOC richness) were made for Margalef's family richness and Equitability. For % ETOC, the mean difference between before and after spray values was statistically different in SZ and USZ ($p < 0.05$) but not DSZ, whereas the mean difference for the Shannon-diversity index was statistically different in USZ and DSZ ($p < 0.05$) but not SZ.

Table 5.10: Mean differences, standard deviations, paired t-test and p-values calculated for each biological metric for all three sampling sites in the Swartkops River before and after glyphosate spray

Biological metrics	**Sampling sites**		
	SZ	**USZ**	**DSZ**
SASS5 score	-9.82 ±2.03 (t=8.36, p = 0.01)	24.13 ±9.15 (t=4.57, p = 0.04)	26.32 ±6.12 (t=7.45,p = 0.02)
Number of taxa	-1.58 ±0.38 (t= 7.31, p = 0.02)	2.82 ±1.70 (t= 2.86, p = 0.10)	6.70 ±0.54 (t=21.46, p = 0.00)
ASPT	-0.28 ±0.20 (t=2.41, p = 0.14)	0.51 ±0.18 (t=4.93, p = 0.04)	0.52 ±0.70 (t=1.28, p = 0.33)
% Chironomidae + Oligochaeta	-16.58 ±5.19 (t=5.53, p = 0.03)	-19.05 ±5.37 (t=6.14, p = 0.03)	-30.07 ±10.19 (t=5.11, p = 0.04)
% ETOC	-12.37 ±3.72 (t=5.77, p = 0.03)	20.58 ±4.34 (t=8.22, p = 0.01)	10.10 ±4.80 (t=3.65, p = 0.07)
ETOC richness	-1.27 ±0.63 (t=3.47, p = 0.07)	3.12 ±2.37 (t=2.28, p = 0.15)	2.60 ±0.91 (t=4.94, p = 0.04)
Margalef's family richness index	-0.40 ±0.17 (t=4.12, p = 0.06)	0.31 ±0.44 (t=1.22, p = 0.35)	0.87 ±0.15 (t=9.98, p = 0.01)
Shannon index	0.21 ±0.14 (t=2.69, p = 0.12)	0.28 ±0.03 (t=18.33, p = 0.00)	0.63 ±0.15 (t=7.21, p = 0.02)
Equitability	0.09 ±0.04 (t=4.27, p = 0.06)	0.05 ±0.04 (t=2.17, p = 0.16)	0.12 ±0.04 (t=5.29, p = 0.03)

5.3.4 Response of biological metrics to physicochemical water quality variables before and after glyphosate spray

Canonical Correspondence Analysis (CCA) of before and after glyphosate spray revealed a strong relationship between biological metrics and physicochemical water quality variables. However, multi-collinearity analysis found most of both the biological and physicochemical variables were redundant, having high R-squared values (R^2> 0.8 implies redundancy). Thus, based on the multi-collinearity analysis, % ETOC, ETOC richness and SASS5 scores were selected as biological data and tested against dissolved oxygen (DO), electrical conductivity (EC), total inorganic nitrate (TIN), turbidity and pH as environmental data. The CCA ordination plot before glyphosate spray (Figure 5.9) showed that DO was the most important variable that influenced % ETOC and ETOC richness at USZ. However, pH was found to be the principal variable influencing SASS5 scores at DSZ. None of the selected biological metrics was associated with the suite of physicochemical variables found at DSZ and SZ. The CCA ordination plot (Figure 5.10) after glyphosate spray showed that DO was the most important variable that impacted on % ETOC, ETOC richness and SASS5 scores at USZ. None of the tested biological indices was found at DSZ and SZ after glyphosate spray, a situation similar to that before glyphosate spray CCA ordination plot.

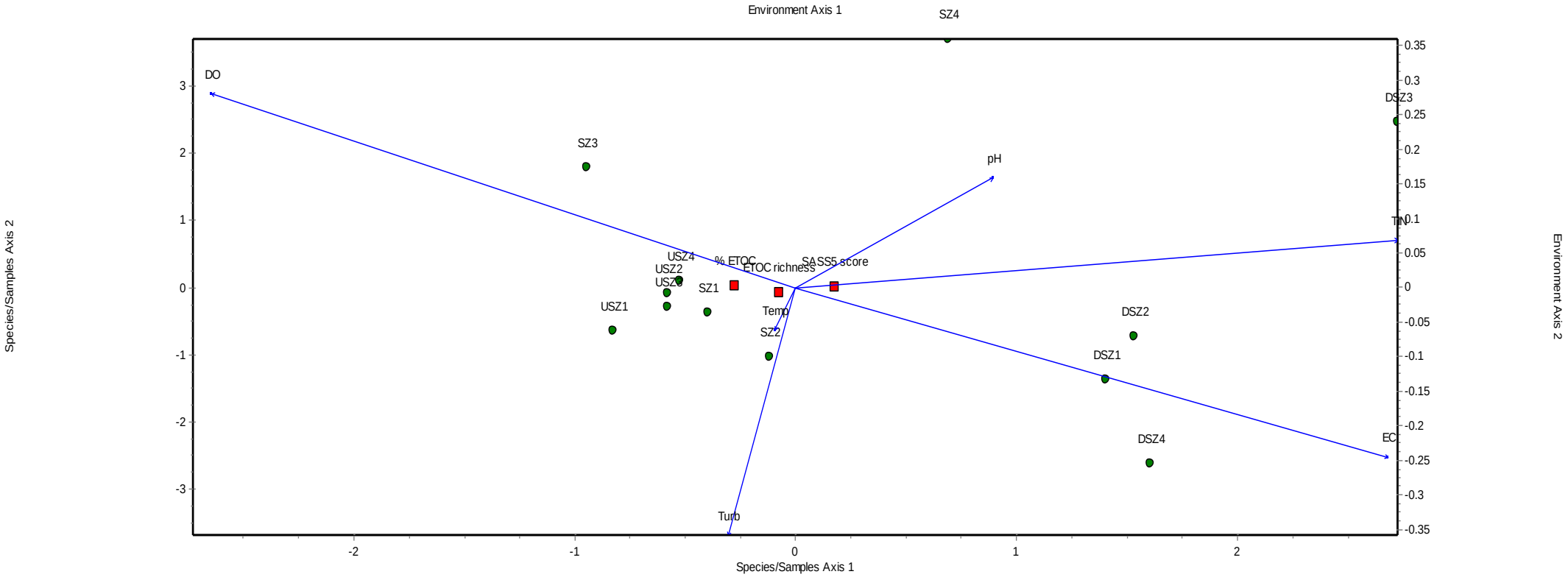

Figure 5.9: Canonical Correspondence Analysis (CCA) ordination plot for selected seasonally measured biological metrics and physicochemical variables before glyphosate spray at the three sampling sites. Letters represent sites (SZ = spray zone; USZ = upstream spray zone; DSZ = downstream spray zone) and numbers attached to letters represent different sampling seasons (1 = Autumn; 2 = Winter; 3 = Spring; 4 = Summer)

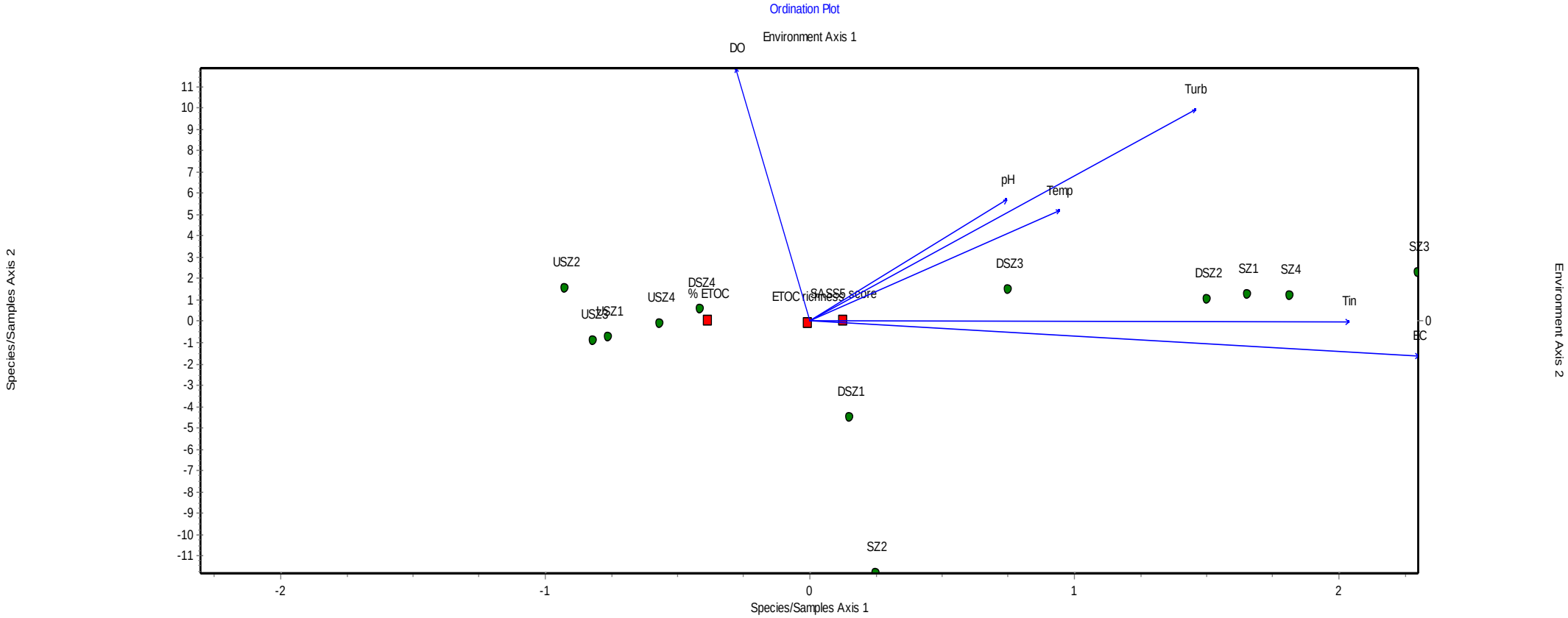

Figure 5.10: Canonical Correspondence Analysis (CCA) ordination plot for selected weekly measured biological metrics and physicochemical variables after glyphosate spray at the three sampling sites. Letters represent sites (SZ = spray zone; USZ = upstream spray zone; DSZ = downstream spray zone) and numbers attached to letters represent different sampling seasons (1 = Week 1; 2 = Week 2; 3 = Week 3; 4 = Week 4)

5.3.5 River health assessment of sampling sites before and after glyphosate spray

River health status of each sampling site before and after glyphosate spray was assessed using SASS5 scores and ASPT values. The biological bands provided in Table 5.2 were used for the interpretation of results. The mean total SASS5 score before glyphosate spray revealed that the overall water quality category was "good" for USZ, and "critically modified" for SZ and DSZ (Table 5.11). However, the mean ASPT value placed the overall water quality category as "fair" for USZ, and "critically modified" for SZ and DSZ (Table 5.12). Similar overall water quality categories were observed for after spray with glyphosate. The only change in classification was SZ which decreased from "poor" to "critically modified" in terms of SASS score.

Table 5.11: Before glyphosate spray water quality categories for each of the three sampling sites in the Swartkops River based on SASS5 and ASPT biological bands for the southern eastern coastal belt (lower zone) ecoregion

Biological index	**Sampling site**		
	SZ	**USZ**	**DSZ**
SASS5 score			
Spring	Poor	Good	Critically modified
Summer	Fair	Good	Critically modified
Autumn	Fair	Good	Critically modified
Winter	Critically modified	Good	Critically modified
Overall quality	**Poor**	**Good**	**Critically modified**
ASPT			
Spring	Critically modified	Fair	Critically modified
Summer	Critically modified	Fair	Critically modified
Autumn	Critically modified	Poor	Critically modified
Winter	Critically modified	Fair	Critically modified
Overall quality	**Critically modified**	**Fair**	**Critically modified**

Table 5.12: After glyphosate spray water quality categories for each of the three sampling sites in the Swartkops River based on SASS5 and ASPT biological bands for the southern eastern coastal belt (lower zone) ecoregion

Biological index	Sampling site		
	SZ	**USZ**	**DSZ**
SASS5 score			
Week 1	Critically modified	Good	Critically modified
Week 2	Critically modified	Fair	Critically modified
Week 3	Critically modified	Good	Critically modified
Week 4	Critically modified	Good	Critically modified
Overall quality	**Critically modified**	**Good**	**Critically modified**
ASPT			
Week 1	Critically modified	Fair	Critically modified
Week 2	Critically modified	Fair	Critically modified
Week 3	Critically modified	Fair	Critically modified
Week 4	Critically modified	Poor	Critically modified
Overall quality	**Critically modified**	**Fair**	**Critically modified**

5.4 Discussion

5.4.1 Spatio-temporal variations between before and after glyphosate spray data sets

Although the before-spray sampling were performed seasonally (Spring, Summer, Autumn and Winter) and the after-spray weekly for only four weeks (Summer, 2011), the basis for comparison of the two data sets was not the time difference, but rather the number of data points. Dallas (2007), regardless of time difference, used the number of data points of SASS5 Scores and ASPT values to generate RHP data for the various ecoregions of South Africa. Thus, the before-spray data comprising four data points (Spring, Summer, Autumn, Winter) were compared to four data points of after-spray (Week 1, Week 2, Week 3, Week 4) in this context.

5.4.2 Physicochemical water quality condition before and after glyphosate spray

Analysis of physicochemical water quality variables before spray revealed distinct demarcation among the three sampling sites (SZ, USZ and DSZ). The USZ (positive control was least impacted, followed by SZ and DSZ (negative control). Similar observations were made after spray with glyphosate.

However, comparisons of before and after spray data (Table 5.9), revealed that most water quality variables, including dissolved oxygen (DO), turbidity, pH, temperature and five days biochemical oxygen demand (BOD_5) in both SZ and DSZ, were not significantly different, which seemed to suggest that the spray did not affect these variables. Thus, the deteriorated water quality variable at SZ and DSZ might be due to high organic loading and industrial effluent discharges as observed in the levels BOD_5, nitrite-nitrogen (NO_2-N), nitrate-nitrogen (NO_3-N), ammonium-nitrogen (NH_4-N), orthophosphate-phosphorus (PO_4-P), electrical conductivity (EC) and DO. Such observations might be attributed to effluents from the Uitenhage and Despatch Waste Water Treatment Works (WWTW) (Odume, 2011; Odume and Muller, 2011). The DSZ exuded an obnoxious odour throughout the sampling period, which may have been caused by anaerobic decomposition of organic matter producing gases such as methane and hydrogen sulphide, which are harmful to sessile macroinvertebrates (Odume, 2011). This probably contributed to the poor macroinvertebrate metrics that were observed in the SZ and DSZ, rather than the glyphosate spray. Odume (2011) contends that the nutrient levels recorded at the SZ and DSZ could cause mesotrophic and eutrophic conditions when compared to the South African water quality guidelines for aquatic ecosystems. Odume and Muller (2011) and Odume *et al*. (2012) further argued that the relatively high electrical conductivity (EC) at SZ and DSZ might have resulted from discharges of effluents from WWTWs and the automotive industry around these sites. In addition to these, the natural geology and marine origin of the Swartkops River catchment's soil could result in naturally high EC levels. Since macroinvertebrates are particularly sensitive to changes in EC (Dallas and Day, 2004) the disappearance of sensitive taxa from the SZ and DSZ could be attributed to high levels of EC. Thus, it is likely that the contribution of glyphosate spray to poor water quality of the SZ was not significant.

Eutrophication is a primary water quality issue in most freshwater ecosystems around the world with its impact assuming a global concern as it adversely affects the structure and functioning of affected ecosystems (Hart, 2011). Bulk nutrient loading (mostly nitrogen and phosphorus compounds) of many inland South African reservoirs derives from wastewater return flows, which leads to algal blooms (Hart, 2011).

The result from this study revealed that nitrate-nitrogen (NO_3-N), nitrite-nitrogen (NO_2-N), orthophosphate-phosphorus (PO_4-P), phytoplankton chlorophyll *a* and periphyton chlorophyll *a* did not reveal any statistically significant differences for the three sampling sites of the Swartkops River before and after glyphosate spray. Therefore, it is possible to speculate that effect of glyphosate on growth of water hyacinth at the SZ was not significantly different from the non-sprayed sites.

5.4.3 Macroinvertebrate community structure before and after glyphosate spray events

Aquatic macroinvertebrates are used in biomonitoring programmes because they exhibit varying degrees of sensitivity to environmental water quality deterioration. The presence or absence of macroinvertebrates therefore reflects the water quality status and aquatic health of the environment in which they live. Thus, from the perspective of environmental water quality, the presence of macroinvertebrates perceived to be sensitive to water quality deterioration at a particular site may signify good quality (Arimoro and Muller, 2010; Fouche and Vlok, 2010; Odume, 2011).

During before and after glyphosate spray events, macroinvertebrates sampled from all three sites (SZ, USZ and DSZ) were quite distinct between the sites. Families such as Baetidae, Baetidae 2sp, Hydropsychidae and Hydropsychidae 2sp were commonly found in USZ (Appendices M and N). These taxa are indicative of largely unimpaired water quality status. On the contrary, the families Simulidae, Oligochaeta and Chironomidae that have a high tolerance to polluted environments were commonly found at SZ and DSZ, although more abundantly at DSZ. The families Chironomidae and Oligochaeta are regarded as highly tolerant among aquatic macroinvertebrates because of their ability to survive in oxygen-depleted environments (Arimoro, 2009). Odume and Muller (2011) argued that most chironomids feed on fine particulate organic matter including algae, and their increase may be related to an increase in periphyton and phytoplankton resulting from high nutrient levels. Thus the dominance of chironomids and oligochaetes at the SZ and DSZ before and after glyphosate spray could be attributed to their ability to thrive facultatively in an environment with high organic loads. This means that glyphosate spray has little or no adverse impact on these families. This observation was supported by chironomid exposure to glyphosate provided in Chapter Four of this thesis.

The before spray results showed that families within the orders Diptera, Coleoptera and Hemiptera were the most ubiquitous groups, and therefore recorded in all three sampling sites throughout the sampling period (Appendix M). Most families within these orders, including Dytiscidae, Elmidae, Gyrinidae, Belostomatidae, Corixidae, Gerridae and Notonectidae are air-breathers capable of replenishing their oxygen supply directly from the atmosphere and were less affected by depleted oxygen levels in water (Odume, 2011). However, after spray results revealed a low presence of these families in the SZ (Appendix N). In fact, the effect was visible a few hours after spray (Appendix O). This observation could be attributed to the fact that these families are air-breathers and therefore it is possible direct spray of glyphosate on the river might have interfered with the breathing systems. Such an observation was confirmed in a laboratory test where members of these families experienced high mortality minutes after exposure to low levels of glyphosate, whereas families such as Chironomidae, Oligochaeta and Simulidae survived for a relatively longer period.

5.4.4 Biological metrics condition before and after glyphosate spray

It is an oversimplification to assume the health status of a river site based solely on the presence or absence of macroinvertebrate taxa. However, subjecting the occurrence of different taxa to multimetric analysis will better evaluate the extent to which such occurrences could reflect the health status of the site. In order to use multimetrics as indicators of river health requires understanding the effects of environmental water quality deterioration on different aspects of aquatic macroinvertebrate communities (Odume and Muller, 2012). In this study, different biological metrics (multimetric) including SASS5 Scores, Number of Taxa, ASPT, composition (% Chironomidae + Oligochaeta and % ETOC), richness (ETOC richness and Margalef's family richness) and diversity (Shannon-diversity index and Equitability) were used to evaluate how different taxa or groups of taxa responded to changes in environmental water quality, particularly glyphosate spray. Before-spray analysis revealed significant differences in all metrics, except ASPT, among all three sampling sites (SZ, USZ and DSZ), while after-spray evaluation showed significant differences in SASS5 scores, number of taxa, % Chironomidae + Oligochaeta and Shannon diversity index in all three sampling sites.

It is therefore possible that macroinvertebrate metrics in the USZ performed better than those in SZ and DSZ because of relatively good environmental water quality in the USZ compared to that of SZ and DSZ. Thus, it could be speculated that glyphosate spray at SZ did not significantly affect the macroinvertebrates community as shown by the measured metrics (i.e. SASS5 Scores, Number of Taxa, ASPT, composition, richness and diversity).

Before and after glyphosate spray river health evaluation revealed that SASS5 scores and ASPT values were higher at USZ but lower at SZ and DSZ throughout the sampling periods. Thus, SASS5 scores and ASPT values categorised the environmental water quality at the USZ as "good" and "fair", respectively, but both of these indices categorised water quality at SZ and DSZ as "critically modified". This (the categorisation of water quality at SZ and DSZ as "critically modified") could be attributed to discharges from the Uitenhage Waste Water Treatment Work and run-off from informal settlements around these sites. This confirms the DWAF (1996b) water resources situational analysis of the Swartkops River and, more recently, a study by Odume (2011) that categorised the sites within the Uitenhage and Despatch areas as critically or seriously modified. Therefore, it is possible to assume that glyphosate spray did not cause the observed water quality status at the SZ, especially when DSZ experienced similar water quality problems.

5.5 Conclusion

This biomonitoring study has shown that glyphosate spray to control water hyacinth in the Swartkops River has negligible to zero impact on the water quality and macroinvertebrate community of the River. This was evident in the similarities of observed outcomes between SZ and DSZ in nearly all water quality variables and macroinvertebrate metrics investigated. The deterioration of environmental water quality and macroinvertebrate communities at SZ could be attributed to other factors such as high organic loading, effluent discharges, industrial and agricultural wastes, as well as chemical run-offs and natural geology of the river bed, rather than glyphosate spray at the site.

However, of particular interest in this study are the transient effects of glyphosate spray on air-breathing macroinvertebrate communities in the Swartkops River since after-spray results revealed a low presence of families of air-breathers in the SZ, an observation supported by a laboratory tests. It will, therefore, be important to consider the short-term lethal effect in environmental management of glyphosate. Thus, the short-term water quality guideline derived in Chapter Four of this thesis is necessary.

CHAPTER SIX

FRAMEWORK FOR ENVIRONMENTAL WATER QUALITY MANAGEMENT OF GLYPHOSATE-BASED HERBICIDES

6.1 Introduction

In the previous three chapters of this thesis, both laboratory and field investigations of glyphosate-based herbicides (Roundup® and Kilo Max WSG, hereafter are both referred to as glyphosate**)** were conducted. In the laboratory investigations, aquatic ecotoxicological methods were used to evaluate responses of the freshwater aquatic shrimp *C. nilotica* exposed to Roundup® at different biological system scales (Chapter Three), and the responses of multiple South African aquatic species exposed to Roundup® through species sensitivity distribution (SSD) (Chapter Four). In the field investigations, the effect of Kilo Max WSG on the physicochemical and biological conditions of three selected sites in the Swartkops River before and after glyphosate spray by Working for Water were evaluated. These are separate studies and need to be integrated in order to achieve the overall aim of this study (i.e. to provide background information for the environmental water quality management of glyphosate-based herbicides in South Africa). This chapter presents a conceptual framework for environmental water quality management of glyphosate-based herbicides based on the findings of the previous three chapters. It begins by discussing how to use the developed water quality guidelines (WQGs) developed (Chapter Four) and biomonitoring (Chapter Five) as tools for environmental water quality management of glyphosate-based herbicides. The chapter continues by discussing a newly developed integrated conceptual framework for environmental water quality management of glyphosate-based herbicides, and ends by discussing possible issues that may affect the environmental water quality management of these herbicides.

6.2 Using water quality guideline (WQG) and biomonitoring as tools for environmental water quality management

6.2.1 WQG as a tool for environmental water quality management

The water quality guidelines (WQGs) derived for glyphosate-based herbicides in Chapter Four, can, and should, be used to inform the application rate and monitoring of such herbicides in South African freshwater ecosystems. For protection of aquatic life, the short-term in-stream concentrations measured just after spray (≤ 4 days) should generally be lower than the short-term guideline. Similarly, the in-stream concentrations measured a longer period after spray (≥ 4 days) should be lower than the long-term guideline. Thus, the derived guidelines for the protection of aquatic life could be used to determine if glyphosate-based herbicides currently sold and used across South Africa pose a potential problem to the environment. However, a major problem that may be encountered in applying the guideline is the difficulties of measuring in-stream concentrations (as experienced in this study).

Although, there are various methods that can be used to measure in-stream concentrations of glyphosate based-herbicides, the expertise and cost involved make it difficult to apply these methods in developing countries such as South Africa in comparison perhaps to Europe, North America and Australasia. For instance, surface waters can be sampled for pesticides using passive sampling (Muschal, 2000). Passive samplers, which can be constructed from low density polyethylene bags containing solvent, are deployed in the river system. As the passive samplers integrate organic contaminates over time, pesticides that occur infrequently and/or at levels below analytical detection limits in the water column are detected (Muschal, 2000). Thus, passive samplers not only integrate organic contaminants over time but they also concentrate them to concentrations higher than in water. However, to obtain a quantitative result of pesticide concentrations calibration requires methods specific to the organic contaminants monitored and the environmental field conditions within which the samplers are placed (Muschal, 2000). In addition to cost, there is high risk of losing these samplers as most in-stream installation in South Africa suffers from tampering and vandalism.

Therefore, in South Africa it is useful, even necessary, to devise other means of assessing the effects of glyphosate-based herbicides on freshwater ecosystems, other than using actual in-stream concentrations. Findings of the investigations reported in Chapter Five of this thesis suggest that biomonitoring, used in a specific manner, could be a useful tool in this regard.

6.2.2 Biomonitoring as a tool for environmental water quality management

Field spraying with a glyphosate-based herbicide to control aquatic weeds can occur at sites that are impacted mainly by the herbicide (single stressor), or by multiple pollutants (multiple stressors). That is, where glyphosate is the main cause of the observed bio-physical conditions or just one of many possible causes. Where the herbicide is evidently the primary stressor differences in conditions before and after spray can be largely attributed to glyphosate. However where multiple pollutants affect ecosystem health, differences between physicochemical and biological conditions before and after spray can be attributed to any of many possible factors, including glyphosate. Differences between these two biomonitoring scenarios are presented in Table 6.1. Results from mainly single-stressor biomonitoring sites are more straightforward to interpret, since the applied chemical (in this case the glyphosate-based herbicide) is the main suspect if there are differences between physicochemical and biological conditions before and after a spray event. However, biomonitoring results from multiple-stressor impacted sites need a different approach because of the complex factors involved. This study develops and suggests the use of "confounding factor analysis" (CFA). The approach is described below using results from biomonitoring in the Swartkops River (Chapter Five). As revealed by the before-spray data (Odume, 2011), the selected sites investigated are severely impacted by multiple stressors, except the reference site (i.e. upstream of spray zone).

Table 6.1: Differences between a mainly single stressor biomonitoring site and multiple-stressor biomonitoring site

Single-stressor biomonitoring site	Multiple-stressor biomonitoring site
Applicable in less impacted site	Applicable to severely impacted site
Relatively good physicochemical and biological conditions	Relatively poor physicochemical and biological conditions
Herbicide is the main cause of effect	Herbicide is one of many causes of effect

6.3 Multiple-stressor biomonitoring site – the confounding factor analysis (CFA) approach

6.3.1 Determining if glyphosate is the confounding factor (causal agent)

In Chapter Five of this thesis, a condition assessment of general physicochemical and biological conditions of the Swartkops River was conducted through biomonitoring of three sites (spray zone, upstream of spray zone and downstream of spray zone) before and after Kilo Max WSG spray. Condition assessment was conducted to determine if there was any impairment to the river's ecology as a result of Kilo Max WSG spray to control water hyacinth. Impairment was assumed by the presence of a confounding factor (CF) at the spray zone (SZ) but absence of the CF upstream and downstream of spray zone (USZ and DSZ). ***A confounding factor is identified when there is an unexplained change in the downstream gradient of measured biological and physicochemical variables***. Bearing in mind the condition of the sampling sites before Kilo Max WSG spray, where USZ had the best condition (reference site), followed by SZ, and then DSZ, confounding is assumed to occur if SZ condition is worse than USZ and DSZ after Kilo Max WSG spray. This means that the condition at the SZ after Kilo Max WSG spray is probably due to an extraneous factor, rather than a "natural" biological or physicochemical factor. Therefore, at this highly polluted site, the complex chemical mix, including the Kilo Max WSG spray, is postulated as the extraneous factor. Thus, if the presence of the extraneous factor (i.e. the confounding factor) in this case is assumed to be Kilo Max WSG (even though it could be attributed to a common source of multiple potentially causal agents, or due to chance associations), then certain outcomes could be expected. These expected outcomes could then be used to develop a model to determine if glyphosate was in fact is the causal agent (Table 6.2). A system of plus (+), minus (−) and zero (0) was used to analyse the data. A plus (+) was used to support a potential confounding factor (i.e., the evidence suggests that the potential confounding factor is actually causing the observed condition to a significant degree); a minus (−) for weakening the potential confounding factor (i.e., the evidence suggests that the potential confounding factor does not contribute to the observed condition to a significant degree); and a zero (0) for no effect on the condition (Table 6.2).

Table 6.2: Models for determining if glyphosate is the causal agent in a multiple stressor biomonitoring sites (CFA = confounding factor analysis; SZ = spray zone; USZ = upstream of spray zone; DSZ = downstream of spray zone)

Model number	SZ	USZ	DSZ	CFA
1	Worse condition	Better condition	Better condition	Presence of CF (+)
2	Same condition	Same condition	Same condition	Absence of CF (0)
3	Better condition	Better condition	Worse condition	Absence of CF (−)
4	Worse condition	Better condition	Worse condition	Absence of CF (−)
5	Better condition	Worse condition	Worse condition	Absence of CF (−)

From Table 6.2, it can be seen that a confounding factor was only identifies where the SZ condition was significantly worse than that of both DSZ and USZ. This means that the condition at the SZ after glyphosate-spray is probably due to an extraneous factor, rather than a "natural" biological or physicochemical factor, as explained earlier.

6.3.2 Analysis of data from multiple stressor biomonitoring sites using the CFA model

One way of combining data within the same and/or across different assessment types is using a weight of evidence (WoE) approach (Cormier and Suter, 2008). The WoE approach involves assembling evidence, weighting evidence, and weighing the body of evidence (Suter and Cormier, 2011). Thus, data from both physicochemical variables and biological metrics before and after spray, which form lines of evidence (LoE), were assigned different weights, using the WoE approach. This is necessary because not all evidence has equal strength and quality (Suter and Cormier, 2011). Appendix P describes different methods for weighing body of evidence including strengths and weaknesses of such methods.

Assembly of evidence

The empirical evidence for evaluating the abiotic and biotic factors before and after spray comes from Chapter Five, where a paired t-test analysis was performed for each physicochemical variable and biological metric for all three sampling sites (i.e. spray zone (SZ), upstream of spray zone (USZ) and downstream of spray zone (DSZ)) in order to determine if there was a significant difference between a measured variable or metric before and after glyphosate spray. The calculated p-values (two-sided) are presented in Tables 6.3

and 6.4 for physicochemical variables and biological metrics, respectively. These were assembled as lines of evidence (LoE).

Table 6.3: Paired t-test analysis p-values calculated for each physicochemical variable before and after glyphosate spray events at the three sampling sites in the Swartkops River. These are tested against the models listed in Table 6.2 to allocate the confounding factor. (CFA = confounding factor analysis; SZ = spray zone; USZ = upstream of spray zone; DSZ = downstream of spray zone)

WQ variables	**Sampling sites**			**Weight for CFA**
	SZ	**USZ**	**DSZ**	
DO (mg/L)	0.72	0.89	0.03	–
pH	0.96	0.37	0.10	0
Temperature (ºC)	0.36	0.15	0.37	0
Electrical Conductivity (mS/m)	0.02	0.19	0.01	–
Turbidity (NTU)	0.61	0.04	0.75	0
BOD_5 (mg/L)	0.69	0.52	0.97	0
Nitrate-nitrogen (NO_3-N) (mg/L)	0.50	0.80	0.84	0
Nitrite-nitrogen (NO_2-N) (mg/L)	0.42	0.06	0.92	0
Ammonium - nitrogen (NH_4-N) (mg/L)	0.05	0.02	0.91	–
Orthophosphate-phosphorus (PO_4-P) (mg/L)	0.92	0.64	0.70	0
Phytoplankton chlorophyll *a* (µg/L)	0.89	0.09	0.07	0
Periphyton chlorophyll *a* (µg/cm^2)	0.34	0.11	0.06	0
Overall score	The evidence suggests that the potential confounding factor has no effect on the physicochemical conditions observed in the Swartkops River.			0

Table 6.4: Paired t-test analysis p-values calculated for each biological metric before and after glyphosate spray events at the three sampling sites in the Swartkops River (CFA = confounding factor analysis; SZ = spray zone; USZ = upstream of spray zone; DSZ = downstream of spray zone)

Biological metrics	**Sampling sites**			**Weight for CFA**
	SZ	**USZ**	**DSZ**	
SASS5 score	0.01	0.04	0.02	0
Number of taxa	0.02	0.10	0.00	–
ASPT	0.14	0.04	0.33	–
% Chironomidae + Oligochaeta	0.03	0.03	0.04	0
% ETOC	0.03	0.01	0.07	–
ETOC richness	0.07	0.15	0.04	–
Margalef's family richness index	0.06	0.35	0.01	–
Shannon index	0.02	0.00	0.02	–
Equitability	0.06	0.16	0.03	0
Overall score	The evidence suggests that the potential confounding factor does not contribute to the observed biological conditions of the Swartkops River to a significant degree.			–

Weighting of evidence

The lines of evidence (LoE) assembled from the condition assessment of the Swartkops River based on the physicochemical and biological variables investigated at the three sampling sites (i.e. spray zone, upstream of spray zone and downstream of spray zone) (Table 6.3 and 6.4) were weighted using a system of plus (+), minus (−) and zero (0). Based on the p-value, a plus (+) was used to support a potential confounding factor (i.e., the evidence suggests that the potential confounding factor is actually causing the observed condition to a significant degree); a minus (−) for weakening the potential confounding factor (i.e., the evidence suggests that the potential confounding factor does not contribute to the observed condition to a significant degree); and a zero (0) for no effect on the condition. The overall score of each category of evidence was obtained by addition of all the weights (plus, minus and zero signs) to give an aggregate (overall) score. Outcomes of the weighted evidence from assessments of physicochemical and biological conditions are presented in Tables 6.3 and 6.4, respectively.

Weighing the body of evidence

Weighted evidence from each category (overall scores) were used to form a body of evidence using the independent applicability method (Suter and Cormier, 2011). The independent applicability is the simplest formal method for weighing a body of evidence, and hinges on the principle that any sound evidence is sufficient enough to demonstrate impairment (Suter and Cormier, 2011). Apart from its recommendation for situations in which protection is the main objective (as was the case in this study), the independent applicability method was employed as a method to weigh the body of evidence in this study because it is transparent and consistent; it was based on the overall evidence and score for each category of evidence (Table 6.5). The weight of the weighed body of evidence was taken as the overall strength and quality of the body of evidence with respect to its logical implication (Suter and Cormier, 2011).

Table 6.5: Weighed body of evidence from the all three categories of evidence

Category of evidence	**Score**	**Possible conclusion**
Physicochemical variables	0	There is no clear evidence that glyphosate spray impacts negatively on the physicochemical conditions of the Swartkops River.
Biological metrics	–	There is no clear evidence that glyphosate spray impacts negatively on the biological conditions of the Swartkops River.
Body of evidence	0	There is no clear evidence that glyphosate spray impacts negatively on the physicochemical and biological conditions of the Swartkops River.

The weight of evidence approach (WoE) had been used to integrate field investigations (i.e. both physicochemical variables and biological metrics before and after spray) in order to provide background information for the environmental water quality management of a glyphosate-based herbicide (Kilo Max WSG) in South Africa. Synthesis of the results shows that glyphosate could not be linked to the poor physicochemical and biological conditions of the Swartkops River.

6.4 Conceptual framework for environmental water quality management of glyphosate-based herbicides

There are different forms of presenting conceptual frameworks such as visuals, mind maps, flow diagrams, text or table formats, but each form has varying degrees of linkage to theory (Ross *et al*., 2006). Conceptual frameworks may be compiled from a combination of literature, discussion, available data and "expert" opinion, depending on their purpose. It can be constructed by conducting systems analysis (more formal) or mind mapping of key elements and linkages (Ross *et al*., 2006). Based on the above discourse (sections 6.2 and 6.3), a proposed integrated conceptual framework for environmental water quality management of glyphosate-based herbicides was developed (Figure 6.1). The proposed conceptual framework has two main "frames". These are the quantitative and the qualitative analyses frames. The quantitative analysis will express actual amount of in-stream glyphosate concentrations, while qualitative analysis will express the presence or absence of glyphosate in freshwater bodies.

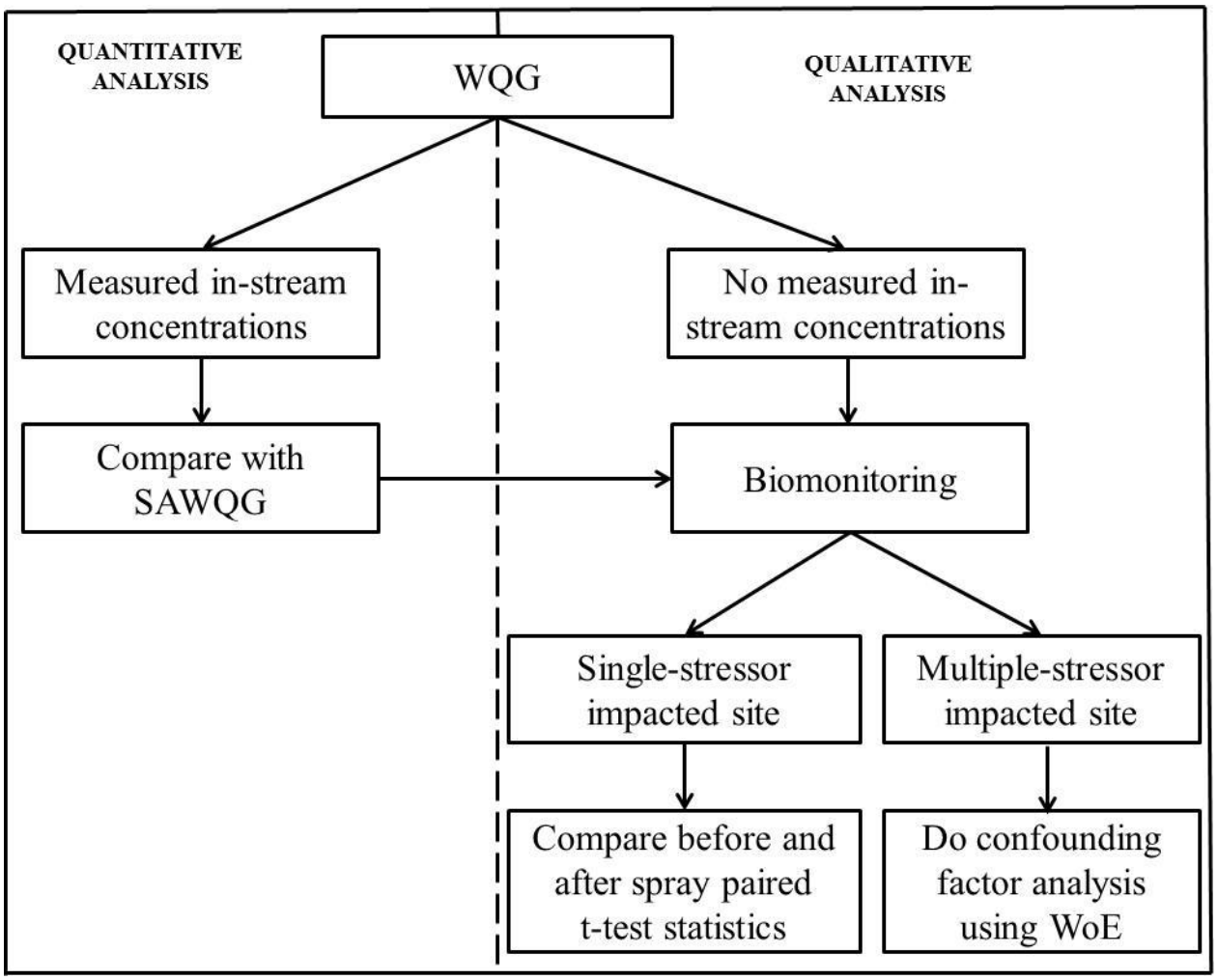

Figure 6.1: Conceptual framework for integrated environmental water quality management of glyphosate-based herbicides.

Quantitative analysis

In managing glyphosate-based herbicides, it is proposed that the first activity, should be quantifying in-stream glyphosate concentrations. This can be done through the various sampling and analytical chemistry methods that may be available. The results are then compared with the derived WQGs for glyphosate. If the in-stream concentration is below the guideline, then there is low potential for adverse environmental impact. Otherwise, appropriate actions should be taken to rectify the situation if the in-stream concentration is above the guideline. Whatever the case, it is always necessary to conduct biomonitoring investigations to ascertain the impact of glyphosate on both physicochemical and biological variables.

Qualitative analysis

In some situations, it will not be possible to measure actual in-stream concentrations due to lack of sampling and analytical chemistry methods, or the focus of management will not be that (i.e. measuring in-stream concentrations), but rather merely to ascertain the absence or presence of glyphosate. Ascertaining the absence or presence of glyphosate through biomonitoring can be used to determine whether glyphosate is the main cause of the observed conditions or only one of many possible causes. Biomonitoring can be conducted in less impacted or severely impacted before the spray as explained in section 6.2.2. Since single-stressor impacted sites have relatively good physicochemical and biological conditions, differences in these conditions before and after spray can be attributed to glyphosate, to a larger extent (section 6.2.2.). Similarly, since multiple-stressor impacted sites are generally poor, differences between physicochemical and biological conditions before and after spray can be attributed to one or combination of many possible factors, including glyphosate. The confounding factor analysis (CFA) can be used to assess the presence or absence of glyphosate in multiple-stressor impacted sites (section 6.2.2.).

6.5 Management issues in environmental water quality management of glyphosate-based herbicides

6.5.1 Over-protection or under-protection

Species sensitivity distribution (SSD) method uses single-species toxicity data to predict hazardous concentrations affecting a certain percentage (HCp) of species in a community (Newman *et al*., 2000). Therefore, SSD methods can be used to generate protective concentrations (PCs) or water quality guidelines for the management of Roundup® and other glyphosate-based herbicides. However, guidelines can be very conservative and therefore, over-protective, as noted by Jooste (2001) and Brown (2005). Over-protective guidelines may have implications for socio-economic development. For example, if a guideline suggests that a certain rate of pesticide application could harm the aquatic environment, it would trigger management actions that may result in reducing the application rate. If, at this new reduced application rate, the pests are not effectively controlled, it may result in extensive loss of agricultural produce.

It is therefore necessary that site-specific investigations are conducted wherever the TVs are exceeded so that the appropriate measures are implemented rather than over-protective

measures. The idea of over-protective guidelines is based on the "precautionary principle". The precautionary principle states that managers should be proactive in taking decisions in advance of scientific certainty to prevent harm to humans and the environment (WHO, 2004). It is an approach that anticipates and prevents harm to human health and the environment, given that science is limited in addressing emerging environmental challenges, including pesticide pollution. However, *in toto* application of the precautionary principle in some areas of water resources management, such as implementing an over-protective guideline, may have serious socio-economic impacts (Jooste, 2001).

Conversely, a flexible guideline may also result in under-protection, which may have negative effects on the environment. For example, the food chain may be detrimentally affected when one species declines because it is under-protected. This may result in increase or decline of other species and a regime shift in the ecosystem. Furthermore, the flexibility of such a guideline may unintentionally allow over use of a particular chemical, which may cause extinction of species due to under-protection. It is therefore imperative to strike a critical balance to prevent deriving a guideline that is either over-protective and is unnecessarily costly to economic development or under-protective and ultimately reduces ecosystem health.

6.5.2 Communication

Communication ensures creating a better understanding within the target audience of a water quality management strategy if the need arises. It also informs the public about change in attitudes, perceptions and behaviours in order to maintain a good water quality. For example, if users are not able to use a water resource because of its poor quality, the result may be social instability. This social instability can be induced as much through the poor water quality impacts as through a simple lack of effective communication of the problem to the affected community. In order to avoid a situation of social instability developing as a result of poor water quality (or any management issue pertaining to a natural resource), the management options adopted by the resource managers must be communicated to the affected community.

Communication can be effected through written and/or audio-visual materials. Written materials include posters, pamphlets, flyers and science journals, while audio-visuals include radio and television broadcasts, interviews, documentaries and special reports. Depending on the target audience, the appropriate means should be used in communicating management issues. Some examples will suffice. Posters are a resource for places where many people gather, such as hospitals, bus stops, subways, community centers, and in the streets. A poster must be attractive and the size should be appropriate to the content to facilitate quick understanding. Science journals target well-defined audiences such as students, professionals in different fields, teachers, and the general public.

Communicating management issues with science journals is effective because the message is the result of a description and analysis of possible solutions to the problem. For example, the source of pollution of a freshwater ecosystem, physicochemical conditions that influence the pollution, impacts on human and environmental health, and the need to be alert to broadcasting levels of pollution, can all be described by the relevant authorities to protect both humans and freshwater ecosystems. However, since the readership of science journals is relatively small, reinforcement by other messages through different media is necessary. Radio and television broadcasting methods are often closest to many people, and are therefore useful in communicating preventive measures for natural and anthropogenic sources of freshwater pollution. Radio and television communicate with a heterogeneous public with different levels of comprehension. Thus, the messages should be made to reach all levels of the audience and capture the interest of each one. This is possible if the target language used is the one that is best understood by the target group. For example, it is most appropriate to use isiXhosa to communicate with the people in and around Mthatha if the Mthatha River is accidentally contaminated with an inflow of excess agro-chemical from the surrounding farmlands that results in a large fish kill. Since most people in Mthatha are Xhosa and speak isiXhosa (their native language), using isiXhosa to communicate information about the incident and about plans to contain it will be more effective.

6.5.3 Management options

Although preference of any particular management direction would chiefly be informed by the outcome of an integrated environmental assessment, successful implementation of any management option depends on integrating different management options. This means it is necessary to consider risks and benefits of alternative management actions together with stakeholder preferences, economic costs, regulatory requirements, legal considerations, and any other factors in the decision-making process. However, the final management option decision could essentially be driven by resource value or technology availability (e.g. Best Available Technology (BAT), Best Available Technology Not Entailing Excessive Cost (BATNEEC), Best Practical Means (BPM)) (Jooste, 2001). The resource-driven approach of decision-making recognises some valued function or process of the resource such as economic activity and ecosystem functioning, while the technology-driven approach is based on the principle that if a technology does not exist to effect a management action, then that action is simply not workable (Jooste, 2001).

The resource-driven management decision-making process places emphasis on stressor effect on ecosystems (e.g. effect of in-stream concentrations on some defined water use). Therefore, the resource-driven management option can be employed in environmental water quality management of pesticides such as glyphosate products, which are usually used to control aquatic weeds in South Africa. The resource-driven management option requires that some environmental quality objectives (EQOs) are set (Jooste, 2001). In practice, technology-driven criteria or guidelines are set and assessed against a likelihood of violating an EQO such as a WQG (for glyphosate-based herbicides). If a WQG is violated, then the resource-driven management option should be adopted rather than the technology-driven management option (Jooste, 2001).

6.5.4 Different levels of protection

Theoretically, the SSD method of deriving WQG is able to extrapolate small datasets to provide protection for 95 % of assemblages or communities, thereby accounting for unknown species sensitivities (Pennington, 2003). Thus, the short-term WQG may be able to protect 95 % of a population from a severe and transient exposure, while the long-term WQG may be able to protect 95 % of a population from exposure throughout their life cycles.

A long-term toxicity WQG was separately derived for the management of Roundup® because it reflects environmental realism where aquatic organisms are exposed to the herbicide for prolonged periods. However, South Africa has different Ecological Reserve categories, which means different levels of protection for each category. Warne *et al*. (2004) recommended different levels of protection to equate the various Ecological Reserve categories for toxicants (Table 6.6). These recommendations are applied in this study to relate the derived WQGs trigger values to Reserve categories of South Africa.

Table 6.6: Recommended protection levels to equate the various ecological integrity classes of the ecological Reserve of South Africa (after Warne *et al*., 2004)

Alternate class description for ecological integrity		**Level of protection**
Excellent (natural)	A	PC > 95
Good	B	PC > 90
	C	
Fair		PC > 80
	D	
Poor	E	PC < 80

Thus, in addition to the PC95 protective concentrations derived in Chapter Four, other different levels of protection, including PC90, PC80 and PC60, were calculated using the SSD Generator (Posthuma *et al*., 2002; USEPA, 2005) for the different ecological integrity classes of South African Ecological Reserves. The levels of protection concentration (PC) percentages were selected based on the default output of the SSD Generator (i.e. the first four in decreasing order. Unlike the BurrliOZ software that calculates PC values as point estimates with 50 % certainty (Warne *et al*., 2004), the SSD Generator used in this study calculates PC values as point estimates together with their corresponding 95 % lower and upper confidence limits (interval estimates). Tables 6.7 and 6.8 present short-term (based on 48-96 h LC50 values) and long-term (based on NEC values) water quality guidelines for different levels of protection of South African freshwater ecosystems from exposure to Roundup®.

Table 6.7: Short-term WQG values associated with different levels of ecological integrity classes (based on 48-96 h LC50 values)

Alternate class description for ecological integrity		Level of protection	Guideline value with 95 % confidence limits (mg/L)
Excellent (natural)	A	PC95	0.076 (0.015-0.383)
Good	B	PC90	0.152 (0.033-0.699)
	C		
Fair		PC80	0.352 (0.083-1.484)
	D		
Poor	E	PC60	1.082 (0.273-4.286)

Table 6.8: Long-term WQG values associated with different levels of ecological integrity classes (based on NEC values)

Alternate class description for ecological integrity		Level of protection	Guideline value with 95 % confidence limits (mg/L)
Excellent (natural)	A	PC95	0.002 (0.000-0.021)
Good	B	PC90	0.005 (0.001-0.044)
	C		
Fair		PC80	0.015 (0.002-0.117)
	D		
Poor	E	PC60	0.065 (0.009-0.461)

In Chapter Four of this thesis, long-term water quality guidelines (WQGs) associated with PC95 protection level were derived using no observed effect concentration (NOEC) and no effect concentration (NEC), despite the limitations of NOEC as discussed in that Chapter. The purpose of using NOEC to derive the guideline was to compare it with the NEC-derived guideline. Interrogations of both guidelines found the NEC-derived guideline (0.002 mg/L) to be more conservative than the NOEC-derived guideline (0.085 mg/L). Even the geometric mean of the NOEC-NEC-derived guideline (0.013 mg/L) was less conservative compared to the NEC-derived guideline. The short-term Canadian water quality guideline is 0.06 mg/L (CCME, 1999) (no long-term guideline currently exists, though Canada practise a system of short-term and long-term), while that of Australia and New Zealand is 1.2 mg/L (ANZECC and ARMCANZ, 2000) (Australia and New Zealand practise a single guideline value system).

Although both short-term WQGs for Canada (0.06 mg/L) and this study (0.076 mg/L) lie within same range as estimated for the current study (0.015-0.383), the Australia and New Zealand guidelines is about 16 time higher than that of this study. Therefore, it was appropriate to use short-term LC50 and NEC values to derive WQGs associated with different protection levels for the purpose of environmental water quality management of glyphosate-based herbicides. These protective concentrations can form part of integrated water resources management (IWRM) in South Africa.

6.6 Conclusion

The ecotoxicological studies under laboratory conditions provided exposure-response relationships that may contribute to assessments of alternative regulatory (management) decisions. Condition assessment also provides paired physicochemical and biological data that can be used to generate a risk model by estimating from current conditions the risks that will continue if no remedial action is taken.

The conceptual framework developed and presented here is intended for use by interdisciplinary research and management teams involved in water resources management in South Africa for the environmental water quality management of glyphosate-based herbicides as part of integrated water resource management (IWRM). However, it may be adopted and applied in the integrated management of other pesticides in South Africa and around the world. Adoption of this framework should be done in a selective way to inform each study type. Thus, it is not necessary to fill every possible aspect and layer of the framework with detailed information if that will not help to provide either valuable contextual information or specific information needed to inform analysis. For example, it will not be necessary to generate evidence on the condition of a system if the objective is to assess the cause of contamination.

CHAPTER SEVEN

GENERAL CONCLUSION

7.1 Introduction

This chapter briefly describes the general conclusions of this study in relation to the overall aim, specific objectives, research question and hypotheses it set out to address. It then gives suggestions about how to apply and implement the findings and conclusions emanating from this study. The chapter concludes by giving recommendations for further studies in order to address specific issues encountered during the course of this study.

7.2 Summary conclusions in relation to the specific objectives

The overall aim of this study was to provide a sound scientific background for the environmental water quality management of glyphosate-based herbicides in South Africa. Thus, specific objectives were set in order to achieve this aim. The following are summary conclusions in relation to each specific objective (specific objectives are in bold type and italicised):

To evaluate the exposure-response relationship of Caridina nilotica exposed to Roundup® under different laboratory exposure conditions

Both lethal (short-term and long-term) and sub-lethal (short-term and long-term) tests revealed that varying concentrations of Roundup® elicit responses at different biological system scales in *C. nilotica*. Lethal exposure to Roundup® involving all three life stages showed that neonates were the most sensitive life stage in both concentration and time-dependent manner. *Caridina nilotica* generally responded to sub-lethal exposure to Roundup® in a concentration-dependent manner in all tested biological system scales including length, weight, moulting (growth), embryos (reproduction) acetylcholinesterase (AChE), lipid peroxidation (LPx) (biochemical), except the gonads. Therefore, all the listed measures could be used as endpoint measures for the detection of Roundup® toxicity to *C. nilotica*.

To evaluate the species sensitivity distribution (SSD) of South African aquatic organisms exposed to Roundup®

Species sensitivity distributions (SSDs) from this study provided an estimate of species representing five taxonomic groupings affected by glyphosate. The results from this study suggest that algae (*C. protothecoides*, *C. sorokiniana*) were most sensitive to 48-96 hour short-term glyphosate exposure, while crustaceans (*D. pulex*, *C. nilotica*) and the mollusc, *Burnupia stenochorias*, were more sensitive than fish (*Oreochromis mossambicus*) and insects (*Baetis harrisoni* and *Tanytarsus flumineus*).

The long-term NOEC results showed the crustacean *Daphnia pulex* to be the most sensitive, while the most insensitive was another crustacean, *C. nilotica*. Between these two extremes of sensitivities lie the combined sensitivities of algae (*C. sorokiniana*, *C. protothecoides*), insects (*Baetis harrisoni, Tanytarsus flumineus*) and the mollusc (*Burnupia stenochorias*), and the fish (*Oreochromis mossambicus*).

The long-term NEC results showed algae (*C. protothecoides*, *C. sorokiniana*) to be the most insensitive group, while the mollusc, *B. stenochorias,* was the most sensitive. The sensitivity of the crustaceans (*D. pulex*, *C. nilotica*) and insects (*B. harrisoni*, *T. flumineus*) as well as the fish (*O. mossambicus*) lies between these two extreme sensitivity ranges. The results revealed that the ranges of sensitivities were not exclusive as there may be overlaps among some or all taxonomic groups.

To evaluate the field effect of a glyphosate-based herbicide on physicochemical and biological conditions of the Swartkops River through biomonitoring

The biomonitoring study revealed that using a glyphosate-based herbicide to control water hyacinth within the Swartkops River had a negligible impact on the physicochemical and biological conditions. Although the physicochemical and biological conditions of the spray zone (SZ) were poor, they were attributed to other factors such as high organic loading, effluent discharges, industrial and agricultural wastes as well as chemical run-offs and natural geology of the river bed, rather than glyphosate spray at the site. Nevertheless, the abundance of most air-breathing macroinvertebrates declined significantly just a few hours after glyphosate spray. This seems to suggest that glyphosate may have a transient effect on this group of macroinvertebrates. Thus, the short-term water quality guideline derived in Chapter Four of this thesis is necessary.

To develop water quality guidelines (WQGs) for the management of glyphosate-based herbicides as part of integrated water resource management (IWRM) in South Africa

Based on the generated SSD curves (second objective), 95% protective concentrations (PC95) were calculated for Roundup®. The PC95 values serve as trigger values (TVs) for implementation of any management strategy. The derived water quality guideline (WQG) short-term trigger value indicating the potential for severe effects to sensitive freshwater organisms during transient events for glyphosate (Roundup®) is 0.021 (0.000-0.695)mg/L. It was calculated using the short-term 48-96 h LC50 values. The derived WQG long-term trigger value for the protection of aquatic life is 0.042 (0.020-0.086) mg/L which was calculated using the long-term NEC values. Different levels of protection, including PC90, PC80 and PC60, were also calculated in order to protect the various ecological integrity classes of the Ecological Reserve of South Africa.

To develop a conceptual framework for the integrated environmental water quality management of glyphosate-based herbicides in South Africa

A conceptual framework that can be used for the integrated environmental water quality management of glyphosate-based herbicides in South Africa was developed as part of integrated water resource management (IWRM). The framework is made up of three blocks (i.e. generating evidence, weighing evidence and management strategy). However, the framework may be adopted and applied to manage environmental water quality of other pesticides in South Africa, and the world over. This framework should be adopted in a selective way to inform each study type.

7.3 Application and implementation of findings and conclusions

This study was motivated by the lack of adequate South African-based ecotoxicological data for managing glyphosate-formulated herbicides, and therefore, by the desire to fill this gap. This then informed the overall aim of this study, which was to provide a background for the environmental water quality (EWQ) management of glyphosate-based herbicides in South Africa, as part of integrated water resources management (IWRM).

The study has provided adequate information for the realisation of this aim based on its findings and conclusions. Therefore, to ensure the successful application of these findings and conclusions, the following suggestions are made:

- The South African Department of Water Affairs (DWA), water resource managers and water scientists/researchers can apply the developed conceptual framework to manage environmental water quality (EWQ) of glyphosate-based herbicides, as part of integrated water resource management (IWRM). The framework can also be adopted in a selective manner (depending on research needs) to manage environmental water quality of other pesticides in South Africa.

- Any programme to implement the conceptual framework presented in this thesis for the environmental water quality management of any pesticide must have environmental water quality management as the underlying concept. This means that such a programme should consider assessing the pesticide in question in all aspects of environmental water quality (i.e. ecotoxicology, physicochemistry and biomonitoring). Laboratory-based ecotoxicological investigations should evaluate the short-term/long-term and lethal/sub-lethal effect conditions of the pesticide. Physicochemistry is necessary to evaluate the pesticide's physical and chemical effects on freshwater ecosystems. This includes measuring the in-stream concentration of the pesticide. Biomonitoring will assess selected biological metrics deemed sensitive to the pesticides. The biological metrics presented in this thesis could be used if data are collected using the SASS5 protocol. Both physicochemistry and biomonitoring are field investigations.

- It is expected that the derived WQGs for Roundup® will be the basis of managing glyphosate-based herbicides found in South African freshwater ecosystems. It is expected that these guidelines will be evaluated by the relevant organs of the South African national Department of Water Affairs (DWA), who will apply (implement) these guidelines until site-specific guidelines are derived.

7.4 Recommendation for further studies

Following this rigorous study, and based on the findings and conclusions emanating from the various investigations that form part of this thesis, it is recommended that:

- Further study is needed on the use of a biological system scales sensitivity distribution (BS3D) approach in guideline derivation programmes. That is, more studies should be done to evaluate using a comprehensive knowledge of one organism to derive a water quality guideline as against multiple species, using species sensitivity distributions (SSDs). The focus of such investigations should be whether BS3D be used as a stand-alone approach or as part of the conventional SSD approach. Further study could be done to provide comprehensive knowledge about other organisms belonging to different taxonomic groups (e.g. fish, algae, insect or mollusc) and then compared with SSD guidelines. Guidelines based on only one species can be used to protect members of the taxonomic group to which the tested species belong.

- Future *C. nilotica* histopathological investigations should consider including other organs such as the gut, gills, hepatopancreas and eyestalk as potential target organs. These are all potential endpoints encountered during the *C. nilotica* histopathological analysis. In addition, future sub-lethal investigations should consider using Roundup® concentrations higher than those used in this study.

7.5 Concluding statement

The aim of this study was to provide a sound scientific background for the environmental water quality management of glyphosate-based herbicides in South Africa. Based on this aim, various investigations were conducted with the underlying research question being "Do glyphosate-based herbicides pose any risks to South African freshwater ecosystems?" To address this question, substantial data were accrued from detailed responses of the freshwater aquatic shrimp *C. nilotica* and the combined but less detailed responses of many South African freshwater aquatic species to Roundup® exposure, and an *in situ* spraying episode of the Swartkops River with Kilo Max WSG.

However, significant questions and uncertainties still remain. Despite these caveats, the combined data sets do contribute to a sound scientific background for the environmental water quality management of glyphosate-based herbicides in South Africa.

GLOSSARY

5-enolpyruvylshikimate-3-phosphate synthase (EPSPS): An enzyme that catalyses the biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan in plants and microorganisms (bacteria and fungi). The enzyme is a target for herbicides such as glyphosate. It belongs to the family of transferase enzymes.

Abiotic: The non-living component of an ecosystem.

Abundance: The number of organisms in a population, combining 'intensity' (density within inhabited areas) and 'prevalence' (number and size of inhabited areas).

Acclimation: The process whereby an organism becomes accustomed.

Acetylcholinesterase (AChE): An enzyme that hydrolyses the neurotransmitter acetylcholine at neuromuscular junctions and cholinergic brain synapses, where its activity serves to terminate synaptic transmission. It is a serine protease, which belongs to the family of carboxylesterase enzymes.

Acid equivalent (a.e): The portion of a formulation (e.g. Roundup®) that theoretically could be converted back to the corresponding or parent acid; or the theoretical yield of parent acid from a pesticide active ingredient which has been formulated as a derivative (examples of derivatives include salts, esters and amines). For example, Roundup® contains 360 g glyphosate (glycine) acid equivalence per litre (480 g isopropylamine salt of glyphosate/litre) active ingredient). Thus, when converting between acid equivalents (a.e.) and active ingredient (a.i.), 1 g a.i will contain 0.75 g a.e.

Active ingredient (a.i): The component of a pesticide formulation responsible for its toxicity (phytotoxicity for herbicides) or ability to control the target pest. It is always identified on the pesticide label; either by common name (e.g. glyphosate and atrazine) or chemical name (e.g. N-(Phosphonomethyl) glycine and 2,4-dichlorphenoxy acetic acid). The active ingredient statement may also include information about how the product is formulated and the amount of active ingredient contained in a litre or gram of formulated product.

For example, the Roundup® label indicates the active ingredient (glyphosate) is formulated as isopropylamine salt, and one litre of Roundup® contains 480g of the active ingredient. Pesticides are regulated primarily on the basis of active ingredients.

Acute toxicity: The harmful effects of a chemical or mixture of chemicals occurring after a brief exposure.

Acute: Occurring within a short period in relation to the life span of the organism (usually four days for fish). It can be used to define either the exposure (acute test) or the response to an exposure (acute effect).

Adverse effect: Change in the morphology, physiology, growth, development, reproduction, or life span of an organism, system, or (sub) population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences.

Agent: A chemical, biological, or physical entity that contacts a target.

Agrochemicals: Synthetic chemicals such as pesticides and fertilizers used in agricultural production.

Assessment factor: Numerical adjustment used to extrapolate from experimentally determined (dose-response) relationships to estimate the agent exposure below which an adverse effect is not likely to occur.

Assessment: Evaluation or appraisal of an analysis of facts and the inference of possible consequences concerning a particular object or process.

Average score per taxon (ASPT): SASS score divided by the number of taxa.

Basal activity (of a biomarker): The level of a biomarker (e.g., acetylcholinesterase and lipid peroxidation) measured in a control organisms.

Battery toxicity testy: The parallel application of a range of different toxicity tests.

BG-11 medium: A universal medium for the culture and maintenance of blue-green algae.

Bioaccumulation: The net accumulation of a substance by an organism as a result of uptake from environmental medium.

Bioassay: Commonly used as synonyms with toxicity test.

Bioavailability: The rate and extent to which an agent can be absorbed by an organism and is available for metabolism or interaction with biologically significant receptors. Bioavailability involves both release from a medium (if present) and absorption by an organism.

Biological system scales sensitivity distribution (BS3D): A statistical or empirical distribution describing the variation among different biological system scales of a single species exposed to a toxic agent.

Biomarker: The use of physiological, biochemical, and histological changes as indicators of exposure.

Biomonitoring (biological monitoring): The regular and systematic use of living organisms to evaluate changes in environmental water quality.

Biota: The living organisms of a region or system.

Biotic: The living component of an ecosystem.

Biotope: An area of uniform environmental conditions.

Catchment: The land area from which a river or reservoir is fed; a drainage basin (known as 'watershed' in American usage).

Cause-effect relationship: The effect or response in question is clearly a direct or indirect result of the exposure of the organism(s) to the toxic agent(s) being examined.

Chronic toxicity: The harmful effects of a chemical or mixture of chemicals occurring after an extended exposure.

Chronic: Occurring after an extended time relative to the life span of an organism (conventionally taken to include at least one tenth of the life span). Long-term effects are related to changes in metabolism, growth, reproduction, or the ability to survive.

Community: An assemblage of populations of different species living in the same area at a given time. Sometimes, a particular sub-grouping may be specified, such as the macroinvertebrate community or the fish community in a river.

Concentration: Amount of a material or agent dissolved or contained in unit quantity in a given medium or system.

Concentration-effect relationship: Relationship between the exposure, expressed in concentration, of a given organism, system, or (sub) population to an agent in a specific pattern during a given time and the magnitude of a continuously graded effect to that organism, system, or (sub) population.

Concentration-response curve: A curve describing the relationship between different exposure concentrations of a material and percentage response of the exposed test population.

Confounding factor (CF): An extraneous (or hidden) factor thought to influence the observed physicochemical and biological conditions at a river site.

Confounding factor analysis (CFA): An assessment to determine if a suspected chemical agent is acting as a confounding factor and influencing the observed physicochemical and biological conditions at a river site.

Contaminant: A substance that is either present in an environment where it does not belong or is present at levels that might cause harmful effects to humans or the environment.

DEBtox version 2.0.1: Software that applies the DEB (dynamic energy budget) theory to ecotoxicological problems. It links (time-varying) external concentrations of a toxicant to the effects on endpoints such as survival, growth and reproduction, over time. Therefore, DEBtox ranks as a toxicokinetic-toxicodynamic (TKTD) modelling approach. In this way, DEBtox can be used to analyse toxicity data over time, and to predict effects under untested conditions.

Direct effect: an effect resulting from an agent acting on the assessment endpoint or other ecological component of interest itself, rather than on other components of the ecosystem (Posthuma *et al.,* 2002).

Ecology: the study of the interrelationships between organisms and their environments.

Ecosystem function: A biological, chemical or biological processes taking place in an ecosystem.

Ecosystem health: The level of functional efficiency of an ecosystem.

Ecosystem structure: The composition of the biological community in an ecosystem and the interrelationships between the individual populations of species.

Ecosystem: The interacting system of biological community (biotic factors) and its non-living environment (abiotic factors) (personal definition).

Ecotoxicity: The property of a compound to produce adverse effects in an ecosystem or one of its components.

ECOTOXicology database (ECOTOX): A database hosted by the USEPA that provides single chemical toxicity information for aquatic and terrestrial life.

Ecotoxicology: Study of the effects of toxic chemicals across all levels of biological organisation, including molecular, individual organisms, populations, communities and ecosystems. It is a multidisciplinary field, which integrates toxicology, ecology, and statistics.
Effect assessment: Combination of analysis and inference of possible consequences of the exposure to a particular agent based on knowledge of the dose-effect relationship associated with that agent in a specific target organism, system, or (sub) population.

Effect concentration (ECx): Concentration that causes designated effect criterion in x% of the population after a specified period of exposure. The EC values and their 95% confidence limits are usually derived by statistical analysis of effects in several test concentrations after a specified period of exposure.

Effect: Change in the state or dynamics of an organism, system, or (sub) population caused by the exposure to an agent.

Electrical conductivity (EC): The measure of electrical current conducted which depends on the ions in solution, and is also therefore a measure of the total quantity of salts dissolved in water.

Endpoint: A response measure in a toxicity test, i.e., the value(s) derived from a toxicity test that characterise the results of the tests (e.g., NOEC, LC50 or EC50).

Environmental water quality: The value or usefulness of water in a freshwater ecosystem, determined by the combined effects of its biological, chemical and physical chemical constituents, and varying from user to user.

Eutrophication: The process whereby high levels of nutrients result in the excessive growth of plants.

Expert judgement: Opinion of an authoritative person on a particular subject.

Exposure assessment: Evaluation of the exposure of an organism, system, or (sub) population to an agent (and its derivatives).

Exposure duration: The length of time over which continuous or intermittent contacts occur between an agent and a target.

Exposure route: The way in which an agent enters a target after contact (e.g., by ingestion, inhalation, or dermal absorption).

Exposure: Concentration or amount of a particular agent that reaches a target organism, system, or (sub) population in a specific frequency for a defined duration.

Extrapolation: Calculations, based on quantitative observations in exposed test species, of predicted concentration-effect and concentration-response relationships for a substance in a biota (or humans). This may include interspecies extrapolations and extrapolation to susceptible groups of individuals.

Fate: Pattern of distribution of an agent, its derivatives, or metabolites in an organism, system, compartment, or (sub) population of concern as a result of transport, partitioning, transformation, or degradation.

Fauna: Collective term for the animals living in a particular area or period.

Flora: Collective term for the plants living in a particular area or period.

Half-life: The time required for half of a chemical compound to break down in the environment. Half-lives of chemical compounds vary widely based on environmental factors, and the amount of chemical remaining after a half-life will always depend on the amount of the chemical originally applied. Some chemicals may degrade into compounds of toxicological significance. For example, the primary environmental degradate of glyphosate in and water is aminomethylphosphonic acid (AMPA), which is thought to contribute to the overall toxicity of the herbicide.

Hazard assessment: A process designed to determine the possible adverse effects of an agent or situation to which an organism, system, or (sub) population could be exposed. The process includes hazard identification and hazard characterisation. The process focuses on the hazard, in contrast to risk assessment, where exposure assessment is a distinct additional step.

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system, or (sub) population is exposed to that agent.
HC_5: Hazard concentration for 5% of the species.

HC_P: Hazard concentration for p% of the species.

Histopathology: The use of histological techniques to study pathological alterations in biological tissues. Histopathology is recognised as a biomarker of environmental pollution and is widely used in water quality monitoring programmes.

Immobility: The quality of not moving; remaining in place.

Indigenous: Living or growing naturally in a particular area, but not naturally confined only to that area.

Indirect effect: An effect resulting from the action of an agent on components of the ecosystem, which in turn affect the assessment endpoint or other ecological component of interest.

In-stream concentration: Concentration of chemical compounds measured from samples taken from the study sites.

Integrated water resource management: a system whereby individual management tools, standards, guidelines and good environmental practices are combined in a coordinated manner in order to derive maximum benefit from water resources for the present and future generations.

Lethal concentration (LCx): Concentration that results in an x% mortality of exposed population after a specified duration of exposure. The LC values and their 95% confidence limits are usually derived by statistical analysis of effects in several test concentrations after a specified period of exposure.

Lethal: Causing death by direct action.

Line of evidence: A set of data and associated analysis that can be used, alone or in combination with other lines of evidence, to estimate risks. Each line of evidence is qualitatively different from any others used in the risk characterisation. In ecotoxicological assessments, the most commonly used lines of evidence are based on biological survey, toxicity tests of contaminated media, and toxicity tests of individual chemicals.

Lowest Observed Effect Concentration (LOEC): The lowest concentration of a chemical agent used in a toxicity test that has a statistically significant adverse effect on the exposed population of test organisms compared with the controls.

Measurement endpoint: Measurable (ecological or biological) characteristic that is related to the valued characteristic chosen as an assessment point.

Median effect concentration (EC50): Concentration that causes designated effect criterion in 50% of the population after a specified period of exposure.

Median lethal concentration (LC50): Concentration that results in a 50% mortality of exposed population after a specified duration of exposure.

Medium: Material (e.g., air, water, soil, food, consumer products) surrounding or containing an agent.

Mode of action: A mode of action (MoA) describes a functional or anatomical change, at the cellular level, resulting from the exposure of a living organism to a chemical substance. In comparison, a mechanism of action (MOA) describes such changes at the molecular level. However, there is no clear consensus and the term mode of action is often used, especially in the study of pesticides, to also describe molecular mechanisms such as action on specific nuclear receptors or enzymes. A mode of action is important in classifying chemicals as it represents an intermediate level of complexity in between molecular mechanisms and physiological outcomes, especially when the exact molecular target has not yet been elucidated or is subject to debate. A mechanism of action of a chemical could be "binding to DNA" while its broader mode of action would be "transcriptional regulation".

Model: A formal representation of some component of the world or a mathematical function with parameters that can be adjusted so that the function closely describes a set of empirical data.

Multivariate statistical techniques (MST): A method used to analyse data that arises from more than one variable. It essentially models the reality situation involving more than a single variable. Multivariate analysis can be used to process data (usually contained in tables of rows and columns) in a meaningful fashion in order to make intelligent decisions.

No effect concentration: Highest concentration found by experiment or observation that causes no alterations of morphology, functional capacity, growth, development, or life span of target organisms distinguishable from those observed in control organisms under the same defined conditions of exposure.

No observed effect concentration (NOEC): The highest concentration of a material in a toxicity test that has no statistically significant adverse effect on the exposed population of test organisms compared with the controls.

Non-target organisms: Organisms (microbes, plants and animals) that are not intentionally targeted by a pest management strategy. When new pesticides are evaluated for regulation purposes, their impacts on non-target organisms are taken into consideration.

Normalisation: Alteration of a chemical concentration or other property (usually by dividing by a factor) to reduce variance due to some characteristic of an organism or its environment (e.g. division of the body burden of a chemical by the organism's lipid content to generate a lipid-normalised concentration).

No-till cultivation system: A system of planting crops into untilled soil by opening a narrow slot or trench only of sufficient width and depth to obtain proper seed coverage without disturbing the soil through tillage. The technique increases the amount of soil water, biodiversity and organic matter and decreases erosion. However, it requires usage of herbicide "burn-down" herbicide such as glyphosate instead of tillage for seedbed preparation. Thus, no-till is often associated with increased chemical use in comparison to traditional tillage based methods of crop production.

Oogonium: Primordial female germ cells which give rise to the oocytes.

Ovigerous female: A female organism, which is carrying eggs.

Percentile: Proportion expressed as a percentage. The median is the 50^{th} percentile.

Pesticides: A chemical used for killing pests. Major classes include insecticides (insect killers), herbicides (plant killers), and fungicides (fungus killers).

pH: The negative base 10 logarithm of the hydrogen ion activity (pH = 7 is neutral; pH < 7 is acid; pH > 7) is alkaline.

Physiology: Study of the internal processes and activities of organisms.

Phytobenthos: The aquatic ecosystem flora of the region at or near the bottom.

Phytoplankton: Photosynthetic microscopic organisms that inhabit the upper sunlit layer of aquatic ecosystems.

Phytotoxicity: A term that describes the degree of toxic effect by compounds (such as herbicides) on plant growth.

Pollutant: A harmful material that makes an environment less fit for organisms to occupy it.

Pollution: The degradation of natural systems by the addition of harmful substances.

Population: A group of interbreeding individuals of species living in a specific location in a given time.

Precautionary principle: An approach that exercises caution when uncertainties exist, generally assuming a worst-case scenario.

Regression analysis: Method helpful in ascertaining the probable form of the relationship between variables, its objective usually to predict or estimate the value of one variable corresponding to a given value of another variable.

Resource quality: Includes all aspects of water quantity, water quality and aquatic ecosystem quality, the latter including the quality of in-stream and riparian habitats and aquatic biota.

Resource Quality Objectives (RQOs): Numeric or descriptive (narrative) goals for resource quality within which a water resource must be managed. These are given legal status by being published in a Government Gazette.

Resource-directed measures (RDM): An approach to water quality management that takes into account the ecosystem requirements of the water resource, whilst still providing for the needs of other water users.

Response: Change developed in the state or dynamics of an organism, system, or (sub) population in reaction to exposure to an agent.

Riparian vegetation: Variety of plant species found near to a river bank.

Risk: The predicted or actual probability of occurrence of an adverse effect on humans or the environment due to exposure to a chemical substance or mixture.

Runoff: Rainfall that runs over the surface of the ground rather than filtering into it.

SASS score: Sum of the number of families (taxa) present at each sampling site against each taxon present. SASS is the South African Scoring System.

Sedimentation: The process whereby sediments are deposited at the bottom of an aquatic ecosystem.

Shikimate (shikimic acid) pathway: This is a seven step metabolic route used by plants and microorganisms (bacteria and fungi) for the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan). The shikimate pathway is not found in animals, including humans. Therefore, the products of this pathway represent essential amino acids that must be obtained from the animal's diet.

Source-directed controls (SDC): These are regulations of water use, which focus on managing the quality and quantity of water entering a water resource with the primary purpose of ensuring that the objectives that have been set for the water resource (typically defined by the management class and RQOs) are achieved. SDCs include regulatory mechanisms such as water quality standards for waste water, waste water discharges, pollution prevention, and waste minimisation technologies. Additionally, progressive implementation of self-regulation is encouraged. Economic incentive mechanisms are also implemented. Thus, source-directed controls (SDC) aim to control and minimise potential impacts on the water resource so that resource protection objectives (such as RQOs) are achieved.

South African Scoring System (SASS): A system for the rapid bioassessment of water quality of rivers using invertebrates.

Species sensitivity distribution (SSD): A statistical or empirical distribution describing the variation among a set of species exposed to a toxic agent. The set of species may be defined as a taxon, assemblage, or community.

Spermatid: Immature sperm, which derive nutrients from the testicular sertoli cells.

Spermatocyte: A male gametocyte derived from a spermatogonium and at the developmental stage of spermatogenesis during which meiosis occurs. It located in the seminiferous tubules of the testis.

Spermatogonia: Primordial male germ cells which give rise to the spermatocytes.

Spermatozoa: Mature motile sperm cells with flagella for swimming to an egg cell..

Stress response: An organism's response to stress placed on it.

Stressor: A chemical or biological agent, environmental condition, external stimulus or an event that causes burden (stress) to an organism.

Stressor: Any entity, stimulus, or condition that can modulate normal functions of the organism or induce an adverse response (e.g., agent, lack of food, drought).

Sub-lethal: Below the concentration that directly causes death, producing less obvious effects on behaviour, biochemistry and/or physiological function.

Susceptibility: The condition of an organism or other ecological system lacking the power to resist a particular disease, infection, or intoxication. It is inversely proportional to the magnitude of the exposure required to cause the response.

Target: Any biological entity that receives an exposure or a dose (e.g., a human, a human population, or a human organ).

The hydrological cycle: The continuous movement of water on, above and below the surface of the Earth.

Threshold: Dose or exposure concentration of an agent below which a stated effect is not observed or expected to occur.

Tolerance: The ability of an organism to withstand the adverse effects of pollution.

Toxicant: An agent or a substance capable of producing an adverse response in a biological system, seriously injuring structure and/or function or producing death.

Toxicity test: The means by which the toxicity of a chemical or other test substance is determined. A toxicity test is used to measure the degree of response produced by exposure to a specific level of stimulus (or concentration of chemical).

Toxicity: Inherent property of an agent to cause an adverse biological effect.

Trigger value (TV): A protective concentration value used to manage chemical substances in the environment for the protection of aquatic life.

Water quality: The physical, chemical, radiological, toxicological, biological and aesthetic properties of water that determine its fitness for use; or that are necessary for protecting the health of aquatic ecosystems. Water quality is therefore reflected in (a) concentrations of substances (either dissolved or suspended), (b) physicochemical attributes (e.g. temperature), (c) levels of radioactivity, and (d) biological responses to those concentrations, physicochemical attributes, or radioactivity.

Xenobiotic: A foreign chemical or material usually not produced in nature and not normally considered a constitutive component of a specified biological system.

ABBREVIATIONS AND ACRONYMS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AF	Assessment factor
AFDW	Ash-Free Dry Weight
AMPA	Aminomethylphosphonic acid
ANOVA	Analysis of variance
ANZECC	Australian and New Zealand Environment Conservation Council
ARMCANZ	Agriculture and Resource Management Council of Australia and New Zealand
ASPT	Average score per taxon
BAT	Best available technology
BATNEEC	Best available technology not entailing excessive cost
BPM	Best practical means
BS3D	Biological system scales sensitivity distribution
BSA	Bovine serum albumin
CAS	Chemical Abstracts Service
CCA	Canonical Correspondence Analysis
CCME	Canadian Council of Ministers of the Environment
CF	Condition factor
CFA	Confounding Factor Analysis
DDT	Dichlorodiphenyltrichloroethane
DO	Dissolved oxygen
DSZ	Downstream of spray zone
DWA	Department of Water Affairs
DWAF	Department of Water Affairs and Forestry
EC	Electrical conductivity
ECL	Environmental Concern Levels
EDC	Endocrine disrupting chemicals
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
EQO	Environmental quality objectives
ERA	Environmental risk assessment
ESL	Environmentally safe level
EWQ	Environmental water quality

FCE	Feed conversion efficiency
FCR	Feed conversion ratio
FI	Feed intake
GH	Growth hormone
GM	Genetically-modified
GMG-R	Genetically modified glyphosate-resistant
GR	Glyphosate-resistant
GSM	Gravel, sand and mud
H&E	Haematoxylin-eosin
HAE	4-hydroxyalkenals
HC	Hazardous concentration
HPLC	High performance liquid chromatography
HR	High reliability
HT	Herbicide-tolerance
IPA	Isopropylamine
IR	Insect-resistance
IUPAC	International Union of Pure and Applied Chemistry
IWR	Institute for Water Research
IWRM	Integrated water resource management
LHTT	Life history toxicity test
LOD	Limit of detection
LoE	Line of evidence
LOEC	Lowest observed effect concentration
LOQ	Limit of quantitation
LPx	Lipid peroxidation
LR	Low reliability
MAR	Mean Annual Runoff
MDA	Malondialdehyde
MIH	Moult inhibiting hormone
MR	Moderate reliability
MSH	Moult stimulating hormone
MST	Multivariate statistical technique
NEC	No effect concentration
NOEC	No observed effect concentration
NWA	National Water Act
NWRS	National Water Resource Strategy

OD	Optical density
OECD	Organisation for Economic Coorporation and Development
PAH	Polycyclic aromatic hydrocarbons
PC	Protective concentration
PCB	Polychlorinated biphenyls
PCA	Principal Component Analysis
PEP	Phosphoenolpyruvate
PI	Percent inhibition
PLG	Percent length gain
POEA	Polyoxyethylene amine
PWG	Percent weight gain
RDM	Resource-directed measures
RHP	River Health Programme
ROS	Reactive oxygen species
RQO	Resource quality objective
S3P	Shikimate-3-phosphate
SASS5	South African Scoring System version 5
SAWQG	South African water quality guidelines
SDC	Source-directed control
SGR	Specific growth rate
SSD	Species sensitivity distribution
SZ	Spray zone
TBARS	Thiobarbituric acid reactive species
TL	Total length
TMS	Table Mountain Sandstone
TV	Trigger values
UCEWQ	Unilever Centre for Environmental Water Quality
USEPA	United States Environmental Protection Agency
USZ	Upstream of spray zone
WfW	Working for Water
WHO	World Health Organisation
WoE	Weight of evidence
WQG	Water quality guideline
WWTW	Waste Water Treatment Work

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APPENDICES

APPENDIX A: Protocol for Acid-Wash of Glassware

Procedure

1. Take off any stick-on labels, parafilm, etc.
2. Rinse once with tap water.
3. Wash thoroughly with 5% Extran (phosphate free soap).
4. Rinse with tap water.
5. Soak in 10% hydrochloric acid (HCL) for 10 minutes.
6. Rinse well with tap water.
7. Rinse three times with boiling water (slightly cooled).
8. Rinse three times with deionized water.
9. Leave to drip dry on paper toweling next to the sink.

Precautions

1. Use containers provided for acid wash only.

2. Wear protective gloves, laboratory coat and safety goggles when acid washing glassware.

APPENDIX B: Range-finding Tests

Materials and methods

Forty days post hatching (dph) *Caridina nilotica* were exposed to a control and five different Roundup® concentrations: 0.0 (control), 0.1, 1, 10, 100 and 1000 mg/L. Each exposure category had three replicates, with 10 shrimps per replicate. Thus, the total number of shrimps per concentration was 30, with the total of number of shrimps used in this test being 180. The 96-hour static non-renewal was the toxicity method used for this test and shrimps were not fed throughout the experimental periods. The endpoint for this test was mortality. Shrimps were deemed dead if they became immobile and did not respond to a gentle prod or blow from a stream of water. For the duration of the test, dead shrimps were removed twice daily from each experimental unit and recorded. Water quality parameters, including temperature, electrical conductivity (EC), hydrogen ion concentration (pH) and dissolved oxygen (DO) were measured at the beginning and end of the experiment in all concentrations and the control. All mortality data were tested for normality using the Kolmogorov-Smirnov test. Roundup® 48-hour and 96-hour median lethal concentration (LC50) values and their associated 95% confidence limits were calculated for *C. nilotica*, using USEPA Probit analysis programme version 1.5 (USEPA, 1990). One-way analysis of variance (ANOVA) was performed to test the hypothesis that all mean mortality values across different concentrations were equal. In the presence of significant values, Tukey multiple comparison post hoc tests were used to compare the specific difference between any two means. Student's t-test was used to compare mean 48-hour and 96-hour LC50 values of separate age groups in the definitive acute toxicity tests. Dunnett's test was used to estimate the LOEC and NOEC values.

Results

The mean values and their standard deviations (± SD) measured for the water physicochemical parameters were 24.68 ± 0.29 ºC for temperature, 8.47 ± 0.17 for pH, 5.98 ± 0.44 mg/L for DO, and 0.92 ± 0.01 mS/cm for EC. These ranges were all within the acclimated conditions of the culture maintained in the laboratory. Mortalities of *C. nilotica* after 48-hour and 96-hour exposure to different Roundup® concentrations are presented in Table B.1.

Table B.1: Mortality data for 48-hour and 96-hour range-finding tests

Concentration (mg/L)	Number exposed	Number of mortalities	
		48-hour	96-hour
0.0	30	0	0
0.1	30	0	0
1.0	30	0	0
10.0	30	3	3
100.0	30	19	29
1000.0	30	30	30

One-way analyses of variance (ANOVA) of the 48-hour and 96-hour mean mortality values across categories of concentrations were found to be different ($p < 0.05$). However, Tukey pairwise comparison showed that the mean mortality values of shrimps exposed to control, 0.1, 1 and 10 mg/L of Roundup® were not significantly different from each other but significantly lower than mean values of shrimps exposed to 100 and 1000 mg/L. Mean mortality values of shrimps exposed to 100 mg/L were found to be significantly lower than shrimps exposed to 1000 mg/L for the 48-hour exposure test (Figure B.1), but there were no significant differences in mean mortality values for the 96-hour exposure test (Figure B.2). Lethal concentrations (LCs) and corresponding 95% confidence limits of Roundup® for *C. nilotica* were also calculated and are presented in Table B.2 for the 48-hour exposure test and Table B.3 for the 96-hour exposure test, respectively.

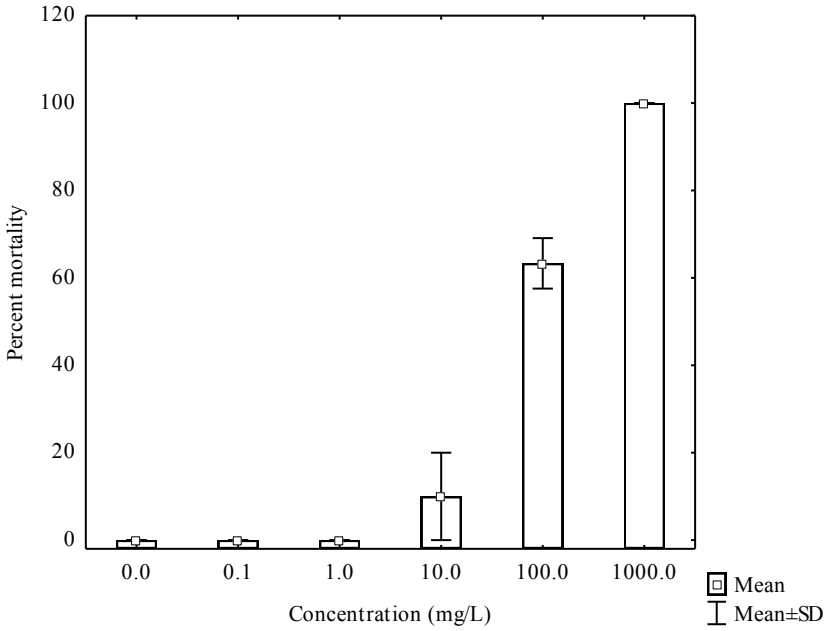

Figure B.1: *Caridina nilotica* 48-hour range-finding test percent mortality after exposure to different Roundup® concentrations

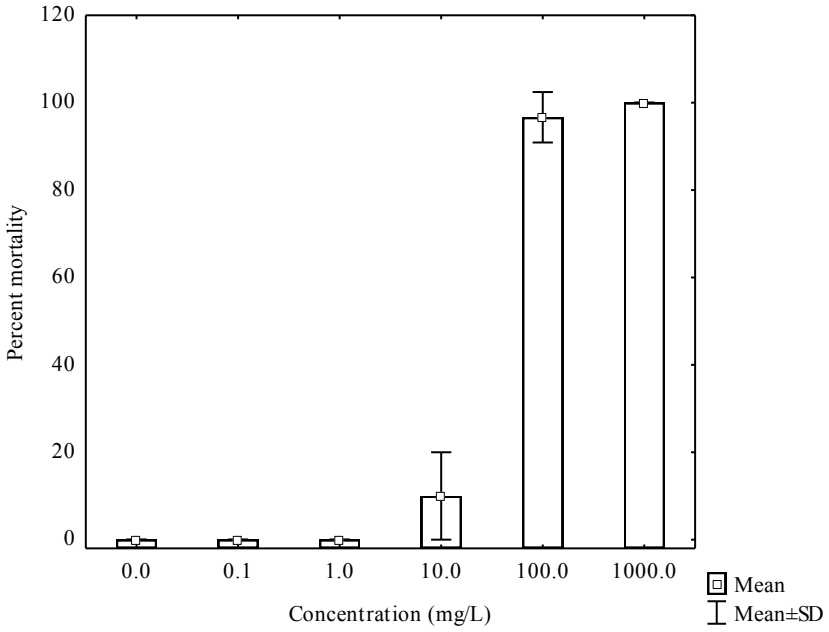

Figure B.2: *Caridina nilotica* 96-hour range-finding test percent mortality after exposure to different Roundup® concentrations

Table B.2: Probit estimate of 48-hour LC values and 95% confidence limits for range-finding test

Point estimate	Exposure concentration	95% confidence limits	
		Lower	Upper
LC1	3.071	0.672	6.980
LC5	7.232	2.314	13.81
LC10	11.419	4.419	20.108
LC15	15.542	6.784	26.120
LC50	57.193	35.444	92.464
LC85	210.468	124.949	485.098
LC90	286.457	162.237	745.041
LC95	452.284	236.112	1423.512
LC99	1065.215	466.999	4900.088

Table B.3: Probit estimate of 96-hour LC values and 95% confidence limits for range-finding test.

Point estimate	Exposure concentration	95% confidence limits	
		Lower	Upper
LC1	4.621	1.860	7.685
LC5	7.646	3.821	11.679
LC10	10.001	5.540	14.780
LC15	11.988	7.065	17.458
LC50	25.784	17.722	39.327
LC85	55.461	36.752	107.153
LC90	66.479	42.928	138.194
LC95	86.953	53.678	202.804
LC99	143.872	80.500	422.232

Discussion

The aim of a long-term toxicity test is to calculate endpoints such as survival, growth, or reproduction that are inhibited at a specific chemical concentration. Thus, if nothing is known about the sensitivity of the test animals to the test chemical, then a geometrically spaced series of test concentrations is used to establish a dose-response relationship for the key endpoints.

One way of establishing a dose-response relationship is to conduct a short-term range-finding test in which the organisms are exposed to a control and three or more concentrations of the test material that differ by a factor of 10 (OECD, 2005). The range-finding test was performed at the beginning of this study because there was no prior study involving the exposure of Roundup® to *C. nilotica*. In this study, the test design followed OECD (2005) recommendations for conducting a range-finding test for cases where no previous knowledge exists for either test chemical or species. Thus, five concentrations and a control were used in the range-finding test, as OECD (2005) recommended a minimum of three concentrations and a control. Furthermore, the test conditions were the same as the definitive acute toxicity tests. The test was deemed successful since there was 0% mortality in the control group. In a separate 96-hour range-finding test involving the mysid *Siriella armata* and *Daphnia magna* prior to a comparative ecotoxicological study, Perez and Beiras (2010) reported 10% control mortality with no need of water renewal or aeration but with daily feeding to prevent mortality of the test animals. The current study confirmed Perez and Beiras (2010) findings that a range-finding test not only helps to select appropriate concentrations for the subsequent definitive test, but also establishes conditions peculiar to the definitive test. However, contrary to Perez and Beiras (2010), this study found that the control group could survive without food for 96 hours.

Conclusion

The LC50 values of this study are associated with corresponding broad 95% confidence limits and represent preliminary results to establish the extent of Roundup® toxicity to *C. nilotica*. These data were very useful in selecting appropriate Roundup® concentrations for conducting the subsequent definitive short-term lethal and sub-lethal toxicity tests. The data also provided knowledge of conditions under which such tests could be carried out successfully.

APPENDIX C: Protocol for Histology

Fixative

Preparation of Davidson's alcohol formalin acetic acid fixative (AFA fixative)

- 330 mL 95% ethanol
- 220 mL 100% formalin (saturated aqueous solution of formaldehyde gas, 37-39% solution)
- 115 mL glacial acetic acid
- 335 mL distilled water
- Store at room temperature

Embedding

Day one

- 70% ethanol 1 hour
- 80% ethanol 1 hour
- 90% ethanol 1 hour
- 95% ethanol 1 hours
- 100% ethanol 1 hours
- 100% ethanol 1 hours
- Leave in 100% ethanol overnight

Day two

- Xylene 1 hour
- Xylene 1.5 hours
- Paraplast wax 1 hour (under vacuum)
- Paraplast wax 1.5 hours (under vacuum)
- Paraplast wax 2 hours (under vacuum)
- Embed in Paraplast wax and leave to set

Tissue sectioning

1. Paraffin blocks containing tissues were rimmed to the surface.
2. 5-µm sections were cut using a disposable knife blade.

3. A flexible paint brush was used to pick sections from the knife and these sections were floated out on the water bath at 45º C. This allowed removal of folds and expansion of the sections.
4. The flattened sections were then placed on the pre-treated glass slides with 2% aminopropyl triethoxy-saline in acetone.
5. The glass slides, with the tissues on them were air-dried for a minimum of 24 hours.

Staining (Garvey's modified Mayer's Hematoxylin-Eosin Staining)

Preparation of Garvey's modified Mayer's hematoxylin solution

1. 45 g potassium aluminium sulphate was dissolved in 900 mL distilled water contained in 1000-mL volumetric flask with the aid of heat.
2. 2.5 g hematoxylin was dissolved in 100 mL absolute ethanol contained in 100-mL volumetric flask.
3. The above solutions were then combined by transferring the hematoxylin-ethanol solution into the alum-distilled water solution.
4. 0.2 g sodium iodate and 1.0 g citric acid was added to the combined solutions and mixed well using the glass rod.

Preparation of eosin Y alcoholic stock solution, 1% (w/v)

1. Eosin Y: 10 g (or 1 g)
2. Distilled water: 200 mL (or 20 mL)
3. 95% ethanol: 800 mL (or 80 mL)
4. The 1% alcoholic stock solution was diluted with 80% ethanol in the ratio 1:3 to produce a working solution of strength 0.25%.

Staining procedure

1. Place slides containing sections in a slide holder
2. Deparaffinise and rehydrate sections:

Xylene	5 minutes
Xylene	5 minutes
Xylene	5 minutes

(*Blot excess xylene before going into ethanol*)

Absolute ethanol	2 minutes
Absolute ethanol	2 minutes

95% ethanol	2 minutes
95% ethanol	2 minutes
Distilled water	1 minute

(*Blot excess water from slide holder before going into haematoxylin*)

3. Filter haematoxylin to remove oxidized particles
4. Haematoxylin staining

Haematoxylin	5 minutes
Tap water	3 minutes
1% acid alcohol	1 minute
Tap water	3 minutes
70% ethanol	3 minutes

(*Blot excess water from slide holder before going into eosin*)

5. Eosin counter-staining

0.25% Eosin	1 minute
Tap water	0.5 – 1 minute

6. Dehydration

95% ethanol	2 minutes
95% ethanol	2 minutes
Absolute ethanol	2 minutes
Absolute ethanol	2 minutes

(*Blot excess ethanol from slide holder before going into xylene*)

7. Clearing

Xylene	5 minutes
Xylene	5 minutes
Xylene	5 minutes

Mounting

1. Place a drop of DPX mountant on the slide using a glass rod (ensure no bubbles).
2. Angle the coverslips and let them fall gently onto the slide
3. Allow DPX mountant to spread beneath the coverslip, covering all tissues.

Results

- Nuclei: Blue-black
- Cytoplasm: Varying shades of pink

- Muscle fibres: Deep pinky red.
- Fibrin: Deep pink
- Red blood cells: Orange/red

APPENDIX D: Acetylcholinesterase Assay Protocol (After McLoughlin *et al*., 2000)

Sample Preparation

1. Macerate organism in 30 µL 1 % Triton-X-100 in 0.02M PBS (pH 8).
2. Dilute homogenate with 270 µL PBS (pH 8).
3. Centrifuge homogenate at 14 000 g at 4°C for 10 min.
4. In another eppendorf, place 100 µL of homogenate supernatant and dilute to 500 µL with 400 µL 0.1 % Triton-X-100 in 0.02M PBS (pH 8). (Note: You may place another 100 µL of homogenate supernatant in separate eppendorf for stress protein assay)
5. Keep supernatants frozen at -80°C.

Run standard Curve and/or Sample

1. In a microplate, add volumes of chemicals and reagents as shown in the table below (defrost the substrate acetylthiocholine and add to plate last after preheating plate).
2. Quadruplicate blanks, standard curve points and samples.

Chemical	**µL**						
	Blank	**Std1**	**Std2**	**Std3**	**Std4**	**Std5**	**Samples**
Sample supernatant	0	0	0	0	0	0	50
0.1 % Triton-X-100 in 0.02M PBS pH 8	50	45	40	35	30	25	0
Commercial ACHE (1 unit per mL)	0	5	10	15	20	25	0
8mM 5,5-di-thiobis(2-nitrobenzoaic) acid	100	100	100	100	100	100	100
Acetylthiocholine iodide	50	50	50	50	50	50	50

3. Set up machine (select Biotek KC junior, select protocol name: AChE):
   A. Go to: Read Method
      i. Type: Kinetic
      ii. Wavelength: 405
      iii. Kinetic reads: 21 (= 10 min * 2 + 1st reading)
      iv. Interval: 30 sec
      v. Shake method: 1st read
      vi. Intensity: low

vii. Duration: 15 sec

viii. Click on box for incubation; set temperature at 30°C

B. Go to: Template

i. Well type selection: select "BLANKS"

ii. Well type selection: select "SAMPLES" (order in columns)

C. Click: OK

D. Click: Read plate – wait for machine to heat up (when the "OK" is clickable)

E. Place microtiter plate in the machine for 3 min to incubate

F. Add the substrate Acetylthiocholine iodide

G. Click "OK" and Run

H. Check the maximum curves in the programme and limit the number of readings to an amount that gives a straight line.

I. At the end, export "MAXIMUM SLOPE" and "BLANKED DATA" to excel file (include R^2 data)

J. Save excel file and Biotek file, and close KC junior.

AChE Activity Calculations

1. Determine average maximum slope from the four replicates (quadruplicate)
2. Undertake activity calculations as follows:

$$\text{Activity} = \frac{Change\ in\ absorbance(abs)}{Extinction\ coefficient \times light\ pathlength\ (\varepsilon \times L)} \times dilution\ factor(df) \times \frac{10^6}{10^3}$$

$$\text{Activity} = \left(\frac{\Delta abs}{\varepsilon \times L}\right) \times (df) \times 10^3$$

$$\text{Activity}\ \{\mu mole/mL/min\} = [\Delta abs\{min\}/\varepsilon\{molar/cm\} \times df\,(mL) \times 10^3$$

Where

- $df = \frac{Total\ volume\ of\ assay\ in\ each\ well(mL)}{Volume\ of\ the\ enzyme\ or\ sample(mL)}$
- $L = Height(h)(cm)$
- $Volume\ of\ cell = \pi r^2 h$

  $Thus,\ h = (volume) \times \left(\frac{1}{\pi}\right) \times \left(\frac{1}{r^2}\right)$
- $\varepsilon = 1.36 \times 10^4$ mol/*L*/cm (also known as molar absorption coefficient)

Notes:

1. Divide $by\ 10^3$ to change ε from mol/L to mol/mL and multiply by 10^6 to change moles to micromoles; for standard curve, do not use *df* as it's unnecessary).

2. Molar absorption coefficient: = coefficient × concentration × length of tube cotaining the solution.

APPENDIX E: Bradford Protein Assay Protocol (After Ringwood *et al*., 2003)

The protocol measures protein concentration in the range of 0.1 – 1.4 mg/mL.

Run standard curve and or sample

1. Prepare 2 mg/mL BSA stock solution by dissolving 0.02 g BSA in 10 mL milliQ water.
2. Set-up standard concentrations in eppendorf tubes following the table below:

Sample	BSA stock (uL)	MilliQ water (µL)	Standard protein conc. (µg/mL)
Blank	0	100	0
1	5	95	100
2	10	90	200
3	15	85	300
4	20	90	400
5	25	75	500
6	30	70	600
7	35	65	700
8	40	60	800
9	45	55	900
10	50	50	1000
11	55	45	1100
12	60	40	1200
13	65	35	1300
14	70	30	1400

3. In a microplate, add volumes of chemicals and reagents as shown in the table below.
4. Quadruplicate blanks, standard curve points and samples.

Chemical	**µL**						
	Blank	**Std1**	**Std2**	**Std3**	**Std4**	**Std5**	**Samples**
De-ionized water	5	0	0	0	0	0	0
BSA	0	5	5	5	5	5	0
Sample supernatant	0	0	0	0	0	0	5
Bradford dye reagent	250	250	250	250	250	250	250

5. Leave sample to stand for 5 min.
6. Set-up computer for reading
   A. Protocol name: Bradford
   B. Check that
      i. Type: Endpoint
      ii. Wavelength: 595
      iii. Shake method: First read
      iv. Duration: 60 sec.
   C. Go to template
      i. Well type selection: select blanks
      ii. Well type selection: select samples (order in columns)
   D. Click OK
   E. Click OK and RUN
   F. At the end, export blanketed data to excel file
   G. Save excel file and Biotek file and close

Calculate protein content

1. Average quadruplicate absorbance readings for each protein concentration/sample
2. Plot the info on a scatterplot, add trendline and get line equation ($y = mx + c$) and r^2.
3. Determine protein contents for samples.

Calculate specific activity

1. For each sample, divide the result from AChE assay with result from Bradford assay.
2. (μmol/mL/min) / (mg/mL) = mol/L/min/mg protein

APPENDIX F: Step-by-step calculation of ECx values using Microsoft Excel 2010

1. List concentrations in one column (A).
2. List corresponding mortality in next column (B).
3. Change concentrations to log base in next column (C) using Excel as follows: **=log(concentration value)**.
4. Change mortality to percent mortality in next column (D) using Excel as follows: **=(subjects respond/total number of subjects)*100**.
5. Select columns C and D.
6. Go to **insert**, select **scatter**, and click on **with only markers** (a scatter graph appears).
7. Click on any of the points to select them.
8. Right-click, and click on **Add Trendline.**
9. Select the boxes **Display Equation and Chart** and **Display R-squared value on Chart**, and close the display box.
10. The equation, in the form $y = ax + b$, and R-squared value are displayed on the chart.
11. Make ***x*** the subject of the formula, and substitute ***y*** with 50, 20 or 10 if you are estimating EC50, 20 or EC10, respectively (***x*** is the calculated EC value) in Excel as follows: **=(y-b)/a**.
12. Change the value of ***x*** (which is in log form) back to a non-log form as in Excel follows: **=power(10,*x*)**.

APPENDIX G: Mean lengths of Caridina nilotica exposed to different Roundup® concentrations from 40 to 65 days post hatch (dph)

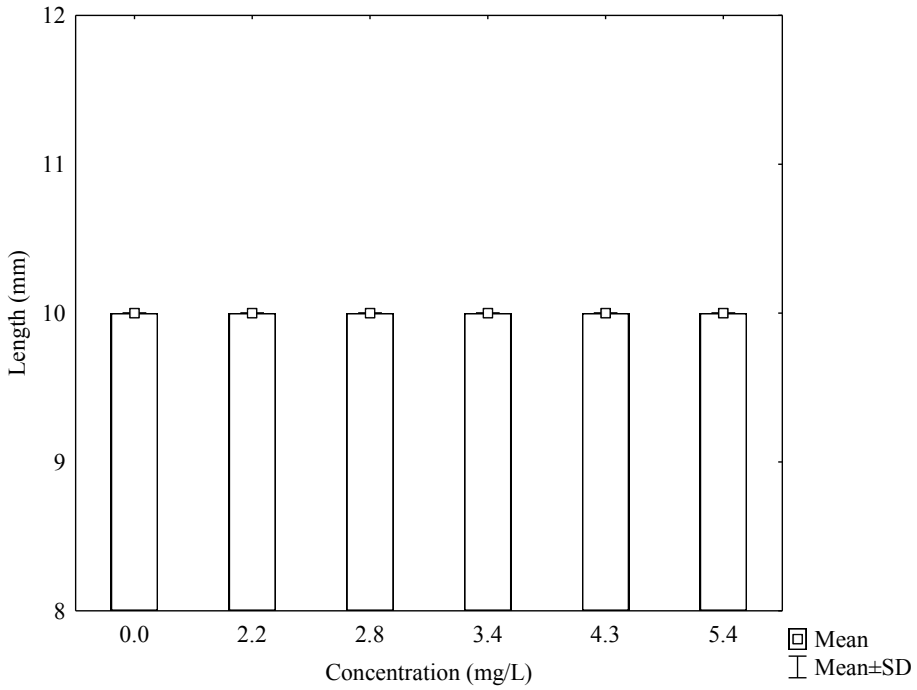

Figure G.1: Mean lengths of 40 dph shrimps at the start of exposure to different Roundup® concentrations

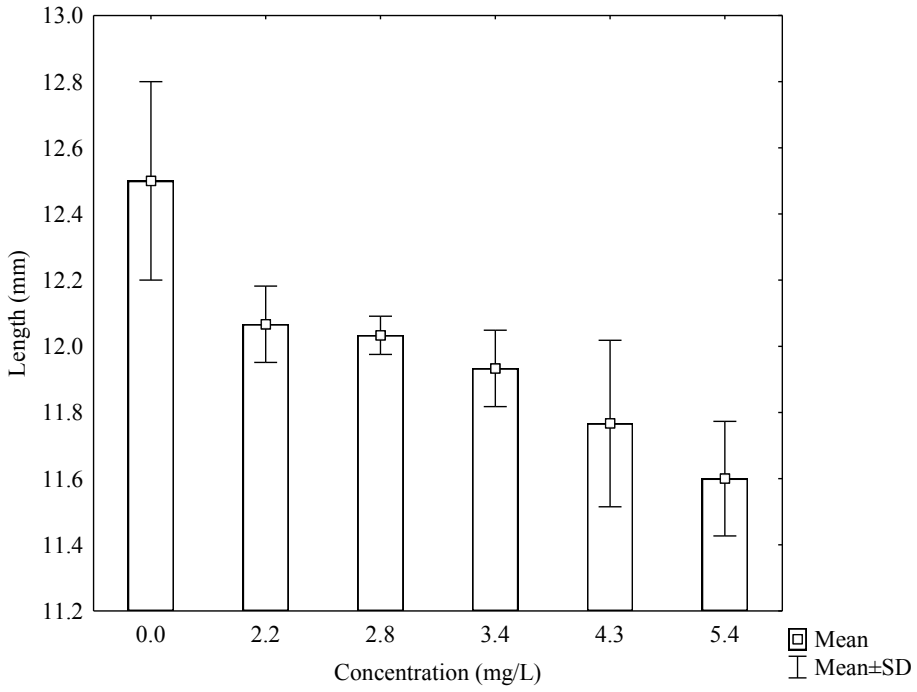

Figure G.2: Mean lengths of 45 dph shrimps after five days' exposure to different Roundup® concentrations

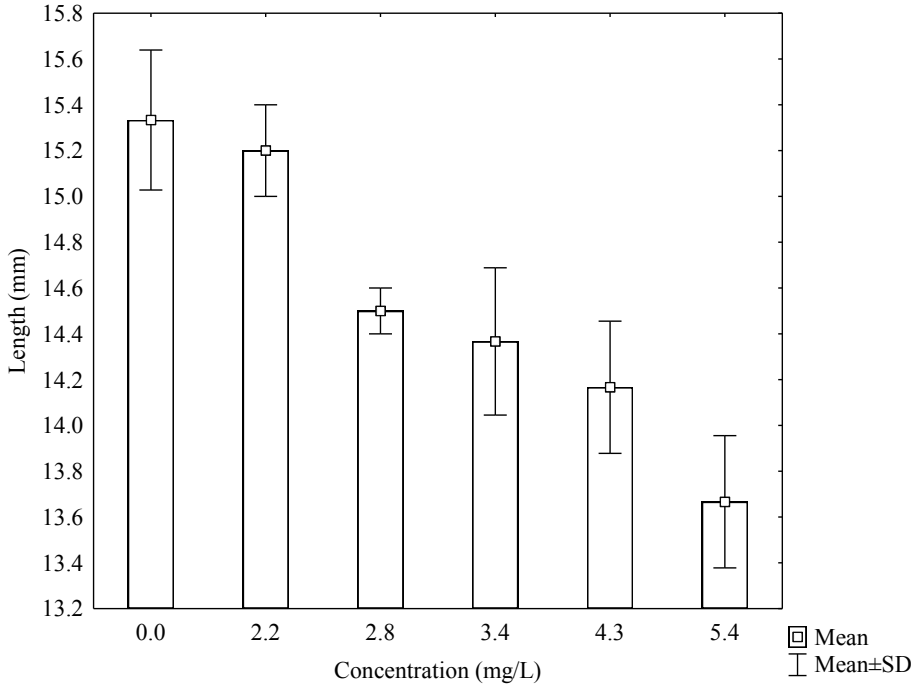

Figure G.3: Mean lengths of 50 dph shrimps after ten days' exposure to different Roundup® concentrations

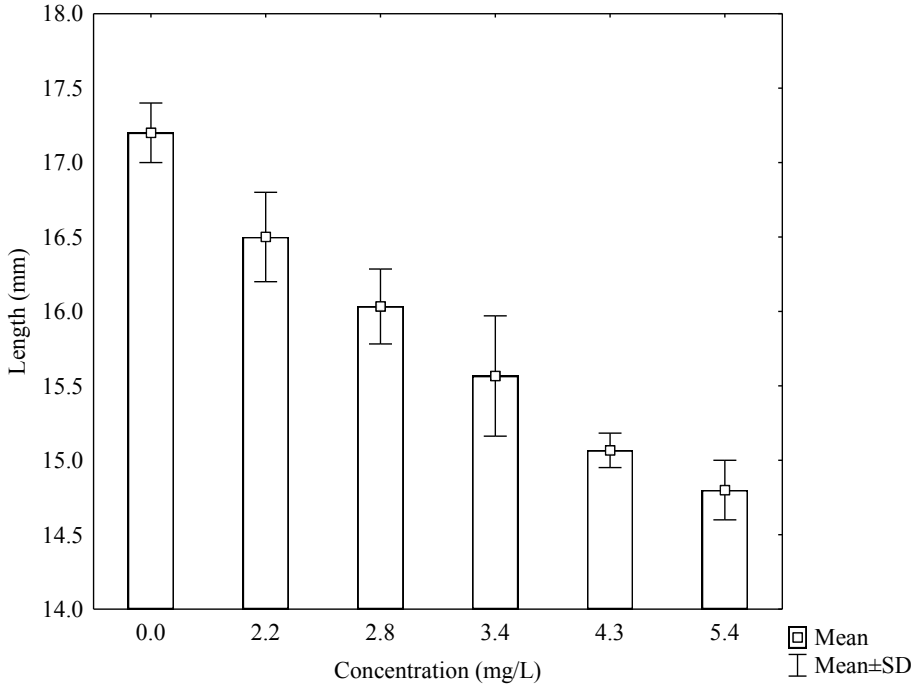

Figure G.4: Mean lengths of 55 dph shrimps after 15 days' exposure to different Roundup® concentrations

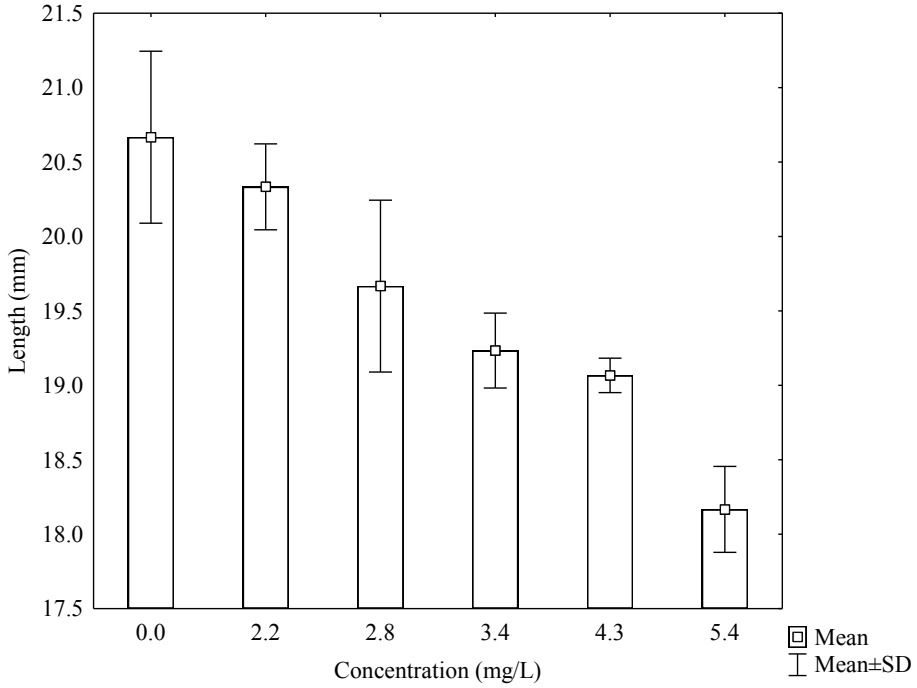

Figure G.5: Mean lengths of 60 dph shrimps after 20 days' exposure to different Roundup® concentrations

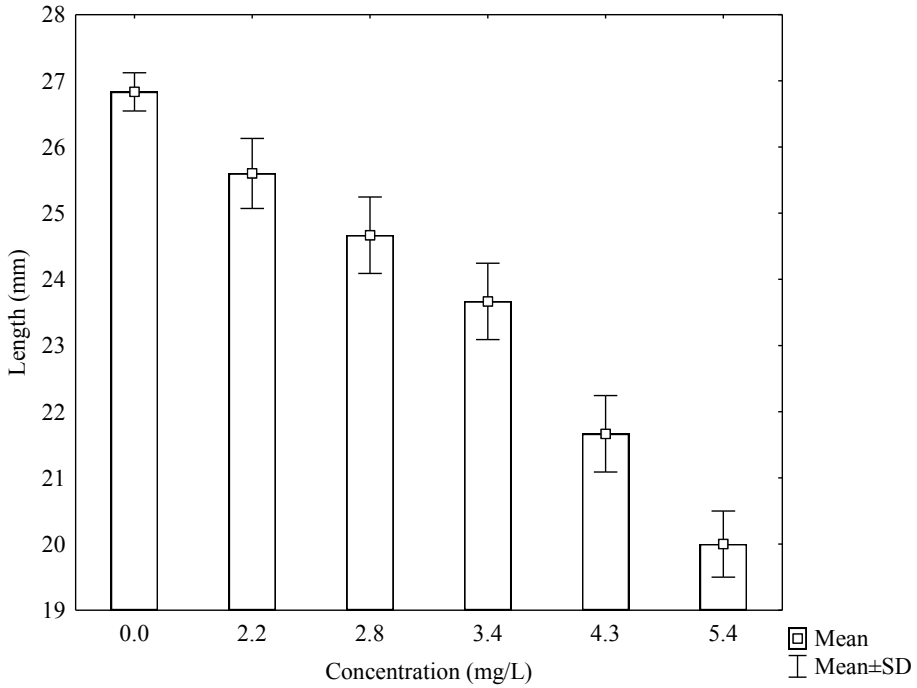

Figure G.6: Mean lengths of 65 dph shrimps after 25 days' exposure to different Roundup® concentrations

APPENDIX H: Mean weights of *Caridina nilotica* exposed to different Roundup® concentrations from 40 to 65 days post hatch (dph)

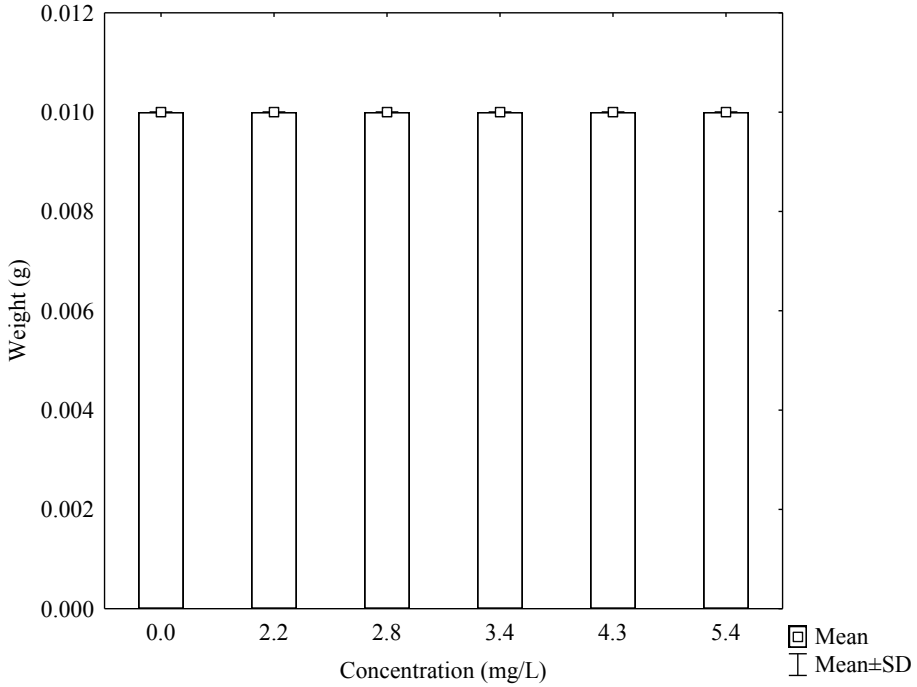

Figure H.1: Mean weights of 40 dph shrimps at the start of exposure to different Roundup® concentrations

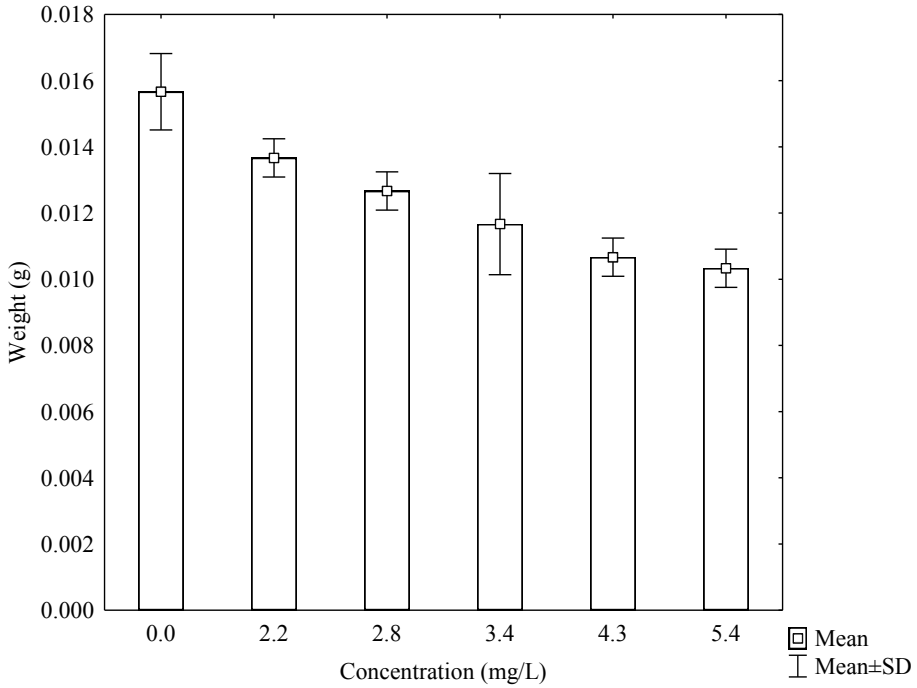

Figure H.2: Mean weights of 45 dph shrimps after 5 days' exposure to different Roundup® concentrations

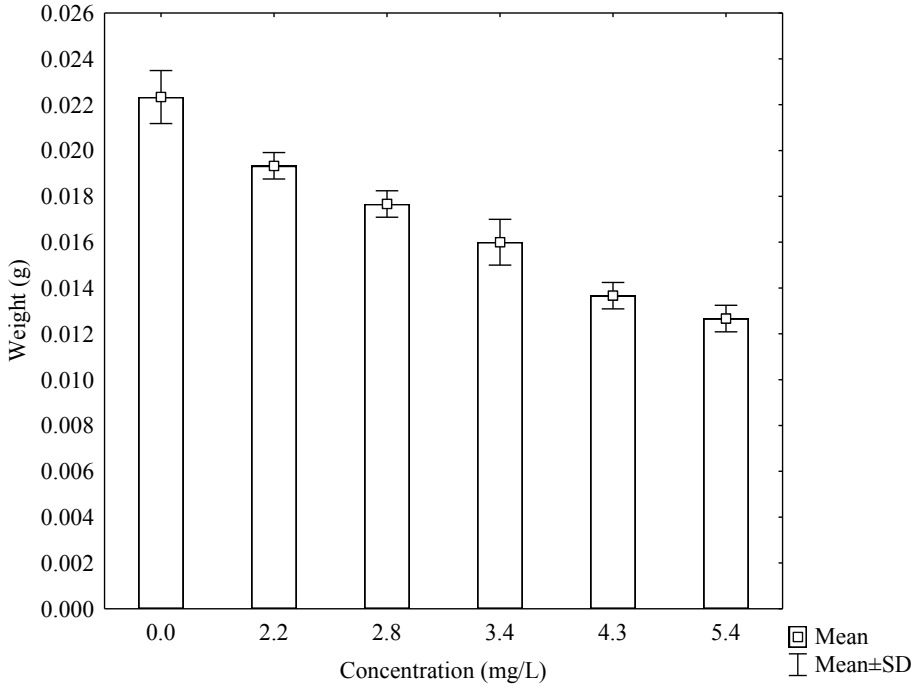

Figure H.3: Mean weights of 50 dph shrimps after 10 days' exposure to different Roundup® concentrations

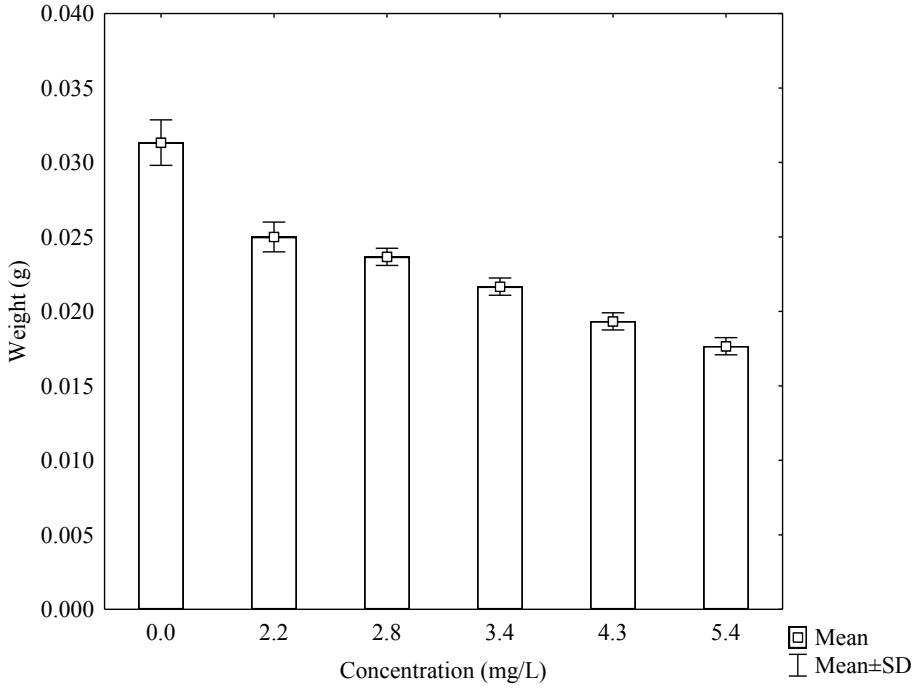

Figure H.4: Mean weights of 55 dph shrimps after 15 days' exposure to different Roundup® concentrations

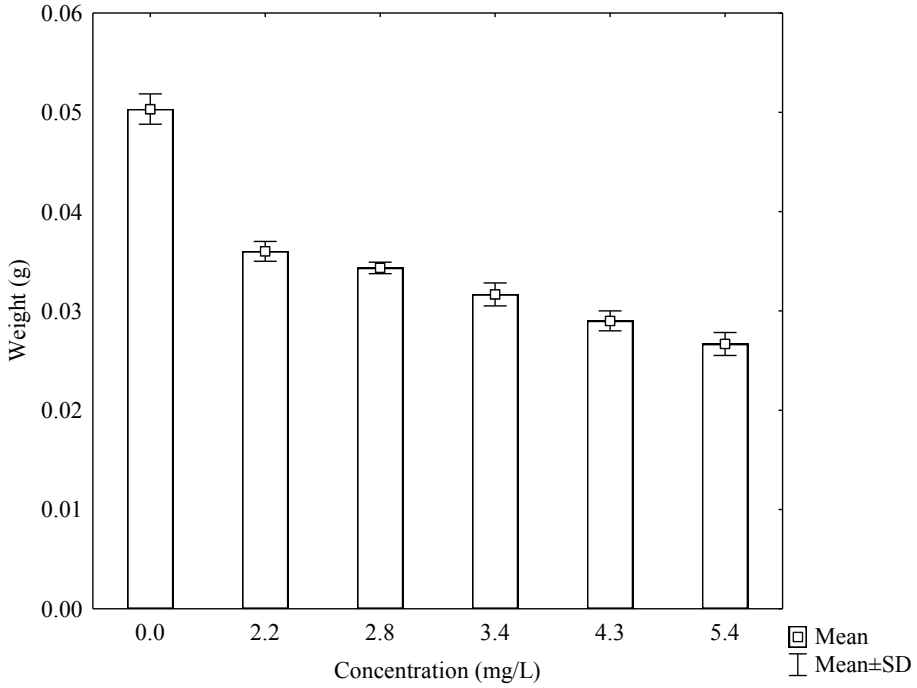

Figure H.5: Mean weights of 60 dph shrimps after 20 days' exposure to different Roundup® concentrations

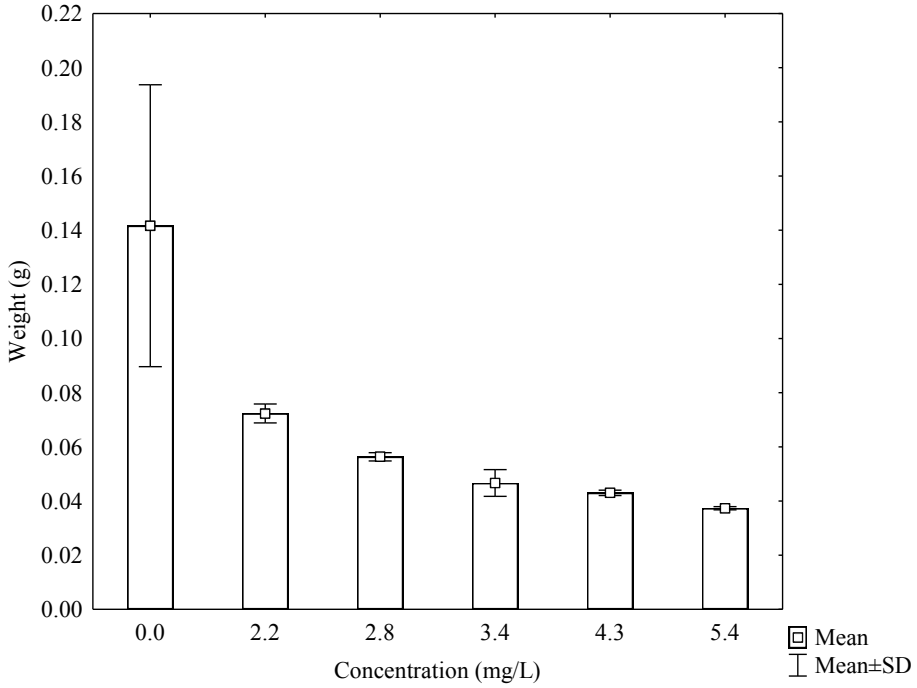

Figure H.6: Mean weights of 65 dph shrimps after 25 days' exposure to different Roundup® concentrations

APPENDIX I: Descriptions of Embryonic Development

Embryonic development was completed by day-17 in the control group and all treatment groups. However, a few embryos hatched in concentrations 0.625 and 1.25 mg/L before day-17. Observed developments as revealed by this study at specific ages of *Caridina nilotica* embryo are described below:

At **day-1**, most of the fertilised eggs in all treatment groups and control were still in the blastula stage with evenly distributed yolk mass. Embryos appeared greenish and had a single layer of chorion protecting the yolk. Cleavage was initiated within this period as was recorded in embryos exposed to 2.5 mg/L Roundup® concentration. Blastomeres were formed and the embryo entered the gastrula stage.

At **day-3**, gastrulation occurred as light patches of yolk cells began to give rise to antennae, antennules and optic rudiments. About 90% of the yolk cells remained in the control and all treatment group embryos.

At **day-6**, body segmentation began with well-developed antennae, antennules and oval black eyes in the control and all treatment group embryos. The caudal papilla also developed. About 70% of the yolk cells remained in the control and all treatment group embryos.

At **day-9**, body segmentation became prominent with somites seen in all treatment group embryos and the control. Appendages and the entire embryo began to move, but this was more prominent in the control than in exposed groups. About 50% of yolk cells remained in the control and all treatment group embryos.

At **day-12**, heartbeat and pleopods of embryos were visible in the control and all treatment groups, and less than 30% of yolk cells remained.

At **day-14**, embryo heartbeat and movements were faster; external features and appendages were prominent; eyes were larger, black and round; and yolk cells were completely absorbed or less than 10% of the yolk remained in all experimental groups. Some embryos in 0.625 and 1.250 mg/L hatched and released neonates (larvae) into the test solution.

At **day-17**, embryos hatched in all experimental groups, releasing neonates that were somatically identical to the adult but significantly smaller. Except in the control group neonates, all exposed group neonates had curved bodies.

APPENDIX J: Preparation of Algal Inoculum and Bg-11 Medium

1. Use a 3 to 4-day old logarithmic phase algal culture.

2. Remove the supernatant medium from the settled algal layer in the algal flask by means of a sterile Pasteur pipette and vacuum pump (do not disturb the algal layer to retain as much of the algal cells as possible).

3. Discard the supernatant medium.

4. Add 50 mL sterile BG-11 with a sterile 25 me pipette to the algal flask (avoid contamination of medium by not touching the flask sides or use a fresh pipette for the second 25 mL medium aliquot).

5. Use the same pipette to re-suspend the algae (pull up and push out, a number of times).

6. Cover the flask with a stopper and leave on work surface for preparation of the algal inoculum.

7. Dispense 9 mL of the BG-11 into a sterile glass tube in a test tube rack, using a sterile 10 mL pipette.

8. Transfer 1 mL of the well-mixed resuspended algae in the flask to the test tube, using a sterile 1 mL pipette (a micropipette can cause contamination).

9. Mix well.

10. Prepare the haemacytometer for cell counting.

Place the well-mixed cell suspension in the tube with a micropipette in the haemacytometer and count the cells microscopically (at least 10 areas).

1. Calculate the number of cells in the resuspended algal culture: Mean cell number x 10^1 (for 10-fold dilution) x 10^4 (factor used for haemacytometer) *(e.g.* 100 x 10 x 10 000 = 10 000 000 cells/mL).

2. Calculate the algal inoculum volume for the test and control wells (excluding blank wells): 200 000 cells are used per mL sample (400 000 cells/well). 0.1 mL algal inoculum is used per well.

Reference

DWAF (Department for Water Affairs and Forestry) (2003). The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach. A discussion document. Institute for Water Quality Studies. Department of Water Affairs and Forestry, Pretoria, South Africa.

APPENDIX K: Step-By-Step Calculations behind Using The Species Sensitivity Distribution (SSD) Generator

1. Calculate the mean and log10 of the mean for each taxon (Obs). A geometric mean may be more appropriate for highly skewed data, but this may give an outlier excessive weight if there are few data. The arithmetic mean is given by: (x1+x2+x3…xn)/n. The geometric mean is given by: (x1*x2*x3…xn)1/n.

2. Convert ranks to proportions: Proportion = (rank-0.5)/Number of Species.

3. Transform proportions to Probit. The Probit is the inverse cumulative distribution function of the normal distribution with a mean of 5 and a standard deviation of 1. A mean of 5 is chosen to ensure that all Probit values are non-negative.

4. Calculate the slope and intercept for Log 10 Mean (X axis) * Probit (Y axis).

5. Calculate the log 10 central tendency (predicted value) for the regression line:
   log 10 Central Tendency = (Probit-Intercept)/Slope. Then: Calculate Prediction Intervals (after Neter *et al*., 1990).

6. Calculate the Mean Squared Error (MSE): For each taxon, subtract the observed log10 mean (Obs) from the log10 central tendency (predicted value), square, add these values and divide by n-2.

7. Calculate the Corrected Sum of Squares (CSSQ): For each taxon, square each Probit value then sum (this is the sum of squares). Next, sum the Probit values for all taxa, square that and divide by the number of taxa (this is the average sum squared). Subtract the average sum squared from the total sum of squares to get the CSSQ.

8. Calculate the Grand Mean - this is the average of all log10 exposure values.

9. Calculate the Point Error: (MSE/(Slope2))*(1+(1/n)+((Pred-Grand Mean)2).

10. Calculate the prediction intervals (PI) using the critical t value (e.g., for n = 5, tCrit = 2.02). Log PI = Log Central Tendency +/- tCrit*(SQRT(Point Error)).
11. Back convert from log value: 10^{value}

Note: * = multiplication sign

References

Neter, J., Wasserman, W. and Kutner, M.H. (1990). *Applied Linear Statistical Models* (3rd edn.). Irwin, Boston, MA, USA. 1184 pp.

Posthuma, L., Suter, G.W. and Traas, T.P. (2002). *Species Sensitivity Distributions in Ecotoxicology.* Lewis Publishers, Boca Raton, FL., USA. 587 pp.

USEPA (United States Environmental Protection Agency) (2005). Methods/indicators for determining when metals are the cause of biological impairments of rivers and streams: species sensitivity distributions and chronic exposure-response relationships from laboratory data. EPA/600/X-05/027. Cincinnati, Ohio, U.S. EPA, Office of Research and Development, National Center for Environmental Assessment.

APPENDIX L: Model Parameters and Statistics for Different Species Sensitivity Distribution (SSD) Curves performed with the Species Sensitivity Distribution Generator

Table L.1: Model parameters **(a)** and statistics **(b)** for species sensitivity distribution (SSD) curve of South African freshwater aquatic organisms based on short-term 48-96 hour LC50 values for glyphosate conducted with The Species Sensitivity Distribution Generator

(a)

Parameters	
Slope	1.206
Intercept	4.706
R^2	0.893
GrandMean	0.244
SumSQ	4.656
CSSQ	4.180
MSE	0.121
Tcrit	1.943
N	8
df	6

(b)

Proportion	**Probit**	**Log Central Tendency**	**SSQ**	**Log Upper PI**	**Log Lower PI**	**Central Tendency**	**Upper PI**	**Lower PI**
0.05	3.355	-1.120	0.131	-0.417	-1.823	0.076	0.383	0.015
0.1	3.718	-0.818	0.116	-0.156	-1.481	0.152	0.699	0.033
0.2	4.158	-0.454	0.104	0.172	-1.079	0.352	1.484	0.083
0.4	4.747	0.034	0.095	0.632	-0.564	1.082	4.286	0.273
0.5	5.000	0.244	0.094	0.839	-0.351	1.754	6.907	0.446
0.7	5.524	0.679	0.098	1.286	0.072	4.774	19.315	1.180
0.8	5.842	0.942	0.104	1.567	0.317	8.747	36.906	2.073
0.9	6.282	1.307	0.116	1.969	0.644	20.258	93.194	4.404
0.95	6.645	1.608	0.131	2.311	0.905	40.532	204.599	8.030

Table L.2: Model parameters **(a)** and statistics **(b)** for species sensitivity distribution (SSD) curve of South African freshwater aquatic organisms based on long-term NOEC values for glyphosate conducted with The Species Sensitivity Distribution Generator

(a)

Parameters	
Slope	1.827
Intercept	5.308
R^2	0.860
GrandMean	-0.191
SumSQ	2.097
CSSQ	1.804
MSE	0.164
Tcrit	1.943
N	8
df	6

(b)

Proportion	**Probit**	**Log Central Tendency**	**SSQ**	**Log Upper PI**	**Log Lower PI**	**Central Tendency**	**Upper PI**	**Lower PI**
0.05	3.355	-1.069	0.076	-0.532	-1.605	0.085	0.294	0.025
0.1	3.718	-0.870	0.068	-0.364	-1.376	0.135	0.433	0.042
0.2	4.158	-0.629	0.061	-0.151	-1.107	0.235	0.706	0.078
0.4	4.747	-0.307	0.056	0.151	-0.766	0.493	1.417	0.172
0.5	5.000	-0.168	0.055	0.289	-0.625	0.678	1.943	0.237
0.7	5.524	0.119	0.058	0.586	-0.349	1.314	3.856	0.448
0.8	5.842	0.292	0.062	0.775	-0.190	1.959	5.951	0.645
0.9	6.282	0.533	0.070	1.045	0.020	3.411	11.103	1.048
0.95	6.645	0.732	0.078	1.276	0.187	5.391	18.885	1.539

Table L.3: Model parameters **(a)** and statistics **(b)** for species sensitivity distribution (SSD) curve of South African freshwater aquatic organisms based on long-term NEC values for glyphosate conducted with The Species Sensitivity Distribution Generator

(a)

Parameters	
Slope	0.927
Intercept	5.844
R^2	0.873
GrandMean	-0.911
SumSQ	13.569
CSSQ	6.925
MSE	0.144
Tcrit	1.943
N	8
df	6

(b)

Proportion	**Probit**	**Log Central Tendency**	**SSQ**	**Log Upper PI**	**Log Lower PI**	**Central Tendency**	**Upper PI**	**Lower PI**
0.05	3.355	-2.687	0.265	-1.686	-3.687	0.002	0.021	0.000
0.1	3.718	-2.295	0.235	-1.353	-3.236	0.005	0.044	0.001
0.2	4.158	-1.820	0.209	-0.932	-2.707	0.015	0.117	0.002
0.4	4.747	-1.185	0.190	-0.337	-2.033	0.065	0.461	0.009
0.5	5.000	-0.911	0.189	-0.067	-1.755	0.123	0.856	0.018
0.7	5.524	-0.345	0.196	0.516	-1.206	0.452	3.280	0.062
0.8	5.842	-0.003	0.209	0.885	-0.890	0.993	7.666	0.129
0.9	6.282	0.472	0.235	1.414	-0.470	2.964	25.930	0.339
0.95	6.645	0.864	0.265	1.864	-0.136	7.312	73.151	0.731

Table L.4: Model parameters **(a)** and statistics **(b)** for species sensitivity distribution (SSD) curve of freshwater aquatic organisms from ECOTOX database based on short-term 24-96 hour LC50 values for glyphosate conducted with The Species Sensitivity Distribution Generator

(a)

Parameters	
Slope	1.267
Intercept	3.165
R^2	0.965
GrandMean	1.448
SumSQ	117.958
CSSQ	25.692
MSE	0.036
Tcrit	1.682
N	44
df	42

(b)

Proportion	**Probit**	**Log Central Tendency**	**SSQ**	**Log Upper PI**	**Log Lower PI**	**Central Tendency**	**Upper PI**	**Lower PI**
0.05	3.355	0.150	0.024	0.412	-0.113	1.412	2.585	0.771
0.1	3.718	0.437	0.024	0.696	0.177	2.732	4.967	1.503
0.2	4.158	0.784	0.023	1.041	0.527	6.078	10.980	3.365
0.4	4.747	1.248	0.023	1.503	0.993	17.706	31.842	9.845
0.5	5.000	1.448	0.023	1.703	1.193	28.060	50.441	15.610
0.7	5.524	1.862	0.023	2.118	1.606	72.780	131.080	40.410
0.8	5.842	2.112	0.023	2.369	1.856	129.540	234.006	71.710
0.9	6.282	2.460	0.024	2.719	2.200	288.172	523.913	158.506
0.95	6.645	2.746	0.024	3.009	2.484	557.721	1021.303	304.565

Table L.5: Model parameters **(a)** and statistics **(b)** for species sensitivity distribution (SSD) curve of freshwater aquatic organisms from ECOTOX database based on long-term NOEC values for glyphosate conducted with The Species Sensitivity Distribution Generator

(a)

Parameters	
Slope	0.889
Intercept	5.544
R^2	0.961
GrandMean	-0.646
SumSQ	29.270
CSSQ	21.350
MSE	0.040
Tcrit	1.740
N	19
df	17

(b)

Proportion	**Probit**	**Log Central Tendency**	**SSQ**	**Log Upper PI**	**Log Lower PI**	**Central Tendency**	**Upper PI**	**Lower PI**
0.05	3.355	-2.461	0.062	-2.029	-2.893	0.003	0.009	0.001
0.1	3.718	-2.053	0.059	-1.632	-2.474	0.009	0.023	0.003
0.2	4.158	-1.558	0.056	-1.147	-1.969	0.028	0.071	0.011
0.4	4.747	-0.896	0.054	-0.492	-1.301	0.127	0.322	0.050
0.5	5.000	-0.612	0.054	-0.208	-1.015	0.245	0.619	0.097
0.7	5.524	-0.022	0.055	0.385	-0.429	0.951	2.428	0.373
0.8	5.842	0.335	0.056	0.747	-0.077	2.163	5.585	0.837
0.9	6.282	0.830	0.059	1.252	0.407	6.757	17.877	2.554
0.95	6.645	1.238	0.062	1.672	0.804	17.311	47.042	6.370

Table L.6: Model parameters **(a)** and statistics **(b)** for species sensitivity distribution (SSD) curve for a combined South African and ECOTOX database LC50 values for glyphosate conducted with The Species Sensitivity Distribution Generator

(a)

Parameters	
Slope	1.058
Intercept	3.670
R^2	0.944
GrandMean	1.257
SumSQ	124.956
CSSQ	42.787
MSE	0.057
Tcrit	1.676
N	52
df	50

(b)

Proportion	**Probit**	**Log Central Tendency**	**SSQ**	**Log Upper PI**	**Log Lower PI**	**Central Tendency**	**Upper PI**	**Lower PI**
0.05	3.355	-0.298	0.055	0.095	-0.691	0.504	1.245	0.204
0.1	3.718	0.046	0.054	0.435	-0.343	1.111	2.720	0.454
0.2	4.158	0.461	0.053	0.847	0.076	2.894	7.027	1.192
0.4	4.747	1.018	0.052	1.400	0.635	10.413	25.140	4.313
0.5	5.000	1.257	0.052	1.640	0.875	18.074	43.611	7.490
0.7	5.524	1.753	0.052	2.136	1.369	56.592	136.891	23.396
0.8	5.842	2.053	0.053	2.438	1.667	112.880	274.111	46.484
0.9	6.282	2.468	0.054	2.857	2.080	294.085	720.112	120.100
0.95	6.645	2.812	0.055	3.205	2.419	648.475	1602.869	262.355

Table L.7: Model parameters **(a)** and statistics **(b)** for *Caridina nilotica* biological system scales sensitivity distribution (BS3D) based on short-term EC50 values

(a)

Parameters	
Slope	1.826
Intercept	2.909
R^2	0.987
GrandMean	1.145
SumSQ	7.691
CSSQ	1.135
MSE	0.017
Tcrit	2.353
N	5
df	3

(b)

Proportion	Probit	Log Central Tendency	SSQ	Log Upper PI	Log Lower PI	Central Tendency	Upper PI	Lower PI
0.05	3.355	0.244	0.010	0.477	0.012	1.755	2.996	1.028
0.1	3.718	0.443	0.008	0.658	0.229	2.775	4.547	1.694
0.2	4.158	0.684	0.007	0.882	0.487	4.833	7.617	3.066
0.4	4.747	1.006	0.006	1.191	0.821	10.147	15.540	6.626
0.5	5.000	1.145	0.006	1.329	0.961	13.966	21.325	9.147
0.7	5.524	1.432	0.006	1.622	1.243	27.056	41.835	17.497
0.8	5.842	1.606	0.007	1.804	1.408	40.362	63.619	25.607
0.9	6.282	1.847	0.008	2.061	1.632	70.289	115.178	42.895
0.95	6.645	2.046	0.010	2.278	1.814	111.133	189.683	65.111

Table L.8: Model parameters **(a)** and statistics **(b)** for *Caridina nilotica* biological system scales sensitivity distribution (BS3D) based on long-term EC10 values

(a)

Parameters	
Slope	1.070
Intercept	5.086
R^2	0.694
GrandMean	-0.081
SumSQ	3.566
CSSQ	3.520
MSE	0.356
Tcrit	2.015
N	7
df	5

(b)

Proportion	Probit	Log Central Tendency	SSQ	Log Upper PI	Log Lower PI	Central Tendency	Upper PI	Lower PI
0.05	3.355	-1.617	0.564	-0.104	-3.131	0.024	0.787	0.001
0.1	3.718	-1.278	0.482	0.121	-2.677	0.053	1.322	0.002
0.2	4.158	-0.867	0.410	0.423	-2.157	0.136	2.651	0.007
0.4	4.747	-0.317	0.360	0.892	-1.527	0.482	7.804	0.030
0.5	5.000	-0.081	0.355	1.121	-1.282	0.830	13.203	0.052
0.7	5.524	0.409	0.377	1.646	-0.828	2.566	44.249	0.149
0.8	5.842	0.706	0.410	1.996	-0.585	5.076	99.070	0.260
0.9	6.282	1.117	0.482	2.516	-0.283	13.078	327.848	0.522
0.95	6.645	1.456	0.564	2.969	-0.057	28.572	931.897	0.876

APPENDIX M: Seasonal Sampling of Macroinvertebrates before Spray with Glyphosate Herbicide

Table M.1: Seasonal sampling of macroinvertebrates before spray with glyphosate herbicide at spray zone (SZ) (R1, R2 and R3 represent first, second and third replicates, respectively)

	Spring			Summer			Autumn			Winter		
TAXON	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
Annelida												
Oligochaeta	37	44	32	72	31	27	19	18	18	23	4	33
Hirudinea	5	8	3	1	4	1	5	2	3	0	1	0
Crutacea	0	0	0	0	0	0	0	0	0	0	0	0
Potamonautida	0	2	1	0	1	0	1	0	1	0	0	0
Ephemeroptera												
Baetidae	1	3	0	1	43	36	0	47	92	0	0	0
Baetidae	0	0	0	80	0	0	279	136	0	0	0	0
Odonata												
Coenagrionidae	36	20	28	16	11	15	23	9	6	0	1	0
Lestidae	0	0	0	3	0	0	0	0	0	0	0	0
Aeshnidae	0	0	1	0	1	3	3	2	1	0	0	0
Cordulidae	2	4	0	0	0	0	0	0	0	0	0	0
Gomphidae	3	0	0	0	0	0	1	0	0	0	0	0
Libellulidae	5	7	4	1	1	1	0	2	1	0	0	0
Hemiptera												
Belostomatidae	3	0	0	0	22	10	11	1	3	0	2	0
Corixidae	0	0	0	156	142	88	67	91	138	1	0	2
Naucoridae	0	0	0	2	0	0	0	0	0	0	0	0
Nepidae	0	0	0	0	0	0	2	1	0	0	0	0
Notonectidae	0	0	0	0	0	0	11	6	3	0	0	0
Veliidae	0	0	0	0	0	0	15	0	0	0	0	0
Gerridae	0	0	0	0	0	0	2	0	0	0	0	0
Coleoptera												
Dytiscidae	7	4	2	26	11	30	18	9	6	1	1	0
Gyrinidae	0	0	0	0	0	0	0	0	0	0	0	0
Hydraenidae	0	0	0	0	0	0	0	0	0	0	0	0
Hydrophilidae	0	0	0	1	0	1	0	0	0	0	0	0
Elmidae	0	1	0	0	0	0	0	0	0	0	0	0
Diptera												
Ceratopogonidae	2	0	0	1	1	1	5	12	25	6	3	3
Chironomidae	165	201	131	528	404	500	218	135	107	14	13	1
Culicidae	3	1	0	18	7	22	1	0	0	5	3	11
Muscidae	0	0	1	0	7	0	0	0	0	0	0	0

Simulidae	10	21	74	14	54	16	1	0	0	0	0	0
Psychodidae	0	0	0	0	0	0	0	0	0	4	0	0
Tabanidae	0	0	0	0	0	0	0	0	5	0	0	0
Tipulidae	0	0	0	3	0	0	0	0	0	0	0	0
Gastropoda												
Ancylidae	2	3	0	6	2	21	3	4	2	0	0	0
Physidae	32	28	18	65	119	68	0	0	1	1	0	0

Table M.2: Seasonal sampling of macroinvertebrates before spray with glyphosate herbicide at upstream spray zone (USZ) (R1, R2 and R3 represent first, second and third replicates, respectively)

	Spring			**Summer**			**Autumn**			**Winter**		
TAXON	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
Annelida												
Oligochaeta	8	19	8	1	7	19	8	8	5	3	7	8
Hirudinea	1	1	0	1	0	1	2	3	1	0	1	0
Crutacea												
Potamonautida	0	0	0	0	0	0	3	2	1	1	0	0
Ephemeroptera												
Leptophlebidae	0	0	1	0	0	0	0	0	0	0	0	0
Baetidae	1	9	5	8	4	12	3	9	5	15	25	28
Baetidae > 2sp	54	0	0	24	0	0	16	0	0	17	0	0
Odonata												
Coenagrionidae	19	10	12	17	7	14	10	2	0	1	5	3
Aeshnidae	0	0	1	0	0	1	0	0	3	0	0	2
Cordulidae	0	0	0	0	0	0	2	0	4	1	2	1
Gomphidae	0	1	0	2	0	0	1	0	0	0	0	0
Libellulidae	11	15	24	5	5	4	12	5	11	4	10	5
Chlorolestidae	2	5	7	0	0	0	3	1	2	0	0	0
Lepidoptera												
Pyralidae	0	0	0	0	0	0	0	1	0	1	0	0
Hemiptera												
Belostomatidae	0	0	0	1	0	2	0	0	0	0	0	0
Corixidae	0	0	0	0	0	0	0	1	1	0	0	0
Naucoridae	0	0	0	0	7	0	0	0	0	0	0	0
Nepidae	0	0	0	1	0	0	0	0	0	0	0	0
Pleidae	0	0	0	0	4	0	0	0	0	0	0	0
Veliidae	0	0	0	10	12	4	6	2	2	0	0	1
Gerridae	0	0	0	0	2	0	0	0	0	1	2	0
Trichoptera												

Ecnomidae	1	2	3	0	0	0	0	0	0	0	0	0
Hydropsychidae	8	10	2	3	6	4	1	3	1	0	5	1
Hydropsychidae >2sp	67	65	9	10	0	0	6	0	0	10	0	0
Leptoceridae	1	0	1	0	0	0	0	0	0	0	0	0
Coleoptera												
Dytiscidae	1	0	0	1	2	4	0	0	0	0	1	2
Gyrinidae	0	0	0	1	0	1	3	2	2	1	3	0
Hydraenidae	5	0	0	0	0	0	0	0	0	0	0	0
Hydrophilidae	0	0	0	4	0	5	1	0	0	0	0	0
Elmidae	0	2	0	0	0	0	0	1	0	0	0	0
Diptera												
Ceratopogonidae	3	2	0	1	3	1	13	2	3	1	0	2
Chironomidae	169	103	107	117	47	35	94	47	17	75	93	148
Culicidae	10	0	1	2	2	3	0	0	1	0	0	0
Muscidae	1	0	1	0	8	0	0	0	0	0	1	0
Simulidae	97	25	2	28	8	3	85	49	6	17	41	66
Anthericidae	0	0	0	0	0	0	0	0	0	0	1	1
Tabanidae	0	0	0	1	0	0	0	0	0	0	0	0
Gastropoda												
Ancylidae	1	0	3	0	1	0	0	0	0	0	0	0
Lymnaeidae	8	9	17	10	28	13	3	14	1	1	4	1
Physidae	2	4	2	4	8	0	4	3	6	2	0	0

Table M.3: Seasonal sampling of macroinvertebrates before spray with glyphosate herbicide at downstream spray zone (DSZ) (R1, R2 and R3 represent first, second and third replicates, respectively)

	Spring			**Summer**			**Autumn**			**Winter**		
TAXON	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
Annelida												
Oligochaeta	181	75	224	163	176	260	80	134	95	22	6	19
Hirudinea	67	37	60	1	0	0	0	0	0	17	133	101
Ephemeroptera	0	0	0	0	0	0	0	0	0	0	0	0
Baetidae	0	1	0	0	0	0	0	0	0	1	0	0
Odonata	0	0	0	0	0	0	0	0	0	0	0	0
Coenagrionidae	0	0	2	0	0	0	0	0	0	2	2	3
Aeshnidae	0	0	0	0	1	0	0	0	0	0	2	1
Hemiptera	0	0	0	0	0	0	0	0	0	0	0	0
Belostomatidae	0	1	0	0	0	0	1	1	0	6	1	2
Corixidae	0	0	0	9	1	10	0	0	0	0	0	0
Pleidae	1	0	2	0	0	0	0	0	0	0	0	0
Veliidae	1	0	0	0	0	0	0	0	0	0	0	0
Notonectidae	0	0	0	0	0	0	0	0	0	0	0	1

Coleoptera	0	0	0	0	0	0	0	0	0	0	0	0
Dytiscidae	1	1	4	0	1	0	0	0	0	0	4	0
Hydrophilidae	1	0	0	3	1	0	1	0	0	0	0	0
Helodidae	0	0	0	0	0	0	0	1	0	0	0	0
Elmidae	0	0	0	4	0	0	0	0	0	0	0	0
Diptera	0	0	0	0	0	0	0	0	0	0	0	0
Chironomidae	14	11	13	48	62	42	15	21	18	139	402	423
Syrphidae	0	0	0	0	0	0	0	2	1	0	0	0
Culicidae	0	0	0	12	0	0	1	108	63	6	4	1
Ephydridae	0	0	0	0	1	0	0	0	0	0	0	0
Muscidae	0	0	0	0	0	2	0	0	1	0	2	1
Simulidae	0	0	5	1	0	0	0	0	0	0	0	0
Gastropoda	0	0	0	0	0	0	0	0	0	0	0	0
Physidae	23	3	15	0	0	0	0	0	0	1	0	4
Planorbidae	0	0	0	6	1	2	0	0	0	0	0	0

APPENDIX N: Weekly sampling of macroinvertebrates after spray with glyphosate herbicide

Table N.1: Weekly sampling of macroinvertebrates after spray with glyphosate herbicide at spray zone (SZ) (R1, R2 and R3 represent first, second and third replicates, respectively).

	Week 1			Week 2			Week 3			Week 4		
TAXON	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
Annelida												
Oligochaeta	65	75	45	107	28	115	66	70	33	49	69	155
Hirudinea	40	34	20	78	71	96	38	77	57	224	86	204
Ephemeroptera												
Leptophlebidae	0	0	0	0	0	0	0	0	0	0	0	0
Baetidae	47	56	36	8	2	18	4	8	7	32	9	14
Baetidae > 2sp	0	0	0	0	0	0	0	0	0	0	0	0
Caenidae	0	0	0	0	0	0	0	0	0	0	0	0
Heptageniidae	0	0	0	0	0	0	0	0	0	0	0	0
Odonata												
Coenagrionidae	0	0	0	0	0	0	1	0	4	12	7	1
Aeshnidae	0	0	0	0	0	0	0	0	0	0	0	0
Gomphidae	0	0	0	0	0	0	0	0	0	0	0	0
Libellulidae	9	0	0	1	0	0	0	0	0	0	0	0
Chlorolestidae	0	12	6	0	0	0	0	0	0	0	0	0
Lestidae	0	0	0	0	0	0	0	0	0	0	0	0
Hemiptera												
Belostomatidae	191	177	106	3	4	0	0	0	1	2	0	0
Corixidae	0	0	0	0	14	2	26	4	19	29	26	18
Nepidae	0	8	8	0	0	0	0	0	0	0	0	0
Pleidae	0	0	0	0	0	0	0	0	0	0	0	0
Veliidae	8	9	3	0	0	0	0	0	0	0	0	0
Gerridae	0	0	0	0	0	0	0	0	0	0	0	0
Hydrometridae	10	0	0	0	0	0	0	0	0	0	0	0
Notonectidae	0	0	0	0	2	0	0	0	0	1	0	0
Trichoptera												
Ecnomidae	0	0	0	0	0	0	0	0	0	0	0	0
Hydropsychidae	0	0	0	0	0	0	0	0	0	0	0	0
Hydropsychidae >2sp	0	0	0	0	0	0	0	0	0	0	0	0
Leptoceridae	0	0	0	0	0	0	0	0	0	0	0	0
Coleoptera												
Dytiscidae	9	6	0	1	4	0	1	1	1	0	0	0
Gyrinidae	12	16	7	0	0	0	0	0	0	0	0	0
Hydrophilidae	0	0	0	1	0	0	1	0	0	0	0	0
Elmidae	0	0	0	33	9	14	0	6	2	2	2	2

Diptera												
Ceratopogonidae	0	0	0	0	0	0	0	0	0	0	0	0
Chironomidae	295	268	190	440	273	198	188	143	271	166	113	210
Culicidae	5	7	2	38	0	19	6	7	19	12	5	26
Muscidae	0	0	1	21	30	54	48	24	37	11	2	4
Simulidae	188	209	178	54	11	106	56	45	52	227	37	182
Gastropoda												
Ancylidae	0	0	0	72	37	109	184	32	115	89	78	120
Lymnaeidae	514	454	398	0	3	4	0	1	0	3	0	0
Physidae	39	32	20	0	1	0	1	0	0	0	0	0

Table N.2: Weekly sampling of macroinvertebrates after spray with glyphosate herbicide at upstream spray zone (USZ) (R1, R2 and R3 represent first, second and third replicates, respectively)

	Week 1			**Week 2**			**Week 3**			**Week 4**		
TAXON	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
Annelida												
Oligochaeta	12	18	21	20	10	13	0	0	7	14	24	0
Hirudinea	6	6	1	0	0	0	4	10	0	1	3	3
Ephemeroptera												
Leptophlebidae	2	3	0	9	10	3	5	2	6	2	1	4
Baetidae	12	11	3	0	0	0	2	4	0	0	8	19
Baetidae > 2sp	52	78	55	111	144	75	68	41	36	19	25	23
Caenidae	0	0	2	3	3	0	0	0	0	0	0	0
Heptageniidae	0	1	0	0	0	3	1	0	1	0	0	0
Odonata												
Coenagrionidae	28	12	11	17	10	2	23	21	8	35	23	23
Aeshnidae	0	1	0	1	0	1	0	0	1	1	1	0
Gomphidae	32	0	0	0	0	0	22	9	0	0	2	2
Libellulidae	2	1	10	2	**3**	8	1	3	0	6	6	12
Chlorolestidae	0	0	2	**0**	0	0	6	2	0	5	3	0
Lestidae	0	1	0	0	0	0	1	1	1	1	0	1
Hemiptera												
Belostomatidae	0	0	1	0	0	3	0	0	2	2	4	0
Corixidae	30	24	23	1	1	0	7	12	6	3	9	4
Nepidae	0	0	0	0	0	0	0	0	0	0	0	0
Pleidae	29	11	7	0	0	0	16	18	5	4	0	0
Veliidae	46	1	1	0	0	0	0	1	1	0	0	1
Gerridae	10	0	0	0	0	0	0	0	0	0	0	1
Hydrometridae	0	0	0	0	0	0	0	0	0	0	0	0
Notonectidae	0	2	0	1	0	2	2	0	2	0	1	0

Trichoptera												
Ecnomidae	0	0	0	8	0	0	2	0	9	4	1	0
Hydropsychidae	5	1	1	1	0	2	0	3	0	7	9	7
Hydropsychidae >2sp	10	12	8	0	0	0	0	1	1	0	0	11
Leptoceridae	1	1	1	0	0	0	0	0	0	0	2	10
Coleoptera												
Dytiscidae	4	0	0	7	4	5	4	2	0	0	6	1
Gyrinidae	179	56	78	106	16	4	102	64	28	88	9	2
Hydrophilidae	0	0	0	0	0	1	1	0	0	0	0	0
Elmidae	0	2	0	0	0	30	0	1	1	0	0	0
Diptera												
Ceratopogonidae	5	2	2	2	6	4	3	1	0	5	10	4
Chironomidae	136	89	124	94	115	91	57	76	45	34	16	15
Culicidae	0	3	4	9	8	7	4	5	1	0	1	0
Muscidae	0	1	0	0	0	0	0	1	0	0	2	0
Simulidae	4	0	18	0	0	0	0	22	12	35	39	22
Gastropoda												
Ancylidae	0	0	0	0	0	0	0	0	0	0	1	0
Lymnaeidae	0	10	9	3	8	0	1	0	5	7	7	17
Physidae	31	36	21	28	31	53	43	45	49	27	50	34

Table N.3: Weekly sampling of macroinvertebrates after spray with glyphosate herbicide at downstream spray zone (DSZ) (R1, R2 and R3 represent first, second and third replicates, respectively)

	Week 1			**Week 2**			**Week 3**			**Week 4**		
TAXON	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
Annelida												
Oligochaeta	22	12	20	6	8	9	0	3	0	4	1	3
Hirudinea	113	0	1	56	0	77	21	0	24	24	33	19
Ephemeroptera												
Leptophlebidae	0	0	0	0	0	0	0	0	0	0	0	0
Baetidae	57	6	0	78	0	0	85	0	64	122	76	111
Baetidae > 2sp	0	0	0	0	1	0	0	0	0	0	0	0
Caenidae	0	0	0	0	0	0	0	0	0	0	0	0
Heptageniidae	0	0	0	0	0	0	0	0	0	0	0	0
Odonata												
Coenagrionidae	107	3	34	9	12	2	3	2	1	30	18	10
Aeshnidae	9	0	0	2	0	0	0	1	0	4	1	1
Gomphidae	8	0	0	0	0	0	0	0	0	0	0	0
Libellulidae	7	0	0	0	0	2	0	0	0	0	0	0
Chlorolestidae	0	0	0	0	0	0	0	0	0	0	0	0

Lestidae	0	0	0	0	0	0	0	0	0	0	0	0
Hemiptera												
Belostomatidae	37	22	5	3	6	8	16	2	19	24	3	2
Corixidae	203	78	56	65	38	43	22	98	110	112	20	79
Nepidae	0	0	0	0	0	0	0	0	0	0	0	0
Pleidae	0	0	0	0	2	0	0	1	1	4	0	0
Veliidae	0	0	0	0	0	0	0	0	0	0	0	0
Gerridae	0	1	0	0	2	0	0	0	2	1	2	0
Hydrometridae	0	0	0	0	0	0	0	0	0	0	0	0
Notonectidae	184	0	2	5	0	2	2	0	0	0	2	2
Trichoptera												
Ecnomidae	0	0	0	0	0	0	0	0	0	0	0	0
Hydropsychidae	0	0	0	0	0	0	0	0	0	0	0	0
Hydropsychidae >2sp	0	0	0	0	0	0	0	0	0	0	0	0
Leptoceridae	0	0	0	0	0	0	0	0	0	0	0	0
Coleoptera												
Dytiscidae	6	13	10	20	0	6	5	16	2	9	10	19
Gyrinidae	0	2	3	0	4	2	0	1	1	0	0	0
Hydrophilidae	0	0	0	2	0	0	0	0	0	3	4	8
Elmidae	0	0	0	0	0	0	0	0	0	0	0	0
Diptera												
Ceratopogonidae	0	0	1	0	2	1	0	0	3	2	1	0
Chironomidae	136	409	246	378	194	173	262	153	361	278	335	220
Culicidae	21	0	1	6	5	0	9	0	0	2	1	7
Muscidae	0	5	1	1	15	1	4	1	0	11	11	11
Simulidae	440	36	105	146	213	44	65	145	84	21	86	126
Gastropoda												
Ancylidae	0	0	0	0	0	0	0	0	0	0	0	0
Lymnaeidae	0	0	0	0	0	0	0	0	0	0	0	0
Physidae	3	14	13	17	2	21	8	9	7	29	34	43

APPENDIX O: Sampling of macroinvertebrates a few hours after spray with glyphosate herbicide

Table O.1: Sampling of macroinvertebrates a few hours after spray with glyphosate herbicide at spray zone (SZ), upstream spray zone (USZ) and downstream spray zone (DSZ) (R1, R2 and R3 represent first, second and third replicates, respectively)

	SZ			USZ			DZS		
TAXON	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
Annelida									
Oligochaeta	35	3	5	15	12	9	1	0	0
Hirudinea	25	3	7	11	3	2	3	0	3
Ephemeroptera									
Leptophlebidae	0	0	0	5	1	0	0	0	0
Baetidae	22	5	8	7	0	0	68	14	5
Baetidae > 2sp	0	0	0	45	56	72	0	0	0
Caenidae	0	0	0	0	2	1	0	0	0
Heptageniidae	0	0	0	0	0	1	0	0	0
Odonata									
Coenagrionidae	0	0	0	17	21	3	8	7	1
Aeshnidae	1	0	0	0	0	0	5	0	1
Gomphidae	1	0	0	18	1	11	0	0	0
Libellulidae	0	0	0	1	2	9	2	0	1
Chlorolestidae	0	0	0	0	4	0	0	0	0
Lestidae	0	0	0	0	1	0	0	0	0
Hemiptera									
Belostomatidae	0	0	0	4	0	2	7	4	0
Corixidae	0	0	0	45	5	16	1	1	1
Nepidae	0	0	0	0	0	0	0	0	0
Pleidae	0	0	0	13	0	21	0	0	0
Veliidae	1	0	0	23	1	1	0	0	0
Gerridae	0	0	0	21	3	16	3	2	6
Hydrometridae	0	0	0	0	0	0	0	0	0
Notonectidae	0	0	0	0	0	1	2	0	1
Trichoptera									
Ecnomidae	0	0	0	0	6	2	0	0	0
Hydropsychidae	0	0	0	1	4	1	0	0	0
Hydropsychidae >2sp	0	0	0	19	8	0	0	0	0
Leptoceridae	0	0	0	0	2	0	0	0	0
Coleoptera									
Dytiscidae	0	0	0	1	1	0	23	8	17
Gyrinidae	0	0	0	126	22	113	0	0	0
Hydrophilidae	0	0	0	0	1	0	0	0	0
Elmidae	0	0	0	0	0	2	0	0	0
Diptera									
Ceratopogonidae	0	0	0	1	8	1	0	0	2

Chironomidae	81	52	45	152	107	68	466	370	434
Culicidae	18	20	17	0	3	0	14	15	14
Muscidae	0	1	1	1	0	1	15	8	2
Simulidae	192	65	54	1	0	5	229	178	143
Gastropoda									
Ancylidae	0	0	0	0	0	0	0	0	0
Lymnaeidae	0	1	1	0	9	12	0	0	0
Physidae	68	23	18	56	23	25	8	3	6

APPENDIX P: Descriptions of Methods for Weighing Body of Evidence Including Strengths and Weaknesses (Adapted From Suter and Cormier, 2011)

Criteria guided judgment

This is a step up in rigour in the application of the assessor's judgment, guided by a set of criteria or issues to consider. The most commonly applied set of considerations is Hill's "criteria" for judging a proposed cause of observed effects. In particular, they are used as issues to consider when determining whether a chemical has a particular effect (e.g. carcinogenic, teratogenic, etc). If the evidence is weighted, the judgment is guided by the weights as well as how many criteria or types of evidence support a hypothesis. Hence, the weight of the body of evidence is the aggregate weight of the relevant pieces and categories of evidence. However, characteristics of the body of evidence as a whole also influence its quality.

Strength: Flexible and transparent.
Weakness: Requires some expert judgment, and takes time.

Check list

Check lists provide a simple and rigorous but inflexible method. That is, a list of dichotomous properties must be checked off before a hypothesis is accepted. The best known example is Koch's postulates, whereby three or four pieces of evidence (depending on the version) must be provided for a pathogen to be proven to cause a disease. Koch's postulates have also been adapted to environmental contamination.

Strength: Quick and transparent.
Weakness: Inflexible and dichotomous.

Independent applicability

Independent applicability is the simplest formal method; any sound evidence is sufficient to demonstrate impairment. This practice is used in enforcement of the U.S. Clean Water Act and other situations in which protection is the paramount goal.

If measurements of any chemical exceed water quality criteria, the system is impaired; if the water is toxic, it is impaired; and if the biotic community fails to achieve biological criteria, the system is impaired. This method recognises that we do not have all three types of evidence in every case, and, even when we do, each type of evidence has weaknesses that may cause an impairment to be missed. For example, most biological criteria are based on invertebrates, but they are less sensitive than fish to some contaminants such as selenium. Similarly, standard toxicity tests of ambient water are short-term sub-chronics that cannot detect the bioaccumulation or reproductive toxicity of selenium in fish. Of the routine methods, only chemical criteria will detect impairment caused by selenium. Hence, although independent applicability has been criticised as overly protective, it is in keeping with legal mandates to protect biological integrity.

Strength: Protective, quick and transparent.
Weakness: Conservative and inflexible.

Numerical indices

Numerical indices based on the ratio to reference are used in some biological condition indices. This was the original method used to combine Chapman's triad of evidence. Data sets may be normalised to a 1-100 scale, averaged within types and then averaged across sites to create an ecotoxicological index. However, reducing the triad or other bodies of evidence to index numbers was not useful. In particular, it destroys the distinct information provided by each type of evidence. For example, it might give the same score to a case in which all lines of evidence were weakly positive as to a case in which none of the measured chemicals were elevated but the sediment was highly toxic and the biotic community was highly degraded.

Strength: Consistent.
Weakness: Pseudo-quantitative and inflexible.

Logic tables

Logic tables combine types of evidence providing a standard conclusion for each possible outcome of a set of standard types of evidence. Most notable among these is the logic triad developed for contaminated sites. The standard body of evidence is:

1. Chemical analyses of the contaminated media which are compared to either reference concentrations or to single chemical toxicity data.

2. Toxicity tests of the contaminated media, which are compared to tests of reference or standard media.

3. Biological surveys of the biotic community inhabiting the contaminated media, which are compared to reference communities.

The logic table is applied to the three types of evidence to determine whether chemical contamination is responsible for biological impairment. That is, it is an ecoepidemiological assessment, combining condition and causal assessments. For example, if the concentrations of chemicals are not elevated, but the sediment is toxic and the biological metrics are below reference levels, the conclusion is that the impairment is due to unmeasured chemicals. This logic is impeccable if the tests and measurements are complete and the quality of the data and its analyses is high. In particular, the logic requires that the right tests and biological metrics are employed. The table allows the possibility of unmeasured chemicals, but not unmeasured modes of toxicity or unmeasured biological effects. Hence, the standard logic is helpful in most cases but will trip up assessors in some cases.

Strength: Transparent and consistent.
Weakness: Inflexible (requires standard set of high quality data).

Sequential logic

Sequential logic is useful if the pieces or categories of evidence can be reduced to a two-part logic (yes, no) or three-part logic (yes, no, uncertain) and may be depicted by a logic diagram. The chief advantage of sequential logic is that data may be generated sequentially, and the process may reach a conclusion without generating or analysing all types of evidence.

Strength: Transparent, consistent and efficient.
Weakness: Inflexible and limited to simple alternatives.

Case-specific logic

Case-specific logic involves a devising logic for interpreting the multiple types of evidence to fit the case at hand. A standard logic may not be used because it does not suit the needs of assessment or because of problems with the standard data, unequal quality or strength of the types of evidence, or the need to include additional types of evidence.

Strength: Flexible.
Weakness: Logic may not be accepted.

Legal weighing of evidence

Legal weighing of evidence is used in courts of law where evidence is weighed by a neutral party. If science courts were established, this approach could be used for high profile or contentious issues without resorting to the actual legal system.

Strength: Flexible and decisive.
Weakness: Influenced by quality of each side's presentation.

Statistical weighing

Statistical weighing, also termed "meta-analysis", includes various statistical methods for combining equivalent quantitative results of multiple similar studies. These include weighted means of estimates, model averaging, etc.

Strength: Quantitative and transparent.
Weakness: Limited to quantitative evidence.

Combined statistical weights

It is possible to have a combined statistical weight of evidence (WoE) since logs of ratios are additive. Therefore, the WoE for different types of evidence may be added to obtain an overall WoE.

Strength: Quantitative and transparent.
Weakness: Limited to quantitative evidence.

Hypothetico-deductive method

Hypothetico-deductive method treats each body of evidence as a hypothesis, deductively generates predictions and chooses the one that performs the best by comparison to reality. A familiar example is the prediction of the path of hurricanes. Alternative models generate predictions, the model that best predicts the storm's behaviour by a statistical or criterion is used to make official predictions for the next time interval, and the process is repeated. More complex cases involving multiple pieces or categories of evidence per hypothesis can be devised as long as they provide deductions of clearly different but verifiable phenomena.

Strength: Convincing and transparent.
Weakness: Seldom applicable.