The effects of selected agricultural chemicals on freshwater microalgae and cladocerans in laboratory studies, with particular emphasis on hormesis

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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DECLARATION

I declare that:

- Except where due acknowledgements have been made, the work is mine alone;
- The work has not been submitted previously, in whole or in part, to qualify for any other academic award;
- The content of the thesis is the result of work, which has been carried out since the official commencement date of the approved research program; and
- Any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Liliana Zalizniak

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Chapters 4-7 are the journal articles, which include input from my supervisor for which I am grateful. These papers also benefited from the comments of anonymous referees.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

a.e.	acid equivalent
ai	active ingredient
ChV	chronic values
CPF	chlorpyrifos
EC_{50}	concentration of a substance causing a particular response (effect) in 50% of population
EEC	expected environmental concentration
GL	gigalitre
Gly	glyphosate
L	litre
LC_{50}	concentration of a substance causing death of 50% of a population
mc	measured concentration
mg	milligram
µg	microgram
nc	nominal concentration
pf	product formulation
RB	Roundup Biactive

LIST OF PUBLICATIONS AND CONFERENCES PRESENTATIONS

Papers published in peer reviewed journals

Zalizniak, L., Nugegoda, D., 2006. Effect of sublethal concentrations of chlorpyrifos on three successive generations of *Daphnia carinata. Ecotoxicology and Environmental Safety*, **64**(2), 207-214.

Zalizniak, L., Nugegoda, D., 2006. Roundup Biactive modifies cadmium toxicity to *Daphnia carinata. Bulletin of Environmental Contamination and Toxicology*, **77**(5), 748-754.

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Refereed abstracts

Zalizniak, L., Nugegoda, D., 2003. Effect of Sublethal Concentrations of Chlorpyrifos on Three Successive generations of *D. carinata.* In: Solutions to Pollutions: Programme Abstract Book, Christchurch, New Zealand, September-October 2003, p. 199. The Society of Environmental Toxicology and Chemistry Asia/Pacific- The Australasian Society of Ecotoxicology, Christchurch, New Zealand.

Nugegoda, D., Zalizniak, L., Heazelwood, P.J., Heffernan, J., 2003. Pesticides and Australian Freshwater Crustaceans. In: Solutions to Pollutions: Programme Abstract Book, Christchurch, New Zealand, September-October 2003, p. 48. The Society of Environmental Toxicology and Chemistry Asia/Pacific- The Australasian Society of Ecotoxicology, Christchurch, New Zealand.

Oral presentations at conferences

An oral presentation was given at the Biennial Conference of the Australasian Society for Ecotoxicology "From Reality to Regulation", 12-14 February 2001, Canberra. The title of the presentation "Effect of Sublethal Concentrations of Glyphosate on *Daphnia carinata*: Population characteristics of three successive generations".

Another presentation titled "Low concentrations of agrochemicals in the environment: are they safe? A case study of chlorpyrifos and glyphosate" was given at the 12th International Symposium on Toxicity Assessment, Skiathos, Greece, 12-17 June, 2005.

An oral presentation "Effects of low concentrations of glyphosate and chlorpyrifos on links in a freshwater trophic chain" was given at the Biennial Conference of the Australasian Society for Ecotoxicology, Melbourne, 26-29 September 2005.

Also several posters were presented at Conferences of Australasian Society for Ecotoxicology and Australian Society of Limnology.

Papers submitted for publication in peer reviewed journals, that are currently under review

Zalizniak, L., Nugegoda, D. Chlorpyrifos and glyphosate at low concentrations stimulate growth of freshwater algae *Chlorella pyrenoidosa* and *Pseudokirchneriella subcapitata.*

Zalizniak, L., Nugegoda, D. Effects of two formulations of a herbicide glyphosate on *Daphnia carinata* in multiple-generation toxicity tests.

CHAPTER 1

SUMMARY

This thesis examines the effects of 3 selected pesticides on a model freshwater food chain of a producer and consumer. Chapter 2 reviews the specific features of the Australian environment and why native Australian species should be used in evaluating the effects of toxicants to Australian biota, especially the effects of pesticides. Toxicity of the herbicide glyphosate (two formulations – technical grade and Roundup Biactive RB) and insecticide chlorpyrifos CPF to aquatic biota are examined. The importance of studying the toxicity of low (environmentally realistic) concentrations of pesticides to non-target organisms is introduced. Based on these the aims of the project are specified.

Chapters 3.1 and 3.2 are literature reviews on the toxicity of glyphosate and chlorpyrifos to aquatic organisms.

In Chapter 4 the requirements for the maintenance of algal and *Daphnia carinata* cultures are presented. Three species of algae were used in various experiments and for maintenance of *D. carinata* cultures: *Chlorella vulgaris, Chlorella pyrenoidosa* and *Pseudokirchneriella subcapitata*. Batch cultures were used for maintenance of the algae, grown in flasks on a light-table. Sub-culturing was conducted once a week or as required. Two media were used for maintenance of these cultures: Tamiya (Vasser 1989) and Keating (1985). The most widespread daphnid in Australia, *D. carinata* is considered to be one of the most suitable for toxicity testing of contaminants entering Australian freshwaters. Little data is available on the culture requirements of the

species, and this chapter evaluates the efficacy of different food types for culture of *D. carinata.* Different types of food were tested: *Chlorella vulgaris* cultured in two different media - Keating and Tamiya, *Chlorella pyrenoidosa* cultured in the same two media, and a suspension of trout pellets. Intrinsic rates of natural increase of individual cultures of *D. carinata* were determined from "life tables". The best food from among those tested in terms of providing adequate survival and fecundity of *D. carinata* were *C. pyrenoidosa* cultured in either Keating or Tamiya medium. Two different procedures of individual cultures are proposed for the maintenance of *D. carinata* for use in toxicity testing using different culture volumes.

In Chapter 5 the results of a series of 72-h toxicity tests with algae are presented. The effects of two formulations of the herbicide glyphosate (technical grade and Roundup Biactive®) and the insecticide chlorpyrifos on the growth of *Chlorella pyrenoidosa* and *Pseudokirchneriella subcapitata* were studied, and the EC_{50} values determined. With glyphosate and Roundup Biactive® the 72-h EC_{50} were: *C. pyrenoidosa* = 788 and 763 mg/L, and *P. subcapitata* = 429 and 397 mg/L, while hormesis was observed when *P. subcapitata* was exposed at concentrations equal to 7% and 4% of EC_{50} respectively. No such effect was noted for *C. pyrenoidosa*, although it is possible that this effect may be present at very low concentrations, which were not tested in this study. For chlorpyrifos the 72-h EC_{50} was well above environmentally realistic concentrations for both algae (3736 for *C. pyrenoidosa* and 2060 μg/L for *P. subcapitata*). However at concentrations 0.3-5 μg/L (with a maximum at 0.06% of EC_{50}) hormesis was observed for both species, where growth rate exceeded that of control by as much as 20% for *C. pyrenoidosa* and 40% for *P. subcapitata*. *P.*

subcapitata was more sensitive to all toxicants tested, and it was recommended as a test species for pesticides in preference to *C. pyrenoidosa.*

In Chapter 6 the effects of sublethal concentrations of chlorpyrifos (ranging from 0.005 µg/L ('0.01 LC_{50}') to 0.500 µg/L ('1 LC_{50}')) on population characteristics of individual culture of *Daphnia carinata* were investigated over 21 days with subsequent testing of the next two generations. The endpoints for the first and second generations observed were: survival, fecundity, time to first brood and number of offspring per female. The results were incorporated into the computation of the intrinsic rate of natural increase for daphnids in each of the treatments. Exposure to chlorpyrifos affected survival and fecundity of animals in the first generation. In the second generation the most affected endpoint was time to the first brood with an indication of hormesis. LC_{50} tests were then conducted using animals of the third generation from each of the exposures in individual tests. Despite the absence of a negative effect of chlorpyrifos in the second generation, results of testing the third generation showed a constant significant decline in LC_{50} in the order of control daphnids through to '0.1 LC_{50}' pre-exposed daphnids ('0.1 LC_{50}', or 0.05 µg/L being the highest concentration in which animals survived exposure to the toxicant in the second generation).

In Chapter 7.1 the long-term toxicity of glyphosate (technical grade and formulation Roundup Biactive) to three successive generations of *D. carinata* was investigated. The experimental protocol was the same as for chlorpyrifos testing (Chapter 6). Glyphosate was tested in two different media: sea salt solution and M4 medium specially designed for daphnids, while Roundup Biactive was tested in M4 medium.

Results indicated that glyphosate and Roundup Biactive had relatively low toxicity to *Daphnia.* Hormesis was evident in sea salt medium exposures in the first and second generations of daphnids with technical grade glyphosate. When exposed to glyphosate and Roundup Biactive in M4 medium animals showed no indication of hormesis. It is hypothesized that glyphosate may have compensated for the lack of microelements in the sea salt medium, and possible mechanisms discussed.

In Chapter 7.2 the modifying effect of glyphosate on the toxicity of cadmium to *Daphnia carinata* was studied in long-term (21 days) exposures with two generations of cladoceran. It was found that low concentration of glyphosate (in the form of Roundup Biactive [RB]) reduces toxicity of Cd, and the performance of daphnia is enhanced in terms of animals' size, survival, fecundity, and consequently the intrinsic rate of natural increase in both generations of animals in the presence of glyphosate. However when the third generation was tested for their sensitivity to Cd in the 48-h LC_{50} experiments there was no difference between RB-free and RB-spiked treatments in pair wise comparison, indicating that no adaptation mechanisms were involved in the enhancement.

In Chapter 8 the overall discussion of the results with respect to observed hormesis is presented. The implications for the effects of the pesticides on environmental freshwater food chains are discussed and recommendations on modifying pesticide use are provided.

CHAPTER 2

INTRODUCTION

2.1 STATE OF THE AUSTRALIAN ENVIRONMENT

Among the inhabited continents Australia is the driest with over 80% of its land having an average rainfall of less than 600 mm/year. Large variations in climate and rainfall throughout Australia result in a great range of natural environments - from temperate south to tropical north with vast arid regions in the centre. Australia's inland aquatic ecosystems provide resources for multiple uses such as agriculture and industry and other human activities (potable water, fishing, recreation etc). To maintain the health of the aquatic environment it is essential to review the available knowledge on the current state of the environment, real and predicted impacts due to natural processes and human involvement, and based on such data to develop management tools to minimise the impact and its consequences.

The key findings of *Australia: State of the Environment 1996* (State of the Environment Advisory Council 1996) highlighted that Australia's inland waters are under increasing pressure from over-extraction, pollution, algal blooms, catchment modification, habitat destruction and flow regulation. Since 1996, the pressures on many inland waters have increased, with a substantial increase in water extraction, continued clearing of catchment and riparian vegetation, increases in the area of land affected by dryland salinity and increases in pesticide use (Australia: State of the Environment 2002).

According to *Australia: State of the Environment 2001 Report* 'the total water use in Australia for 1996/97 was 24 100 GL (NLWRA 2001), an increase of 65% from 1985 (AWRC 1987). Seventy-nine per cent of water was extracted from surface waters (19 100 GL), while 21% was extracted from groundwater resources (5000 GL) (NLWRA 2001a). Seventy-five per cent of water extracted is used for irrigation, with irrigation water use increasing by 76% between 1985 and 1996/97 (NLWRA 2001a). Most of the growth in irrigation has occurred in New South Wales and Queensland, with the area of irrigated land doubling in these states over the last twenty years. Urban and industrial water use has also increased by 55% (NLWRA 2001a) between 1985 and 1996/97'. Increased water usage puts additional pressure on the remaining (depleted) water resourses in terms of maintaining healthy freshwater ecosystems, and making them increasingly sensitive to any pollution.

The Report recognises that 'pesticides are possibly the most widespread pollutants, which are used extensively in agriculture with cotton, rice, sugar cane and horticultural crops. Since 1990, at least 20 fish kills in New South Wales rivers have been attributed to pesticides. Integrated pest management and best management practices for pesticide use are gradually being implemented and a new generation of more selective, less toxic pesticides is also being introduced. However, based on the experience of the past 20 years, pesticide use is likely to increase, potentially causing continuing pollution of inland waters'. In recognition of this, the current study was focused on the pesticides routinely used in large quantities in the Australian environment.

2.2 SPECIFIC FEATURES OF THE AUSTRALIAN AQUATIC ENVIRONMENT

Most ecotoxicological research and the subsequent setting of water quality criteria and related issues are based upon the data gathered in the Northern hemisphere and related to northern aquatic environments. However, the validity of application of these data to other geographical areas with distinctively different features is questionable (Hart 1982, Hobbs *et al.* 2004, Maltby *et al.* 2005).

Williams (1972) defined several distinctive features of Australian inland waters, which include:

1. The inapplicability of the concept of a standard composition for average fresh water;
2. The predominance of sodium and chloride ions in fresh waters;
3. The high proportion of saline/fresh standing water bodies;
4. The high concentration of phosphorus as phosphate in many lakes and reservoirs;
5. The absence of dimictic lakes, the presence of warm monomictic lakes (holomixis occur once, not twice as in the north, and takes place in winter at temperatures above $4^{o}C$). The presence of unique thermal pattern in some highland lakes;
6. The pronounced seasonal and secular fluctuations in discharge values for rivers;
7. The high faunal endemicity

8. The absence of a well-defined seasonal terrestrial leaf-fall that affects the ecology of stream biota.

Because of the specific climatic regime (low and uncertain rainfall, absence of permanent snowfields where rivers can be replenished), topography (mostly flat planes with vast deserts in the centre of the continent), and high evapotranspiration rates, most of Australia lacks rivers or permanent standing waters. As a result of variable rainfall, Australian rivers have highly variable flows, which in turn have impact on the biota.

A large part of the aquatic Australian biota is endemic. Around 130 fish species that are endemic developed unique reproductive strategies adapted to variable flow and periods of drought. Many of the Australian freshwater invertebrates are also endemic, and their community compositions are different from those in the Northern hemisphere. Because of the seasonal nature of northern species, they have their population peaks at different times to avoid competition. This does not happen in Australia due to less pronounced seasons, consequently species interactions are different from those in the Northern hemisphere (Hart 1982).

All the above features make it necessary that toxicity values for *Australian* ecosystems be obtained using *Australian* native species, and related issues, such as the development of water quality criteria for Australia, be based on these data, and not on toxicity data derived from testing species from the Northern hemisphere. The reason why Australian and New Zealand Water Quality Guidelines rely predominantly on the Northern hemisphere data is lack of appropriate Australian data.

In the current study the use of a cosmopolitan cladoceran is proposed (*Daphnia carinata* King) in conjunction with cosmopolitan algal species, which are also native to Australia (unicellular green freshwater algae *Chlorella pyrenoidosa* and *Pseudokirchneriella subcapitata*), to investigate the effects of low concentrations of agricultural chemicals on non-target organisms in prolonged exposures since these species are found in Australian environmental trophic chains.

2.3 AGROCHEMICALS IN THE AUSTRALIAN ENVIRONMENT

The herbicide glyphosate (see Appendix 1 for properties and Fig. 2.1 for structure) was proposed for the study to investigate its effects on algae (potential target organisms) and a cladoceran (non-target organism), which feeds on these algae. Another agrochemical (that is also used in household applications in Australia) is the insecticide chlorpyrifos. Chlorpyrifos (see Appendix 1 for properties and Fig. 2.2 for structure) is highly toxic to crustaceans (of which daphnia is a representative), because they are closely related to insects. Though chlorpyrifos is not toxic to plants and algae, it is expected to influence their growth (though it is not known to what extent) due to its phosphorus content. Both chemicals are expected to influence the algae-cladoceran interactions due to their effects on at least one of the trophic links. Both agrochemicals are widely used in Australia and worldwide.

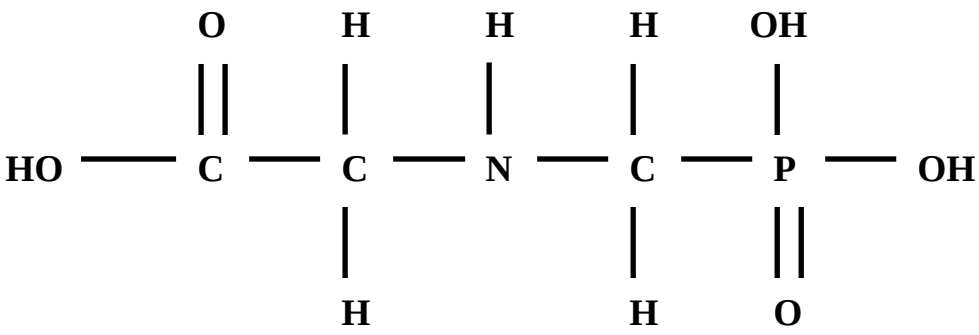

Figure 2.1 Structure of glyphosate.

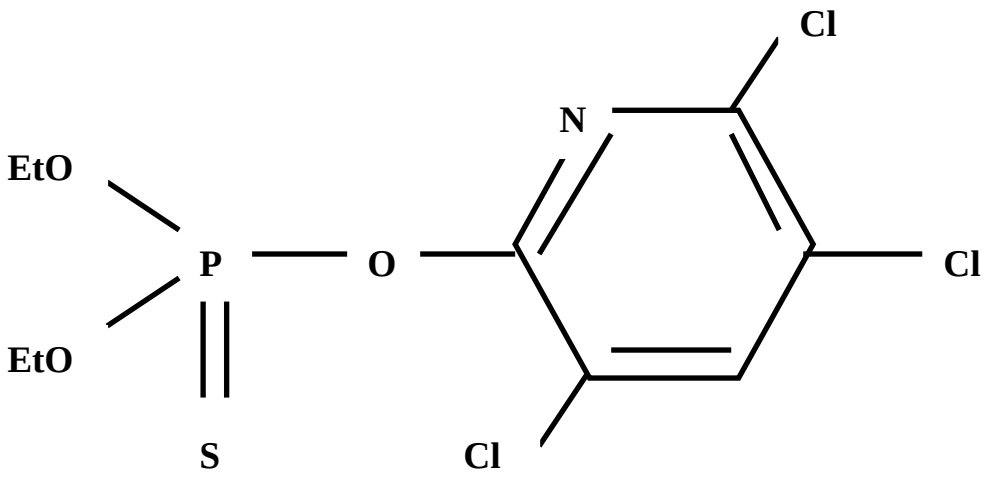

Figure 2.2 Structure of chlorpyrifos.

2.3.1 CHLORPYRIFOS IN THE AQUATIC ENVIRONMENT

CPF was introduced worldwide in 1965 to replace organochlorines and is one of the most widely used chemical organophosphate insecticides in the market today (Dow Agro Sciences www.dowagro.com). About 40 million kg of CPF is manufactured per year and it is an active ingredient in about 800 products in the USA (www.dowagro.com). There are 13 licensed producers of chlorpyrifos-based pesticides in Australia (NRA 2000). During the mid-1990s, 4-5.5 million kg were used annually in non-agricultural situations in over 17% of households in Australia. Agricultural usage estimates even more, with annual application of 4.5-10 million kg (NRA 2000). The National Water Quality Assessment Program (NAQWA) has been monitoring major watersheds in the US since 1991. The data reveals that concentrations of four organophosphorus pesticides (chlorpyrifos among them) exceed water quality criteria for aquatic life protection more often than other pesticides (de Vlaming *et al.* 2004).

Recently, the US EPA and the manufacturers of CPF agreed to eliminate nearly all household applications of the insecticide, but agricultural use continues worldwide, including Australia. For example, chlorpyrifos together with lindane, endosulfan and DDT was still the major concern in some parts of South Australia (Liston and Maher 1997). Chlorpyrifos was also detected in irrigation districts of New South Wales (Bowmer *et al.* 1998, Cooper 1996, Muschal 1998). It is estimated that in the sugar cane growing regions of Queensland, 74 500 kg of chlorpyrifos is used annually, which constitutes around 90% of all insecticide use by that industry (Hamilton and Haydon 1996). As a consequence of its widespread use, chlorpyrifos was detected in

Australian waterways at concentrations up to 0.525 mg/L (Humphrey and Klumpp 2000), while the recommended level of protection of 80 and 95% of species for fresh waters is 1.2 and 0.00004 µg/L respectively (ANZECC & ARMCANZ 2000).

Schulz (2001) studied a rainfall-induced runoff of pesticides from orchards into the Lourens River in South Africa, and found that as a result of such an event the pesticide contamination levels (including chlorpyrifos) were extremely high – they exceeded the national water quality standards and those established by the US EPA and may result in acute toxic effects on aquatic invertebrates and fish. He also conducted a probability analysis of 10-y rainfall data and found that such an event occurs approximately every 7 months. Considering that similar climatic conditions exist in Australia, a possibility of such events occurring here is very high (Muschal and Warne 2003).

In Australia not only agricultural but also household use of chlorpyrifos still continues. In March 1999 ecological and human health risk assessment of chemicals in sewage treatment plant discharges to the Hawkesbury-Nepean river system (NSW, Australia) found chlorpyrifos in the effluent of 2 inland sewage plants at levels that would constitute a risk to aquatic life (Sydney Water 2000). In addition this insecticide is commonly used in urban areas and appears in urban stormwater runoffs in the USA (Bailey *et al.* 1995), and the same pattern is expected to be present in Australia.

Organophosphorus pesticides such as chlorpyrifos are considered to be non-persistent in the environment; however, experimental research with ^{14}C-labelled chlorpyrifos

has shown that this pesticide may persist for relatively long periods of time through sediment-water partitioning (Carvalho *et al.* 2002).

2.3.2 GLYPHOSATE IN THE AQUATIC ENVIRONMENT

Glyphosate was first reported as an herbicide in 1971. Three related products are now manufactured by Monsanto and Zeneca. In pure chemical terms glyphosate is an organophosphate because it contains carbon and phosphorous. However, it does not affect the nervous system in the same way as organophosphate insecticides, and is not a cholinesterase inhibitor, but rather it acts on various enzyme systems inhibiting amino acid metabolism in what is known as the shikimic acid pathway (Duke 1988). This pathway exists in higher plants and micro-organisms but not in animals.

Glyphosate product sales are currently worth approximately US$1,200 million annually worldwide and represent about 60% of global non-selective herbicides sales (Agrow 1995). The total world herbicide market was worth about US$14,285 million in 1995 (British Agrochemical Association 1996).

In UK arable agriculture, glyphosate was the 12th most extensively used pesticide active ingredient; the 5th most extensively used herbicide by weight with 251 tonnes being used; and 38th most widely applied herbicide, being applied over 334,529 ha annually in 1994 (MAFF 1995). In the US nearly 8,500 tonnes was being used on 5-8 million hectares annually in the years leading up to 1991(US EPA 1993).

The toxicity of glyphosate to mammals and birds is generally relatively low. Fish and aquatic invertebrates are more sensitive to glyphosate and its formulations. Some soil invertebrates including springtails, mites and isopods are also adversely affected by glyphosate (www.pesticideinfo.org). Of nine herbicides tested for their toxicity to soil micro-organisms, glyphosate was found to be the second most toxic to a range of bacteria, fungi, actinomycetes and yeasts (Carlisle and Trevors 1988).

However, while glyphosate alone has low toxicity, the formulation of glyphosate with the surfactant polyoxyethylene amine (POEA), which is widely used, is significantly more toxic (Wan *et al.* 1989; Servizi *et al.* 1987).

In Australia the recommended maximum level of glyphosate to ensure protection of 99 and 80% of aquatic freshwater life are 0.37 and 3.6 mg/L respectively (ANZECC & ARMCANZ 2000). Though glyphosate is considered non-persistent, it can last in the aquatic environment for a considerable time (up to ten weeks) and thus has the potential to affect non-target species with a short life cycle, such as cladocerans. Based on the scientific data available on glyphosate toxicity to aquatic animals, major organizations (USEPA 1993, WHO 1994) conclude that glyphosate and its formulation Roundup can be used with minimal risk to the environment. However it is possible that though glyphosate might not be toxic to animals, it still affects them at concentrations found in the environment.

In Australia most formulations of glyphosate have been banned from use in or near water because of their toxic effects on tadpoles and to a lesser extent on adult frogs. There is also concern about long-term sublethal effects of the herbicide on frogs

(Mann & Bidwell 1999). However, new formulations such as Roundup Biactive are excluded from the ban (Agrow 1996). Only few studies have been conducted on the toxicity of Roundup Biactive to aquatic biota (Mann and Bidwell 1999), and more data is required, especially on its long-term sublethal effects.

2.4 AIMS OF THE PROJECT

In order to determine the effects of low (environmentally realistic) concentrations of agricultural pesticides on non-target organisms, and considering all the issues presented above, the aims of the PhD project were as follows:

1. To investigate the effects of the herbicide glyphosate and its formulation Roundup Biactive on the growth of two Australian species of freshwater unicellular green algae *Chlorella pyrenoidosa* and *Pseudokirchneriella subcapitata* with special attention to the effects at environmentally realistic concentrations.
2. To study the effects of the insecticide chlorpyrifos on non-target organisms – algae *Chlorella pyrenoidosa* and *Pseudokirchneriella subcapitata* at low environmentally realistic concentrations especially with respect to hormesis and consequent potential algal blooms.
3. To study the effects of low concentrations of chlorpyrifos on population characteristics of *Daphnia carinata* in long-term multiple generation exposures.
4. To investigate the lethal and sublethal effects of low concentrations of glyphosate and its formulation Roundup Biactive on the growth and

reproduction of a native Australian cladoceran *Daphnia carinata* King in long-term exposures and using multiple-generation toxicity tests.

5. To establish the interdependence (if any) between the two trophic links when exposed to environmentally realistic concentrations of the above pesticides, and to evaluate the consequences of these exposures on an ecosystem.
6. To provide recommendations based on the results of the project on minimising detrimental effects of chronic exposure to environmentally realistic concentrations of the investigated pesticides on aquatic ecosystem health.

CHAPTER 3

EFFECT OF GLYPHOSATE AND CHLORPYRIFOS ON AQUATIC ORGANISMS - LITERATURE REVIEW

3.1 GLYPHOSATE TOXICITY TO DIFFERENT ORGANISMS

3.1.1 General issues

Various National environment agencies including Environment Canada (Peterson *et al.* 1994), USEPA, EU and the Australian National Registration Authority (NRA 1997) use an Expected Environmental Concentration (EEC) in evaluating the hazard of pesticides to non-target aquatic organisms. This concentration is calculated by assuming an overspray of a 15 cm deep water-body at the label application rate (Peterson *et al.* 1994). The EEC is then related to the EC_{50} for a given aquatic test organism.

In Canada, Vision® (containing 356 g/L of glyphosate as an active ingredient) is a major forest management herbicide, representing 81% of all herbicides sprayed on the forests. Because of the aerial method of application it can enter aquatic systems. Once in there, its half-life can vary from several days to ten weeks depending on the pH of the water (Trotter *et al.* 1990, cited in Morgan and Kiceniuk 1992). The Canadian Water Quality Guidelines recommend IMAC (Interim Maximum Accepted Concentration) for protection of aquatic life to be 65 μg/L. However, on occasion, the

glyphosate levels were found to be up to 270 μg/L in some water bodies (Morgan and Kiceniuk 1992).

In Australia the recommended level of glyphosate to protect 99-80% of aquatic freshwater life is 0.37-3.6 mg/L respectively (ANZECC and ARMCANZ 2000).

Water quality parameters can affect the toxicity of glyphosate. Folmar *et al.* (1979) reported that increased temperature and pH both result in an increased toxicity of RB to rainbow trout. They also found that solutions of Roundup aged for up to 7 days in reconstituted water did not change in toxicity to midge larvae, rainbow trout, or bluegills. This indicates that the chemical can accumulate to dangerous levels in environmental waters if there are repeated applications within short time intervals.

There are few studies of effects of glyphosate, which may be of importance to human health. For example, Marc *et al.* (2004) demonstrated that various glyphosate-based herbicides induced cell cycle dysfunction.

3.1.2 Sediment-associated toxicity of glyphosate

Hartman and Martin (1984) demonstrated that the presence of suspended sediment in water significantly increased the acute toxicity of Roundup to *Daphnia pulex* (48-h EC_{50} for daphnia was 3.2 mg/L with suspended sediment and 7.9 mg/L without it) and decreased its toxicity to *Lemna minor*.

3.1.3 Effects of glyphosate on algae

Thomas *et al.* (1986) reported that water samples from arsenal waste sites were highly toxic to vascular plants, but were either stimulatory to or had no effect on *S. capricornutum* (now called *Pseudokirchneriella subcapitata*). Toxicants presented in the waste were suspected to be herbicides (including glyphosate) and their mixtures.

Anton et al. (1993) found that glyphosate was not toxic to the freshwater green alga *Chlorella pyrenoidosa* (see Table 3.1, entries 5-7). However Saenz et al. (1997) found in their study that much lower concentrations of glyphosate and its formulation Rondo caused inhibition of chlorophyll a synthesis in two green algae *Scenedesmus acutus* and *S. quadricauda* (Table 3.1, entries 9-12). Glyphosate inhibits the synthesis of the chlorophyll precursor 5-aminolevulinic acid (ALA) (Duke, 1988).

According to Shikha and Singh (2004) photosynthetic electron transport and O_2 evolution were initially stimulated by glyphosate at 50-200 mg/L, but were inhibited by higher concentrations 200-400 mg/L. Hernando *et al.* (1989) investigated chlorophyll and carotenoid content, greening process, photosynthetic and respiration rates and photosynthetic pigment content of *Chlorella pyrenoidosa* when grown in glyphosate concentrations raging from 0.1 mM to 1 mM (17 mg/L to 170 mg/L). The highest concentration inhibited growth completely; other concentrations reduced growth and photosynthetic pigment content. Glyphosate inhibited chlorophyll synthesis and reduced carotenoids. Oxygen evolution was also strongly inhibited. They concluded that glyphosate acts as an electron inhibitor, affecting both photosystems.

Peterson *et al.* (1994) estimated the Expected Environmental Concentrations for glyphosate formulations to be around 3 mg Gly/L. Authors found that among ten species of algae tested only diatoms and one cyanobacterium were sensitive to glyphosate. It appears that some algal species are more sensitive to glyphosate than others, and the variation is orders of magnitude. According to Saenz *et al.* (1997), the EEC for glyphosate is higher than the concentrations producing negative effects in algae in their study, and therefore the use of glyphosate formulation in aquatic environments may cause harmful effects on long-term development of *S. quadricauda* populations (as well as some others).

Christy *et al.* (1981) calculated EC_{50} (growth rate) of *Chlorella sorokiniana* to be 17.7 mg/L. Gardner *et al.* (1997) studied the effect of Rodeo® on growth of the freshwater green alga *Ankistrodesmus.* They found that the 96-h EC_{50} for this species was 74 mg/L (Table 3.1, entry 8). Maule and Write (1984) calculated the 96-h EC_{50} values of glyphosate for several algal species and found it to be non-toxic to microalgae. The most sensitive species tested was *Chlorococcum hypnosporum* with an EC_{50} of 68 mg/L, the least sensitive with an EC_{50} of 590 mg/L was *Chlorella pyrenoidosa.* Hess (1980) reported that a concentration of 1000 mg/L of glyphosate reduced the growth rate of *Chlamydomonas* to 30% of the control.

Hartman and Martin (1984) found that glyphosate did not produce any inhibitory effects on sprouting or early growth of sago pondweed *Potamogeton pectinatus* when treated with the concentrations up to 10.0 mg/L. However it stimulated plant growth at 1.0 mg/L. Schaffer and Sebetich (2004) found that low concentrations (0.125-12.5

mg/L) of Rodeo (a formulation of glyphosate) induced primary productivity of a phytoplankton community up to 168% of the control value.

3.1.4 Effect of glyphosate on freshwater fauna (single species data)

Most toxicity testing of glyphosate have been conducted using different species of fish (see Table 3.1, entries 91-142), e.g. goldfish *Carassius auratus* (Anton *et al.* 1994), rainbow trout *Oncorhyncus mykiss* (Anton *et al.* 1994, Morgan & Kiceniuk 1992), coho *Oncorhynchus kisutch* (Wan *et al.* 1989, Servizi *et al.* 1987, Mitchell *et al.* 1987), chum *Oncorhynchus keta* (Wan *et al.* 1989), chinook *Oncorhyncus tshawytsha* (Wan *et al.* 1989, Mitchell *et al.* 1987), pink salmon *Oncorhyncus gorbuscha* (Wan *et al.* 1989), rainbow trout *Salmo gairdneri* (Wan *et al.* 1989, Servizi *et al.* 1987, Mitchell *et al.* 1987, Folmar *et al.* 1979), carp *Cyprinus carpio* (Neskovic *et al.* 1996), sockeye salmon *Oncorhynchus nerka* (Servizi *et al.* 1987), mosquitofish *Gambusia yucatana* (Rendon-van Osten *et al.* 2005*).*

There has been an extensive study of the toxicity of different formulations of glyphosate to several species of Australian frogs (Mann & Bidwell 1999) (Table 3.1, entries 51-72) and other species of amphibians (Table 3.1, entries 73-90), and a few studies involving cladocerans (Table 3.1, entries 31, 36, 39-43, 45-47, 49) and other types of invertebrates and protozoans (Table 3.1, entries 21-30, 32-35, 37-39, 50). In general, not much attention was paid to the effects of glyphosate and its formulations on freshwater fauna. This is probably because glyphosate is considered to be non-toxic to animals, since they lack the metabolic pathway, along which the chemical reacts (the shickimate pathway is found only in plants). However some results

suggest that though glyphosate might not be toxic to animals, it still affects them at concentrations found in the environment.

Morgan and Kiceniuk (1992) examined the effects of a two-months exposure to glyphosate, as Vision®, on the growth, behaviour, and gill and liver histopathology of rainbow trout. Concentrations tested were 6.25, 25 and 100 μg/L nominal concentration, the measured concentrations were 4.25, 8 and 45.75 μg/L respectively. There were no significant differences between control and treated animals in terms of all endpoints observed at all treatment concentrations, except one aspect of agonistic behaviour. At the highest tested concentration animals demonstrated higher frequency of aggressive behaviour – wigwags. It should be noted that this effect was observed at concentrations much lower than those found in some water bodies after spray application (Trotter *et al.* 1990).

Wan *et al.* (1989) found that the toxicity of glyphosate and its formulations depends on the type of dilution water used. Overall they found that variation of 96-h LC_{50} values for MON 0818, MON 8709 and Roundup® is in the same order of magnitude irrespective of water types. For glyphosate these values can vary by an order of magnitude depending on water type, with water hardness and pH being the most important contributing factors. Roundup®, MON 8709 and MON 0818 are more toxic to young salmonids in hard waters than they are in soft waters, while the reverse is true for glyphosate.

Edginton *et al.* (2004) compared toxicity of Vision® to several species of amphibians at different pHs and determined that it was more toxic to three species out of four at

pH=7.5 than at pH=6, and the larval stage was more sensitive than the embryonic stage. Together with pH, other environmental factors such as availability of food can exacerbate chemical effects of Vision®, as was determined by Chen *et al.* (2004) in their experiments with *Simocephalus vetulus* and tadpoles of *Rana pipiens.* For both species, significant effects of the herbicide were measured at concentrations lower than the calculated worst-case value for EEC (1.4 mg/L ae), while high pH (7.5) increased the toxicity of herbicide to *S. vetulus.* Thompson *et al.* (2004) confirmed that amphibians are among the most sensitive organisms to glyphosate. However after conducting an *in situ* study they concluded that there was no risk to amphibians from glyphosate (as Vision®) application at recommended rates. Smith (2001) found that Kleeraway ® Grass was toxic to the tadpoles of two species of frogs: chorus frog *Pseudacris triseriata* and plains leopard frog *Rana blairi* at a concentration of 0.75 mg/L (as IPS equivalent) – about half of them died within 24 hours. However, further exposure of surviving animals to this concentration did not have any negative effect on their growth and development. Lajmanovich *et al.* (2003) found that glyphosate formulation Glyfos® induced death in 80% of tadpoles of *Scinax nasicus* at a concentration of 3.07 mg/L, with 75% malformed (craniofacial and mouth deformities, eye abnormalities and bent tails) in a 96-h exposure. Howe *et al.* (2004) observed that Roundup Original, Roundup Transorb and POEA (surfactant) significantly negatively affected growth and development of several species of amphibians in a chronic exposure to sublethal concentrations (0.6 mg/L of ae) of these compounds.

3.1.5 Effect of glyphosate on water communities.

Simenstad *et al.* (1996) conducted an intensive 119-day experiment in southern Willapa Bay, Washington, to evaluate the potential effects of a mixture of glyphosate (Rodeo®, 4.7 L/ha) and an associated surfactant, alkylarylpolyoxyethylene (AAPOE, X-77® Spreader, 1L/ha) on mudflat benthic communities. They concluded that there were no indications of either short- or long-term effects on the mudflat community after aerially applying this concentration of herbicide and surfactant. Though this study did not address either sublethal or indirect ecological effects of the herbicide application, there was an observed decrease in the exotic eelgrass *Zostera japonica*, that might be a longer-term, subtler response by the mudflat community. (Calculation of glyphosate concentration according to the Environment Canada procedure (Peterson *et al.,* 1994) gives us the concentration of active ingredient not more than 1.7 mg/L at the time of application, which in an estuarine environment will quickly decrease even further).

Perschbacher *et al.* (1997) studied the effect of sprayed herbicides (glyphosate among them) on the water communities (plankton productivity, zooplankton populations) and water quality. Though they did not provide all data in their paper, they stated that there were no significant differences between control and treated mesocosms, when sprayed with glyphosate at a rate of 0.43 kg/ha (see Table 3.1, entry 143). Similarly Kilbride and Paveglio (2001) conducted a 3-year study on effects of repeated applications of Rodeo to control smooth cordgrass *Spartina* sp. in Willapa Bay on aquatic biota. They concluded that under worst-case conditions short- and long-term detrimental effects of these applications would be highly unlikely.

In contradiction other researchers suggest that glyphosate could affect aquatic communities. For example, Bengtsson *et al.* (2004) measured the grazing rate of *Daphnia pulex* when pre-exposed to glyphosate via two vectors – water and food *Scenedesmus* spp. (exposure concentration in both cases was 50 mg/L), and found that the grazing rate was greatly reduced (40%) when exposed via the food route, suggesting greater toxicity of glyphosate to *Daphnia* than when exposed directly via water.

3.1.6 Bioconcentration of glyphosate

No bioaccumulation, biomagnification or persistence in a biologically available form is reported for glyphosate.

3.1.7 Toxicity of glyphosate formulations: active ingredient vs. surfactant

A number of researchers (Wan *et al.* 1989; Servizi *et al.* 1987) indicated that the surfactants in Roundup are more toxic to aquatic flora and fauna than the active ingredient glyphosate: MON8709 (Table 3.1, entries 120-124), MON 0818 (part of MON 8709, Table 3.1, entries 36, 105-107, 130-134). Mitchell *et al.* (1987) compared toxicity values for Rodeo herbicide alone and for Rodeo herbicide with X-77 surfactant as recommended for application by the manufacturer Monsanto. They found that the 96-h LC_{50} value of Rodeo/X-77 mixture was more than 4 times lower

than Rodeo without the surfactant (130 mg/L of active ingredient and 580 mg/L respectively, Table 3.1, entries 112-114).

Tsui and Chu (2003) tested several formulations of glyphosate and Roundup surfactant using a number of marine and freshwater organisms (bacterium, algae, protozoans and cladocerans) to assess their relative toxicity. They found that polyoxyethylene amine (POEA) surfactant was the most toxic (normalized as acid equivalent) among four compounds tested, up to 6 times more toxic than Roundup and up to 360 times more than the isopropylamine salt of glyphosate (a usual active ingredient of glyphosate-based herbicides) (see Table 3.1, entries 1-4, 13-28 and 42-49 for comparison). Marc *et al.* (2005) also found POEA was highly toxic to the embryos of sea urchin *Sphaerechinus granularis* – irreversible damage and deaths occurred at concentrations >30 mg/L. Howe *et al.* (2004) confirmed that among several formulations of glyphosate and their surfactants, POEA was the most toxic, negatively affecting development of amphibians at a concentration 0.6 mg/L ae, and was lethal to 50% of *Rana clamitans* at a concentration 2.2 mg/L ae.

Several other authors have confirmed the toxicity of the surfactant to be higher than that of the active ingredient. These include Alberdi *et al.* (1996), who investigated the toxicity of RON-DO® in 48-h toxicity testing using *Daphnia magna* and *D. spinulata* (see Table 3.1, entries 40-41). RON-DO formulation contained 48% of glyphosate as isopropylamine salt and 15% of surfactant (oxide-coco-amide-propyl-dimethyl-amine). EC_{50} values were 66.18 mg/L for *D. spinulata* and 61.72 mg/L for *D. magna.* In comparison Henry *et al.* (1994) found 48-h LC_{50} value for *D. magna* to be 218 mg/L when exposed to Rodeo herbicide (Table 3.1, entry 39). Henry *et al.* (1994) also

found that the surfactant X-77 used in some glyphosate formulations was about a 100 times more toxic to *D. magna* than Rodeo (48-h LC_{50} is 2 mg/L for X-77). In general X-77 Spreader® was 83-136 times more toxic than Rodeo when tested using different species of animals (Table 3.1, entries 37-39). Similarly Folmar *et al.* (1979) found that glyphosate contributed only a small percentage of the toxicity of Roundup® and that the surfactant in the formulation was the primary toxic agent (see also entries 33 and 95-98 of Table 3.1).

Mann and Bidwell (1999) determined the acute toxicity of technical grade glyphosate acid, glyphosate isopropylamine, and three glyphosate formulations to adults of one species and tadpoles of four species of southwestern Australian frogs in 48-h static/renewal tests (Table 3.1, entries 51-72). They found that among the tested formulations Roundup® Herbicide was the most toxic to the tadpoles (between 2.9 and 11.6 mg/L glyphosate acid equivalent [AE]). Touchdown® Herbicide was slightly less toxic (from 9.0 to 16.1 mg/L AE). All other formulations and technical grade glyphosate were practically non-toxic. These authors concluded that the surfactants in test formulations were the major contributing factor to their toxicity, and they should be studied further.

Everett and Dickerson (2003) studied the toxicity of technical grade glyphosate and formulation Roundup to ciliates *Tetrahymena thermophila* and *Ichthyophthirius multifiliis* and also concluded that Roundup was at least 100 times more toxic than technical grade glyphosate, confirming the toxicity of the products used in formulation.

Table 3.1 Glyphosate toxicity to different organisms (1979-2004). A.e. (acid equivalent), ai-active ingredient, pf-product formulation, ChV-chronic values (calculated as a geometrical mean between LOEC and NOEC), all mg/L

#	Glyphosate formulation 1	Species 2	Effect measured 3	Parameter		Reference 6
				Name 4	Value 5	
BACTERIA AND ALGAE						
1	Glyphosate acid	Marine bacterium *Vibrio fischeri*	Luminescence emission	15-min IC_{50}	17.5 (15.8-19.5) ae	Tsui and Chu, *2003*
2	Isopropylamine salt of glyphosate	Marine bacterium *Vibrio fischeri*	Luminescence emission	15-min IC_{50}	162 (150-177) ae	Tsui and Chu, *2003*
3	Polyoxyethylene amine (Surfactant in Rodeo®)	Marine bacterium *Vibrio fischeri*	Luminescence emission	15-min IC_{50}	10.2 (9.80-10.7) ae	Tsui and Chu, *2003*
4	Rodeo®	Marine bacterium *Vibrio fischeri*	Luminescence emission	15-min IC_{50}	24.9 (23.9-26.0) ae	Tsui and Chu, *2003*
5	Glyphosate 36%	Alga *Chlorella pyrenoidosa*	Growth inhibition	96-h EC_{50} NOEC 96-h	396-423 a.i. 108 a.i.	Anton *et al., 1993*
6	Glyphosate, technical grade, 38%	Alga *Chlorella pyrenoidosa*	Growth inhibition	96-h EC_{50}	380 a.i.	Anton *et al., 1993*
7	Glyphosate, technical grade, 54.9%	Alga *Chlorella pyrenoidosa*	Growth inhibition	96-h EC_{50}	1082 a.i.	Anton *et al., 1993*
8	Rodeo®	Green alga *Ankistrodesmus*	Growth	96-hr EC_{50}	74 ± 47 a.i.	Gardner *et. al., 1997*
9	Ron-do	Alga *Scenedesmus acutus*	Chlorophyll *a* inhibition	NOEC LOEC ChV 96-h EC_{50}	3.2 Gly 4.08 Gly 3.61 Gly 9.08 Gly (8.4-9.7)	Saenz *et al, 1997*
10	Ron-do	Alga *Scenedesmus quadricauda*	Chlorophyll *a* inhibition	NOEC LOEC ChV 96-h EC_{50}	1.25 Gly 2.5 Gly 1.76 Gly 9.09 (8.06-10.2) Gly	Saenz *et al, 1997*

11	Glyphosate	Alga *Scenedesmus acutus*	Chlorophyll *a* inhibition	NOEC LOEC ChV 96-h EC_{50}	2 Gly 4 Gly 2.82 Gly 10.2(10.4-11.2) Gly	Saenz *et al, 1997*
12	Glyphosate	Alga *Scenedesmus quadricauda*	Chlorophyll *a* inhibition	NOEC LOEC ChV 96-h EC_{50}	0.77 Gly 1.55 Gly 1.09 Gly 7.2(4.4-8.9) Gly	Saenz *et al, 1997*
13	Glyphosate acid	Freshwater alga *Selenastrum capricornutum*	Absorbance at 680 nm	96-h IC_{50}	24.7 (22.8-16.7) ae	Tsui and Chu, *2003*
14	Isopropylamine salt of glyphosate	Freshwater alga *Selenastrum capricornutum*	Absorbance at 680 nm	96-h IC_{50}	41.0 (29.4-59.1) ae	Tsui and Chu, *2003*
15	Polyoxyethylene amine (Surfactant in Rodeo®)	Freshwater alga *Selenastrum capricornutum*	Absorbance at 680 nm	96-h IC_{50}	3.92 (1.57-9.58) ae	Tsui and Chu, *2003*
16	Rodeo®	Freshwater alga *Selenastrum capricornutum*	Absorbance at 680 nm	96-h IC_{50}	5.81 (2.36-8.14) ae	Tsui and Chu, *2003*
17	Glyphosate acid	Marine alga *Skeletonema costatum*	Absorbance at 675 nm	96-h IC_{50}	2.27 (0.82-11.1) ae	Tsui and Chu, *2003*
18	Isopropylamine salt of glyphosate	Marine alga *Skeletonema costatum*	Absorbance at 675 nm	96-h IC_{50}	5.89 (3.14-10.4) ae	Tsui and Chu, *2003*
19	Polyoxyethylene amine (Surfactant in Rodeo®)	Marine alga *Skeletonema costatum*	Absorbance at 675 nm	96-h IC_{50}	3.35 (2.02-5.40) ae	Tsui and Chu, *2003*
20	Rodeo®	Marine alga *Skeletonema costatum*	Absorbance at 675 nm	96-h IC_{50}	1.85 (0.33-10.49) ae	Tsui and Chu, *2003*

PROTOZOAN						
21	Glyphosate acid	Freshwater protozoan *Tetrahymena pyriformis*	Culture growth	40-h IC_{50}	648 (430-1280) ae	Tsui and Chu, *2003*
22	Isopropylamine salt of glyphosate	Freshwater protozoan *Tetrahymena pyriformis*	Culture growth	40-h IC_{50}	386 (95.2-2020) ae	Tsui and Chu, *2003*
23	Polyoxyethylene amine (Surfactant in Rodeo®)	Freshwater protozoan *Tetrahymena pyriformis*	Culture growth	40-h IC_{50}	4.96 (2.90-8.98) ae	Tsui and Chu, *2003*
24	Rodeo®	Freshwater protozoan *Tetrahymena pyriformis*	Culture growth	40-h IC_{50}	29.5 (11.3-66.0) ae	Tsui and Chu, *2003*
25	Glyphosate acid	Marine protozoan *Euplotes vannus*	Culture growth	48-h IC_{50}	10.1 (6.47-14.5) ae	Tsui and Chu, *2003*
26	Isopropylamine salt of glyphosate	Marine protozoan *Euplotes vannus*	Culture growth	48-h IC_{50}	64.09 (19.0-325) ae	Tsui and Chu, *2003*
27	Polyoxyethylene amine (Surfactant in Rodeo®)	Marine protozoan *Euplotes vannus*	Culture growth	48-h IC_{50}	5.00 (4.62-5.42) ae	Tsui and Chu, *2003*
28	Rodeo®	Marine protozoan *Euplotes vannus*	Culture growth	48-h IC_{50}	23.5 ae	Tsui and Chu, *2003*
INVERTEBRATES						
29	Glyphosate (commercial grade, 41%)	Rotifer *Brachionus calyciflorus*	Survival	24-h LC_{50}	28.0	Xi & Feng, *2004*
30	Rodeo® X-77	Leech *Nephelopsis obscura*	Survival	96-h LC_{50}	1177 (941-1415) 14.1 (10.7-19.0)	Henry *et. al., 1994*
31	Roundup® Herbicide (MON 2139 surfactant)	Water flea *Daphnia magna*	Survival	48-h LC_{50}	3.0 (2.6-3.4) a.i.	Folmar *et al., 1979*

32	Technical grade glyphosate (MON0573)	Midge larvae *Chironomous plumosus*	Survival	48-h LC_{50}	55 (31-97) a.i.	Folmar *et al., 1979*
33	Surfactant (MON0818)	Midge larvae *Chironomous plumosus*	Survival	48-h LC_{50}	13 (7.1-24) a.i.	Folmar *et al., 1979*
34	Roundup® Herbicide (MON 2139 surfactant)	Midge larvae *Chironomous plumosus*	Survival	48-h LC_{50}	18 (9.4-32) a.i.	Folmar *et al., 1979*
35	Roundup® Herbicide (MON 2139 surfactant)	Scud *Gammarus pseudoiemnaeus*	Survival	24-h LC_{50} 48-h LC_{50} 96-h LC_{50}	>100 a.i. 62 (40-98) a.i. 43 (28-66) a.i.	Folmar *et al., 1979*
36	Roundup (480 g/L of glyphosate as isopropylamine salt)	Cladoceran *Daphnia pulex*	Immobilization	96-h LC_{50} (as Roundup) 96-h LC_{50} (as glyphosate) 96-h LC_{50} (as MONO818)	25.5 7.8 3.8	Servizi *et al., 1987*
37	Rodeo® X-77	Amphipod *Hyalella azteca*	Survival	96-h LC_{50}	720(399-1076) 5.3 (4.3-6.7)	Henry *et. al., 1994*
38	Rodeo® X-77	Midge *Chironomus riparius*	Survival	48-h LC_{50}	1216(996-1566) 10.0 (8.2-13.1)	Henry *et. al., 1994*
39	Rodeo® X-77	Cladoceran *Daphnia magna*	Survival	48-h LC_{50}	218(150-287) 2.0 (1.5-2.7)	Henry *et. al., 1994*
40	RON-DO (48% of glyphosate as isopropylamine salt)	Cladoceran *Daphnia spinulata*	Immobilization	24-h EC_{50} 48-h EC_{50}	94.87(89.1-101) ai 66.18(61.1-71.8) ai	Alberdi *et. al., 1996*
41	RON-DO (48% of glyphosate as isopropylamine salt	Cladoceran *Daphnia magna*	Immobilization	24-h EC_{50} 48-h EC_{50}	95.96(91.5-101.1) ai 61.72(58.8-64.2) ai	Alberdi *et. al., 1996*

42	Glyphosate acid	Freshwater crustacean *Ceriodaphnia dubia*	Survival	48-h LC_{50}	147 (141-153) ae	Tsui and Chu, *2003*
43	Isopropylamine salt of glyphosate	Freshwater crustacean *Ceriodaphnia dubia*	Survival	48-h LC_{50}	415 (339-508) ae	Tsui and Chu, *2003*
44	Polyoxyethylene amine (Surfactant in Rodeo®)	Freshwater crustacean *Ceriodaphnia dubia*	Survival	48-h LC_{50}	1.15 (1.04-1.27) ae	Tsui and Chu, *2003*
45	Rodeo®	Freshwater crustacean *Ceriodaphnia dubia*	Survival	48-h LC_{50}	5.39 (4.81-6.05) ae	Tsui and Chu, *2003*
46	Glyphosate acid	Marine crustacean *Acartia tonza*	Survival	48-h LC_{50}	35.3 (30.9-40.3) ae	Tsui and Chu, *2003*
47	Isopropylamine salt of glyphosate	Marine crustacean *Acartia tonza*	Survival	48-h LC_{50}	49.3 (38.4-63.1) ae	Tsui and Chu, *2003*
48	Polyoxyethylene amine (Surfactant in Rodeo®)	Marine crustacean *Acartia tonza*	Survival	48-h LC_{50}	0.57 (0.50-0.65) ae	Tsui and Chu, *2003*
49	Rodeo®	Marine crustacean *Acartia tonza*	Survival	48-h LC_{50}	1.77 (1.33-2.34) ae	Tsui and Chu, *2003*
50	Roundup	Freshwater mussel *Utterbackia imbecillis*	Survival	24-h LC_{50}	18.3 ± 12.9	Conners & Black, *2004*
AMPHIBIANS						
51	Technical grade glyphosate acid	Frog (tadpole) *Litoria moorei*	Survival	24-h LC_{50} 48-h LC_{50}	127(90-180) 121(111-133)	Bidwell & Gorrie, *1995*
52	Technical grade glyphosate acid	Frog (adult) *Crinia insignifera*	Survival	24-h LC_{50} 48-h LC_{50}	89.6(73.6-108.6) 83.6(67.4-103.6)	Bidwell & Gorrie, *1995*
53	Roundup® Herbicide (MON 2139 surfactant)	Frog (adult) *Crinia insignifera*	Survival	24-h LC_{50} 48-h LC_{50}	52.6(39.3-70.5) ae 146(109-196) pf 49.4(40.5-60.2) ae 137(113-167) pf	Bidwell & Gorrie, *1995*

54	Roundup® Herbicide (MON 2139 surfactant)	Frog (tadpole) *Litoria moorei*	Survival	24-h LC_{50} 48-h LC_{50}	12.7(9.0-18.0) ae 35.3(25.0-50.0) pf 11.6(10.3-13.1) ae 32.2(28.6-36.4) pf	Bidwell & Gorrie, *1995*
55	Technical grade glyphosate acid	Frog (tadpole) *Litoria moorei*	Survival	24-h LC_{50} 48-h LC_{50}	88.6(79.8-98.3) 81.2(76.7-85.9)	Mann & Bidwell, *1999*
56	Glyphosate isopropylamine	Frog (tadpole) *Lymnodynastes dorsalis*	Survival	24-h LC_{50} 48-h LC_{50}	>400ae, >587pf >400ae, >587 pf	Mann & Bidwell, *1999*
57	Roundup® Herbicide (MON 2139 surfactant)	Frog (tadpole) *Lymnodynastes dorsalis*	Survival	24-h LC_{50} 48-h LC_{50}	4.6(4.1-5.2) ae 12.8(11.4-14.4) pf 3.0(2.8-3.2) ae 8.3(7.8-8.9) pf	Mann & Bidwell, *1999*
58	Touchdown® Herbicide (4 LC-E)	Frog (tadpole) *Lymnodynastes dorsalis*	Survival	24-h LC_{50} 48-h LC_{50}	14.7(14.0-15.4) ae 44.4(42.3-46.6) pf 12.0(11.4-12.6) ae 36.2(34.4-37.9) pf	Mann & Bidwell, *1999*
59	Roundup® Biactive (MON 77920)	Frog (tadpole) *Lymnodynastes dorsalis*	Survival	24-h LC_{50} 48-h LC_{50}	>400 ae >1111 pf >400 ae >1111 pf	Mann & Bidwell, *1999*
60	Glyphosate isopropylamine	Frog (tadpole) *Litoria moorei*	Survival	24-h LC_{50} 48-h LC_{50}	>343 ae >503 pf >343 ae >503 pf	Mann & Bidwell, *1999*
61	Roundup® Herbicide (MON 2139 surfactant)	Frog (tadpole) *Litoria moorei*	Survival	24-h LC_{50} 48-h LC_{50}	3.1(2.8-3.4) ae 8.6(7.8-9.4) pf 2.9(2.6-3.2) ae 8.1(7.2-8.9) pf	Mann & Bidwell, *1999*
62	Touchdown® Herbicide (4 LC-E)	Frog (tadpole) *Litoria moorei*	Survival	24-h LC_{50}	10.4(9.7-11.1) ae 31.4(29.4-33.6) pf	Mann & Bidwell, *1999*

63	Roudup® Biactive (MON 77920)	Frog (tadpole) *Litoria moorei*	Survival	24-h LC_{50} 48-h LC_{50}	333(305-363) ae 925(847-1008) pf 328(296-363) ae 911(822-1008) pf	Mann & Bidwell, *1999*
64	Glyphosate isopropylamine	Frog (tadpole) *Heleioporus eyrei*	Survival	24-h LC_{50} 48-h LC_{50}	>373 ae >548 pf >373 ae >548 pf	Mann & Bidwell, *1999*
65	Roundup® Herbicide (MON 2139 surfactant)	Frog (tadpole) *Heleioporus eyrei*	Survival	24-h LC_{50} 48-h LC_{50}	8.6(7.8-9.5) ae 23.9(21.7-26.4) pf 6.3(5.6-7.1) ae 17.5(15.6-19.7) pf	Mann & Bidwell, *1999*
66	Touchdown® Herbicide (4 LC-E)	Frog (tadpole) *Heleioporus eyrei*	Survival	24-h LC_{50} 48-h LC_{50}	16.6(14.1-19.6) ae 50.2(42.5-59.3) pf 16.1(13.7-18.9) ae 48.7(41.5-57.1) pf	Mann & Bidwell, *1999*
67	Roudup® Biactive (MON 77920)	Frog (tadpole) *Heleioporus eyrei*	Survival	24-h LC_{50} 48-h LC_{50}	>427 ae >1186 pf >427 ae >1186 pf	Mann & Bidwell, *1999*
68	Glyphosate isopropylamine	Frog (tadpole) *Crinia insignifera*	Survival	24-h LC_{50} 48-h LC_{50}	>466 ae >684 pf >466 ae >684 pf	Mann & Bidwell, *1999*
69	Roundup® Herbicide (MON 2139 surfactant)	Frog (tadpole) *Crinia insignifera*	Survival	24-h LC_{50} 48-h LC_{50}	>5.1 ae >14.2 pf 3.6(3.3-4.1) ae 10(9.2-11.4) pf	Mann & Bidwell, *1999*
70	Touchdown® Herbicide (4 LC-E)	Frog (tadpole) *Crinia insignifera*	Survival	24-h LC_{50} 48-h LC_{50}	13.1(12.3-14.0) ae 39.6(37.2-42.2) pf 9.0(8.4-9.7) ae 27.3(25.5-29.3) pf	Mann & Bidwell, *1999*

71	Roudup® Biactive (MON 77920)	Frog (tadpole) *Crinia insignifera*	Survival	24-h LC_{50} 48-h LC_{50}	>494 ae >1372 pf >494 ae >1372 pf	Mann & Bidwell, *1999*
72	Roundup® Herbicide (MON 2139 surfactant)	Frog (metamorph) *Crinia insignifera*	Survival	24-h LC_{50} 48-h LC_{50}	88.7(68.6-114) ae 246(191-318) pf 51.8(42.1-63.8) ae 144(117-177) pf	Mann & Bidwell, *1999*
73	Glyfos®	Tadpole *Scinax nasicus*	Survival	24-h LC_{50} 48-h LC_{50} 72-h LC_{50} 96-h LC_{50}	4.78 (4.23-5.35) 3.62 (3.28-5.02) 3.23 (3.07-3.36) 2.64 (2.19-2.84)	Lajmanovich *et al., 2003*
74	Vision®	Amphibian larvae *Rana clamitans*	Survival	96-h LC_{10} 96-h LC_{50}	1.2-1.78 a.e. 2.70-4.34 a.e.	Wojtaszek *et al., 2004*
75	Vision®	Amphibian larvae *Rana pipiens*	Survival	96-h LC_{10} 96-h LC_{50}	3.26-7.31 a.e. 4.25-11.47 a.e.	Wojtaszek *et al., 2004*
76	Roundup Original®	Amphibian *Rana pipiens*	Survival	24-h LC_{50} 96-h LC_{50}	3.7 (3.5-3.9) ae 2.9 ae	Howe *et al., 2004*
77	Roundup Original®	Amphibian *Rana pipiens*	Survival	24-h LC_{50} 96-h LC_{50}	>8 ae 6.5 (6.1-6.8) ae	Howe *et al., 2004*
78	Roundup Original®	Amphibian *Rana sylvatica*	Survival	24-h LC_{50} 96-h LC_{50}	5.6 (5.2-6.1) ae 5.1 (4.9-5.4) ae	Howe *et al., 2004*
79	Roundup Original®	Amphibian *Rana sylvatica*	Survival	24-h LC_{50} 96-h LC_{50}	>8 ae >8 ae	Howe *et al., 2004*
80	Roundup Original®	Amphibian *Bufo americanus*	Survival	24-h LC_{50} 96-h LC_{50}	4.2 ae <4 ae	Howe *et al., 2004*
81	Roundup Original®	Amphibian *Bufo americanus*	Survival	24-h LC_{50} 96-h LC_{50}	>8 ae 8 ae	Howe *et al., 2004*
82	Roundup Original®	Amphibian *Rana clamitans*	Survival	24-h LC_{50} 96-h LC_{50}	2.0 (1.9-2.2) ae 2.0 (1.9-2.2) ae	Howe *et al., 2004*
83	Roundup Original®	Amphibian *Rana clamitans*	Survival	24-h LC_{50} 96-h LC_{50}	>8 ae 7.1 (6.6-7.6) ae	Howe *et al., 2004*

84	Glyphosate technical	Amphibian *Rana clamitans*	Survival	24-h LC_{50} 96-h LC_{50}	>17.9 ae >17.9 ae	Howe *et al., 2004*
85	POEA	Amphibian *Rana clamitans*	Survival	24-h LC_{50} 96-h LC_{50}	2.4 (2.2-2.5) ae 2.2 (2.1-2.4) ae	Howe *et al., 2004*
86	Roundup Biactive®	Amphibian *Rana clamitans*	Survival	24-h LC_{50} 96-h LC_{50}	>17.9 ae >17.9 ae	Howe *et al., 2004*
87	Touchdown®	Amphibian *Rana clamitans*	Survival	24-h LC_{50} 96-h LC_{50}	>17.9 ae >17.9 ae	Howe *et al., 2004*
88	Glyfos BIO®	Amphibian *Rana clamitans*	Survival	24-h LC_{50} 96-h LC_{50}	>17.9 ae >17.9 ae	Howe *et al., 2004*
89	Glyfos AU®	Amphibian *Rana clamitans*	Survival	24-h LC_{50} 96-h LC_{50}	9.0 (8.7-9.4) ae 8.9 (8.6-9.2) ae	Howe *et al., 2004*
90	Roundup Transorb®	Amphibian *Rana clamitans*	Survival	24-h LC_{50} 96-h LC_{50}	2.3 (2.2-2.4) ae 2.2 (2.1-2.4) ae	Howe *et al., 2004*
FISH						
91	Technical grade glyphosate (MON0573)	Rainbow trout *Salmo gairdneri*	Survival	24-h LC_{50} 96-h LC_{50}	140 (120-170) a.i. 140 (120-170) a.i.	Folmar *et al., 1979*
92	Technical grade glyphosate (MON0573)	Fathead minnow *Pimephales promelas*	Survival	24-h LC_{50} 96-h LC_{50}	97 (79-120) a.i. 97 (79-120) a.i.	Folmar *et al., 1979*
93	Technical grade glyphosate (MON0573)	Channel catfish *Ictalurus punctatus*	Survival	24-h LC_{50} 96-h LC_{50}	130 (110-160) a.i. 130 (110-160) a.i.	Folmar *et al., 1979*
94	Technical grade glyphosate (MON0573)	Bluegills *Lepomis macrochirus*	Survival	24-h LC_{50} 96-h LC_{50}	150 (120-190) a.i. 140 (110-160) a.i.	Folmar *et al., 1979*
95	Surfactant (MON0818)	Rainbow trout *Salmo gairdneri*	Survival	24-h LC_{50} 96-h LC_{50}	2.1 (1.6-2.7) a.i. 2.0 (1.5-2.7) a.i.	Folmar *et al., 1979*
96	Surfactant (MON0818)	Fathead minnow *Pimephales promelas*	Survival	24-h LC_{50} 96-h LC_{50}	1.4 (1.2-1.7) a.i. 1.0 (1.2-1.7) a.i.	Folmar *et al., 1979*
97	Surfactant (MON0818)	Channel catfish *Ictalurus punctatus*	Survival	24-h LC_{50} 96-h LC_{50}	18 (8.5-38) a.i. 13 (10-17) a.i.	Folmar *et al., 1979*
98	Surfactant (MON0818)	Bluegills *Lepomis macrochirus*	Survival	24-h LC_{50} 96-h LC_{50}	3.0 (2.5-3.7) a.i. 3.0 (2.5-3.7) a.i.	Folmar *et al., 1979*

99	Roundup® Herbicide (MON 2139 surfactant)	Rainbow trout *Salmo gairdneri*	Survival	24-h LC_{50} 96-h LC_{50}	8.3 (7.0-9.9) a.i. 8.3 (7.0-9.9) a.i.	Folmar *et al., 1979*
100	Roundup® Herbicide (MON 2139 surfactant)	Fathead minnow *Pimephales promelas*	Survival	24-h LC_{50} 96-h LC_{50}	2.4 (2.0-2.9) a.i. 2.3 (1.9-2.8) a.i.	Folmar *et al., 1979*
101	Roundup® Herbicide (MON 2139 surfactant)	Channel catfish *Ictalurus punctatus*	Survival	24-h LC_{50} 96-h LC_{50}	13 (11-16) a.i. 13 (11-16) a.i.	Folmar *et al., 1979*
102	Roundup® Herbicide (MON 2139 surfactant)	Bluegills *Lepomis macrochirus*	Survival	24-h LC_{50} 96-h LC_{50}	6.4 (4.8-8.6) a.i. 5.0 (3.8-6.6) a.i.	Folmar *et al., 1979*
103	Roundup® Herbicide (MON 2139 surfactant)	Rainbow trout *Salmo gairdneri*	Survival: Eyed eggs Sac fry Swim-up fry Fingerling (1.0 g) Fingerling (2.0 g)	 24-h LC_{50} 96-h LC_{50} 24-h LC_{50} 96-h LC_{50} 24-h LC_{50} 96-h LC_{50} 24-h LC_{50} 96-h LC_{50} 24-h LC_{50} 96-h LC_{50}	 46 (35-61) a.i. 16 (13-19) a.i. 11 (8.8-13) a.i. 3.4 (2.2-5.3) a.i. 2.4 (2.0-2.9) a.i. 2.4 (2.0-2.9) a.i. 2.2 (0.93-5.2) a.i. 1.3 (1.1-1.6) a.i. 8.3 (7.0-9.9) a.i. 8.3 (7.0-9.9) a.i.	Folmar *et al., 1979*
104	Roundup® Herbicide (MON 2139 surfactant)	Channel catfish *Ictalurus punctatus*	Survival: Eyed eggs Sac fry Swim-up fry Fingerling (2.2 g)	 24-h LC_{50} 96-h LC_{50} 24-h LC_{50} 96-h LC_{50} 24-h LC_{50} 96-h LC_{50} 24-h LC_{50} 96-h LC_{50}	 43 (36-51) a.i. nd 4.3 (3.6-5.1) a.i. 4.3 (3.6-5.1) a.i. 3.7 (3.4-4.1) a.i. 3.3 (2.8-3.9) a.i. 13 (11-16) a.i. 13 (11-16) a.i.	Folmar *et al., 1979*

105	Roundup (480 g/L of glyphosate as isopropylamine salt)	Sockeye salmon *Oncorhynchus nerka* (fingerlings)	Survival	96-h LC_{50} 96-h LC_{50} (as glyphosate) 96-h LC_{50} (as MONO818)	26.7-27.7 8.1-8.4 4.0-4.2	Servizi *et al.*, *1987*
106	Roundup (480 g/L of glyphosate as isopropylamine salt)	Sockeye salmon *Oncorhynchus nerka* (fry)	Survival	96-h LC_{50} 96-h LC_{50} (as glyphosate) 96-h LC_{50} (as MONO818)	28.8 8.7 4.3	Servizi *et al.*, *1987*
107	Roundup (480 g/L of glyphosate as isopropylamine salt)	Rainbow trout (fry) *Salmo gairdneri*	Survival	96-h LC_{50} 96-h LC_{50} (as glyphosate) 96-h LC_{50} (as MONO818)	25.5-28.0 7.8-8.5 3.8-4.2	Servizi *et al.*, *1987*
108	Roundup (480 g/L of glyphosate as isopropylamine salt)	Coho salmon (fry) *Oncorhynchus kisutch*	Survival	96-h LC_{50} 96-h LC_{50} (as glyphosate) 96-h LC_{50} (as MONO818)	42.0 12.8 6.3	Servizi *et al.*, *1987*
109	Roundup (commercial formulation)	Rainbow trout *Salmo gairdneri*	Survival	96-h LC_{50}	12 (5.7-18) a.i.	Mitchell *et al.*, *1987*
110	Roundup (commercial formulation)	Chinook salmon *Oncorhynchus tshawytscha*	Survival	96-h LC_{50}	9.6 (7.9-13) a.i.	Mitchell *et al.*, *1987*
111	Roundup (commercial formulation)	Coho salmon *Oncorhynchus kisutch*	Survival	96-h LC_{50}	11 (5.7-18) a.i.	Mitchell *et al.*, *1987*
112	Rodeo/X-77	Rainbow trout *Salmo gairdneri*	Survival	96-h LC_{50}	130 (120-160) a.i.	Mitchell *et al.*, *1987*
113	Rodeo/X-77	Chinook salmon *Oncorhynchus tshawytscha*	Survival	96-h LC_{50}	140 (120-220) a.i.	Mitchell *et al.*, *1987*

114	Rodeo/X-77	Coho salmon *Oncorhynchus kisutch*	Survival	96-h LC_{50}	120 (68-220) a.i.	Mitchell *et al., 1987*
115	Glyphosate (technical grade)	Coho *Oncorhynchus kisutch*	Survival (range in different types of dilution water)	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	44-210 ai 27-205 ai 27-182 ai 27-174 ai	Wan *et al., 1989*
116	Glyphosate (technical grade)	Chum *Oncorhynchus keta*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	16-202 ai 13-178 ai 10-157 ai 10-148 ai	Wan *et al., 1989*
117	Glyphosate (technical grade)	Chinook *Oncorhyncus tshawytsha*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	24-220 ai 22-220 ai 22-211 ai 19-211 ai	Wan *et al., 1989*
118	Glyphosate (technical grade)	Pink salmon *Oncorhyncus gorbuscha*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	26-380 ai 14-245 ai 14-190 ai 14-190 ai	Wan *et al., 1989*
119	Glyphosate (technical grade)	Rainbow trout *Salmo gairdneri*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	21-220 ai 11-220 ai 11-220 ai 10-197 ai	Wan *et al., 1989*

120	MON 8709	Coho *Oncorhynchus kisutch*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	25-59 product 25-57 product 25-57 product 25-55 product	Wan *et al., 1989*
121	MON 8709	Chum *Oncorhynchus keta*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	25-62 product 25-58 product 23-58 product 23-58 product	Wan *et al., 1989*
122	MON 8709	Chinook *Oncorhyncus tshawytsha*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	33-84 product 33-79 product 33-73 product 33-67 product	Wan *et al., 1989*
123	MON 8709	Pink salmon *Oncorhyncus gorbuscha*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	24-88 product 24-54 product 24-48 product 24-48 product	Wan *et al., 1989*
124	MON 8709	Rainbow trout *Salmo gairdneri*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	31-88 product 20-62 product 17-48 product 17-48 product	Wan *et al., 1989*

125	Roundup®	Coho *Oncorhynchus kisutch*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	14-52 product 13-38 product 13-35 product 13-33 product	Wan *et al., 1989*
126	Roundup®	Chum *Oncorhynchus keta*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	17-31 product 12-27 product 11-25 product 11-20 product	Wan *et al., 1989*
127	Roundup®	Chinook *Oncorhyncus tshawytsha*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	17-41 product 17-33 product 17-33 product 17-33 product	Wan *et al., 1989*
128	Roundup®	Pink salmon *Oncorhyncus gorbuscha*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	17-35 product 17-33 product 17-33 product 14-33 product	Wan *et al., 1989*
129	Roundup®	Rainbow trout *Salmo gairdneri*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	17-33 product 17-33 product 15-33 product 14-33 product	Wan *et al., 1989*

130	75% tallow amine surfactant MON 0818 (Part of MON 8709 – 10% w/w)	Coho *Oncorhynchus kisutch*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	1.8-4.9 ai 1.8-4.6 ai 1.8-4.6 ai 1.8-4.6 ai	Wan *et al., 1989*
131	75% tallow amine surfactant MON 0818 (Part of MON 8709 – 10% w/w)	Chum *Oncorhynchus keta*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	1.5-2.7 ai 1.4-2.7 ai 1.4-2.7 ai 1.4-2.7 ai	Wan *et al., 1989*
132	75% tallow amine surfactant MON 0818 (Part of MON 8709 – 10% w/w)	Chinook *Oncorhyncus tshawytsha*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	2.0-4.9 ai 2.0-3.0 ai 1.9-2.8 ai 1.7-2.8 ai	Wan *et al., 1989*
133	75% tallow amine surfactant MON 0818 (Part of MON 8709 – 10% w/w)	Pink salmon *Oncorhyncus gorbuscha*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	1.7-5.3 ai 1.5-4.5 ai 1.5-4.5 ai 1.4-4.5 ai	Wan *et al., 1989*
134	75% tallow amine surfactant MON 0818 (Part of MON 8709 – 10% w/w)	Rainbow trout *Salmo gairdneri*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	2.0-3.2 ai 2.0-2.7 ai 1.9-2.6 ai 1.6-2.6 ai	Wan *et al., 1989*
135	Vision (356 g/L of glyphosate as N-(phosphonometyl) glycine)	Rainbow trout *Oncorhyncus mykiss*	Survival	96-h LC_{50}	10.42(9.37-11.67) ai	Morgan & Kiceniuk, *1992*

136	Glyphosate commercial formulation 54.9% ai	Goldfish *Carassius auratus*	Survival	96-h NOEC 96-h LC_{50}	3431 ± 137 ai 4183.62 ± 83.5 ai	Anton *et al, 1994*
137	Glyphosate commercial formulation 38% ai	Goldfish *Carassius auratus*	Survival	96-h NOEC 96-h LC_{50}	------ 9500-10000 ai	Anton *et al, 1994*
138	Glyphosate commercial formulation 36 % ai	Goldfish *Carassius auratus*	Survival	96-h NOEC 96-h LC_{50}	2880 ai 9217 ai	Anton *et al, 1994*
139	Glyphosate commercial formulation 54.9% ai	Rainbow trout *Oncorhyncus mykiss*	Survival	96 hr NOEC 96 hr LC_{50}	823.5 ai 4290.8 ai	Anton *et al, 1994*
140	Technical grade 62%	Carp *Cyprinus carpio*	Survival	48 hr LC_{50} 96 hr LC_{50}	645 (632-655) ai 620 (607-638) ai	Neskovic *et al., 1996*
141	Technical grade 62%	Carp *Cyprinus carpio*	Biochemical & histopathological changes	Alkaline phosphatase (AP) activity in liver AP activity in heart Glutamic-oxaloacetic transaminase (GOT) activity in liver and kidney Glutamic-pyruvic transaminase (GPT) activity in kidney GPT activity in serum Gills: epithelial hyperplasia and subepithelial edema Liver: congestion of sinusoid and signs of early fibrosis	Increased at 2.5, 5, and 10 a.i. Increased at 10 a.i. Increased at 2.5 and 5 a.i. Increased at 2.5 a.i. Increased at 5 and 10 a.i. Found at 5 and 10 a.i. 10 a.i.	Neskovic *et. al., 1996*

142	Rival granular	Mosquitofish *Gambusia yucatana*	Survival	96-h LC_{10} 96-h LC_{50} 96-h LC_{90}	9.97 (3.53-13.91) 17.79 (12.19-25.36) 31.71 (22.95-84.71)	Rendon-von Osten *et al., 2005*
COMMUNITIES						
143	Roundup (application spray 0.43 kg a.i./ha)	Plankton communities with fish	DO, pH, T°C, total ammonia and nitrite nitrogen, chlorophyll *a*		No adverse effect found	Perschbacher *et al., 1997*

3.2 TOXICITY OF CHLORPYRIFOS TO DIFFERENT ORGANISMS

3.2.1 General comments

To get the feel what concentrations are environmentally realistic the EEC and TV (trigger value) are presented below. According to the chlorpyrifos manual (Chlorpyrifos 500 insecticide, 4 FARMERS, 1997) the application rate is up to 6 L/ha for some crops. The EEC (Peterson *et al.* 1994) calculated for this rate reaches the value of 2mg/L, while the recommended level of protection of 80 and 95% of species for Australian fresh waters is 1.2 and 0.00004 µg/L respectively (ANZECC & ARMCANZ 2000).

Guilhermino *et al.* (2000) studied the possibility of predicting the acute toxicity of various toxicants (including CPF) to rats from the acute toxicity results for *D. magna.* If the chemical is toxic to daphnia, it is likely (with a probability of 0.83) to be toxic to a rat, if it's not toxic to daphnia, the probability is 0.74 that it is not toxic to a rat. These authors concluded that the results of their study as well as results published by other authors provided good evidence of the applicability of using invertebrate tests as pre-screening methods, thus considerably reducing the number of mammals required in toxicity testing. This shows the importance of studying the toxicity of chemicals on lower taxa not only from environmental point of view but also with implications to higher taxa toxicity.

3.2.2 Sediment-associated toxicity of chlorpyrifos

There are relatively few studies on the effects of sediment-associated CPF. Green and Chandler (1996) assessed the effects of sediment-associated CPF on the population parameters of the marine, infaunal copepod, *Amphiascus tenuiremis*. Total fecundity and weekly fecundity were significantly affected at all concentrations tested (5, 14, and 22 μg-CPF/kg-sed.). Consequently a large reduction was observed in the intrinsic rate of natural increase (r) – 26-52%, and net reproductive rate (R_0) –55-73% of those of the control.

Anderson *et al.* (2003a, 2003b) studied the toxicity of water and sediments in the Salinas River (California, USA) and found them to be toxic, respectively, to cladocerans *Ceriodaphnia dubia* and to the amphipod *Hyalella azteca.* Based on chemical analysis they concluded that the toxicity was due to diazinon and chlorpyrifos, which was present in the river at excessive amounts (up to 0.609 μg/L).

Jergentz *et al.* (2004) monitored agricultural contamination in two streams in the Argentine pampa. The population dynamics and organismic drift of two crustaceans *Hyalella curvispina* and *Macrobrachium borelli* was affected by CPF applications resulting in 100% mortality for both species when a concentration of CPF of 64 μg/kg in the suspended sediment was recorded.

3.2.3 Effects of chlorpyrifos on algae

Because CPF is considered non-toxic to algae, not many studies have been conducted using algal species. Effects of CPF on algae are described in Walsh (1983) (Table 3.2, entry 2) and Van Donk *et al.* (1992) (Table 3.2, entries 1, 3-4). The effects of low concentrations of CPF on algae have not been studied at all. However, considering the phosphorus content of the insecticide, it has the potential to stimulate growth in algal cultures.

3.2.4 Effect of chlorpyrifos on freshwater fauna (single species data)

The toxicity values for CPF to different species are presented in Table 3.2. The results of this extensive literature review suggest that CPF is very toxic to all taxonomic groups of freshwater fauna from invertebrates to fish.

Snell and Carmona (1995) found that the sexual reproductive cycle of rotifers *Brachionus calyciflorus* is affected by a CPF concentration of 300 μg/L, while asexual reproduction remained unaffected (Table 3.2, entry 6). CPF inhibited sexual reproduction in its initial step: sexual female production. The authors concluded that toxicity tests based exclusively on asexual reproduction may not be protective of rotifer life cycles.

Naddy *et al.* (2000) conducted CPF experiments to evaluate the effect of binary combinations of concentration, duration and interval of CPF exposures to *Daphnia magna*. Survival and reproduction of organisms were observed after pulsed exposure to CPF at 0.12, 0.25, 0.5, or 1.0 μg/L. The exposure duration resulting in a 50% survival was 6.5 h at 1.0 μg/L, 12.2 h at 0.5 μg/L, and 48 h at 0.25 μg/L. Daphnids

exposed to two 12-h pulses of CPF at 0.5 μg/L had ≥85% mortality at all pulse intervals tested (0, 3, 7, 14 d). However, if a 12-h exposure is divided in two 6-h pulses with at least 3 days between them, daphnids were able to survive even at 0.5 μg/L of CPF. They also found that animals exposed on day 3, 7, or 14 seemed more sensitive than those exposed as neonates. Naddy and Klaine (2001) investigated further exposure concentrations of 0.5 μg/L (total exposure 12 h) and 1.0 μg/L (total exposure 6 h). Authors concluded that *D. magna* could withstand an acutely lethal CPF exposure provided that there is adequate time for recovery between exposures.

Van der Hoeven and Gerritsen (1997) studied the effect of CPF on young and adult *Daphnia pulex* and their ability to recover after the exposure (Table 3.2, entries 45-50). The NOEC was about 0.05 μg/L. Daphnids were immobilised by CPF several days prior to death, and juveniles were immobilised faster that adults. Immobilised animals died even when no longer exposed. In two cohort experiments no negative effect on reproduction was observed at the highest test concentration at which animals survived (for 0.2 and 0.4 μg/L reproduction was 101 and 121 % respectively). In the third experiment some effect on reproduction was observed at concentrations also affecting survival. In comparison with the control, the mean number of offspring per mobile female at the end of the test (6 days) was reduced significantly: 6-d exposure to 0.4 μg/L, 2-d to 0.8 μg/L , and 1-d to 1.6 μg/L reduced the cumulative number of offspring to 41, 45 and 3% of that of the control, respectively. Laboratory experiments were compared to those in the field. The median effective concentrations for population size were similar to the laboratory experiment. The authors conclude that the effect of CPF on single species *Daphnia* population in the laboratory and the field can be predicted from the effects observed in tests with individuals.

McLeay and Hall (1999) monitored the toxicity of agricultural drainage ditches and the Nicomekl River during a 6-month period, using *C. dubia.* They found that 4% of the ditch water samples were lethally toxic with 6- and 7-day LC_{50}s of 39.9 and 36.5% respectively. Fourteen percent of the ditch water samples and 9% of the river water samples inhibited *C. dubia* reproduction. Chemical analysis of these samples revealed trace quantities of CPF and/or diazinon, which the authors believe responsible for observed mortality. They conclude, that the cause of inhibited reproduction is unknown. Also cited here is the recommended CPF concentration for the short-term exposure for the protection of aquatic life according to Washington State Water Quality Standards, WAC 173-201A (reference unavailable), which is 0.083 μg/L, for long-term – 0.041 μg/L (Davis *et al.*, 1997). These authors also presented toxicity data derived from other publications (Table 3.2, entries 13, 32-33, 51-55, 69, 72).

Varo *et al.* (2002) studied the effects of CPF on two different species of *Artemia.* For acute tests results see entries 60-61 of the Table 3.2. They investigated the changes in ChE activity in CPF-exposed animals, and observed a reduction of up to 80-90% for both species of *Artemia*, when exposed to 1.85 mg/L and 2.22 mg/L of CPF for 24 h. However, both *Artemia* species were resistant to this pesticide and were able to survive with more than 80% ChE inhibition.

Chandler *et al.* (1997) investigated the sublethal effects of CPF on benthic copepod survival, age structure and reproduction. Sediment-associated CPF with concentrations of 21-33 μg/kg of sediment was used in the experiments. They found

no CPF effects on total meiobenthic copepod densities, but the predominant naturally occurring copepod *Microarthridion littorale*, known to be a major prey item for many juvenile fish, was significantly reduced in some CPF-spiked microcosms (>25 ng/g sediment). Other copepods were either unaffected or their growth was enhanced in the presence of chlorpyrifos. *Amphiascus tenuiremis*, known to be adversely affected at this concentration in 96-h static bioassays, increased dramatically in every microcosm. The authors concluded that CPF in their experiment was not bioavailable or degraded with time. This, however, does not explain a significant increase in copepod numbers (up to 64% higher) compared to control.

Roast *et al.* (1999) noted that juvenile (<24 h old) *Neomysis interger* (Western European mysid) are equally tolerant to CPF as adult mysids (96-h LC_{50} in flow-through system 0.19 μg/L and 0.16 μg/L respectively) (Table 3.2, entry 56). Comparison with a standard American species *Americamysis bahia* (Shimmel *et al.* 1983) showed that the European species was more tolerant than the American (0.035 μg/L) (Table 3.2, entry 11), but its tolerance was comparable with other frequently used test species (Table 3.2, entries 10, 15-16). The authors argue that use of indigenous species for toxicity testing and environmental monitoring is more desirable (see also references therein). Verslycke *et al.* (2004) exposed a mysid *Neomysis interger* to environmentally realistic (0.038-0.100 μg/L) concentrations of CPF and observed that their scope for growth (SFG) was significantly affected by the CPF exposures (48, 96 and 168 h). In addition, the protein, sugar, lipid and total energy content in the cellular energy allocation (CEA) assay and the egestion rate in the SFG assay were significantly different in CPF-exposed animals compared with control mysids.

Barron *et al.* (1991) studied dietary intake of CPF in a channel catfish. They concluded that orally administered CPF had limited availability because of extensive metabolism in the liver and possibly in extrahepatic tissues. The same (rapid elimination by biotransformation) applied to waterborne exposure (Barron *et al.* 1993) and intravascular administration of CPF (Barron *et al.* 1991). Holladay *et al.* (1996) exposed Nile tilapia *Oreochromis niloticus* to the environmentally realistic concentration of CPF of 1 μg/L. They found that the immune system of fish was affected by the exposure. Treated animals displayed significantly lower total pronephros (the anterior of head kidney) cell count than did untreated controls. In addition, macrophages isolated from the pronephros of the treated animals had depressed phagocytic function relative to control fish. Toxicity of CPF to the early stages of eastern rainbowfish *Melanotaenia splendid splendida* was investigated by Humphrey and Klumpp (2003). They reported that body length of larvae and otolith perimeter after 14-d exposure were significantly reduced at a CPF concentration of 6.2 μg/L. The increased water temperature greatly increased mortality in CPF exposures, probably because of increased metabolic rate at higher temperatures and consequently the increased uptake of the chemical by the fish. Gruber and Munn (1998) investigated ChE activity of common carp *Cyprinus carpio* from two lakes in the USA. They found that depressed activity in brain tissue was due to ChE-inhibiting insecticides (including CPF) detected in water samples in the weeks prior to tissue sampling. This study indicated that in the regions of intensive agriculture, non-target biota can be exposed to dangerous levels of pesticides for a period of several months, despite the fact that most of the organophosphate and carbamate insecticides break down rapidly in the environment.

Clark *et al.* (1985) investigated acute toxicity of different pesticides to six estuarine fishes in flow-through laboratory tests. Their 96-h LC_{50} values ranged from 1.3 μg/L for juvenile tidewater silverside *Menidia peninsulae* and juvenile California grunion *Leuresthes tenuis* to 520 μg/L for juvenile gulf toadfish *Opsanus beta*. The results show that sensitivity of fish to CPF is lower than that of invertebrates (Table 3.2, entries 74-79, also the references cited therein, entries 12, 70-71, 73). Moore *et al.* (1998) also showed in their study that invertebrates *H. azteca, C. tentans* and *D. magna* were ≥200 times more sensitive to CPF than the vertebrate *P. promelas* (Table 3.2, entries 53-55, 91). Amma and Konar (1996) studied effects of CPF exposure on fish, worm and plankton species (see Table, entries 7, 34, 84-90). The plankton was more susceptible (LC_{95}=8.8 μg/L) than fish and worms (LC_{95}=100.4-129 μg/L). The growth of fish was affected at all concentrations tested (5, 10, 16 and 20 μg/L). Respiratory and feeding rates of fish were significantly reduced at CPF exposure concentrations of 10-20 μg/L. The survival, and reproduction of fish was greatly reduced starting at CPF exposure concentrations of 10 μg/L. Severe histopathological lesions occurred in liver, kidney and gills of fish at CPF exposure concentrations of 10 and 20 μg/L. Water quality parameters (DO, free CO_2, pH, alkalinity, colour and odour) were not affected by CPF. Though these authors conclude that CPF appears to be highly hazardous to fish and fish food organisms, the concentrations of CPF tested in their study are quite high compared to other data (Table 3.2) and are not environmentally realistic. Van Wijngaarden *et al.* (1993) (Table 3.2, entries 5, 8, 15-31, 67, 82-83) reported on 16 single-species toxicity tests, which they performed with invertebrates, fish and a newt. They found that within the crustaceans the most susceptible (*G. pulex*) and the most tolerant species (*P. coxalis*) differ by a factor of

10^3 (Table 3.2, entries 23 and 22). It was also shown that toxicity of CPF to even closely related taxa (*A. aquaticus* and *P. coxalis*) could differ by at least an order of magnitude (Table 3.2, entries 21 and 22). It "indicates that even to closely related species, extrapolation of toxicological data of tested species for ecotoxicological risk cannot as yet be assessed on a scientific basis".

3.2.5 Effect of chlorpyrifos on water communities.

Mani and Konar (1988) studied the effect of CPF exposure on water quality parameters and biota in experimental earthen vats. The vats were exposed six times with 15-d intervals. The duration of exposure is not clear from the paper. Probably it was a single-event application repeated after 15 days six times. There were no significant changes in water quality (DO, CO_2, alkalinity or pH) after 90 days of the experiment, except at an exposure concentration of 20 μg/L. Significant reduction in zooplankton occurred at all exposure concentrations (5, 10, 16 and 20 μg/L), while the phytoplankton population remained unaffected. Chironomid larvae were significantly affected by exposure concentrations >10 μg/L. Monitoring of CPF degradation was not conducted.

Kersting and van Wijngaarden (1992) studied the effect of CPF on a three-level microecosystem. The three subsystems represented different trophic levels – primary producers (algae), herbivores (*Daphnia magna*), and decomposers (bacteria on a sand filter). In a single species chronic exposure they found no negative effect on survival and number of offspring per female at nominal concentrations of CPF up to 0.1 μg/L. It should be noted that in a separate test it was found that the presence of algae

considerably reduced the residence time of CPF in the test solution (half life of 17 h compared to 2-5 days in the acute test without feeding). Therefore, the authors concluded, that the nominal concentration should be considered as an overestimate of the actual concentrations by about 40%. In a microcosm exposure (4.8μg of CPF was introduced to a 6-L first level subsystem, creating the concentration of CPF in the first level of 0.8 μg/L), there was no measurable impact for two weeks on the algae. However after this, the algal biomass started to fluctuate with a strongly increased amplitude and lower level, when compared to those of controls and the pre-treatment period. Initial concentration of CPF in the second level subsystem was around 0.4 μg/L. There was an immediate effect of CPF on *Daphnia* survival. Though the culture recovered after the perturbation with CPF, the *Daphnia* populations became unstable, showing larger oscillations at lower numbers. This pattern continued through till the end of the study (100 days). These effects were consistent with the pH changes in the subsystems. The authors concluded that the observed drop in pH (2 units) could increase the half-life of CPF by an order of magnitude. They speculate that ecosystems can be changed by a perturbation of CPF in such a way that they do not return to their original states whenever the perturbation ceases. Kersting (1995) found that the effect of pH reduction by two units in tests with CPF was due to acetone used as a vehicle for the insecticide (concentration of acetone was 0.07 ml/L). The decrease started 2 weeks after the introduction, and recovery of pH to the pre-treatment level took several months.

Van den Brink *et al.* (1995) studied the effects of chronic low concentrations of chlorpyrifos (0.1 μg/L) in indoor freshwater microcosms (abundance of a species was an end point). They tested whether the safety factor of 0.1 of $L(E)C_{50}$ is sufficient to

protect aquatic communities in a case of chronic exposure. CPF treatment during seven weeks resulted in primary effects on the zooplankton taxa Cyclopoida and *Daphnia galeata* and the macroinvertebrate *Gammarus pulex*, and secondary effects on the rotifer *Keratella quadrata.* The authors concluded that a safety factor of 0.1 appeared to be insufficient to protect the aquatic community. They suggested that a safety factor of 0.01 to be multiplied by the EC_{50} of the most susceptible standard species (or 0.1 of NOEC) is more appropriate for protection in the case of chronic exposure to CPF. Van den Brink *et al.* (1995) studied further the effect of CPF on invertebrate community responses and recovery in outdoor experimental ditches. Crustacea and Insecta showed a rapid concentration-dependent decrease in numbers after application (direct effects). An increase in gastropods and Oligochaeta was observed, suggesting indirect effects. The invertebrate community at all treatment levels was considered to have recovered 24 weeks post treatment. The recovery was found to be dependent on the susceptibility of the taxa and on ecological characteristics (e.g. the length of the life cycle: the longer life cycle, the more time needed for recovery). Authors derived a NOEC of 0.1 μg/L (concentration at time of application, single event) both at the species and the community level.

Van Wijngaarden *et al.* (1996) studied the effect of single event CPF application on experimental outdoor mesocosms. Nominal initial concentrations of 0.1, 0.9, 6, and 44 μg/L were tested. CPF concentrations showed highest spatial and temporal variation within 2 days of treatment. Acute effects were observed on arthropods only and were manifest on day 0, and negligible at the 0.1-μg/L treatment level. The authors concluded that a safety factor 0.1 (48-h LC_{50} of *Daphnia magna* - 1μg/L) may have protected almost all of the species in the mesocosm community against short-

term direct effects, and a safety factor 0.01 probably protected the most susceptible taxa in their study (laboratory 96-h EC_{10} for *Gammarus pulex*, 0.02 μg/L). The question remains, however (they note), whether long-term (in)direct effects on the populations or the community may occur at the 0.1-μg/L treatment level (see also Table 3.2, entries 36-44).

Rose *et al.* (2001) also determined in laboratory experiments that the presence of fish kairomones increased the sensitivity of cladocerans *Ceriodaphnia* sf. *dubia* to CPF with respect to survival, intrinsic rate of natural increase and reproductive parameters. It appears that in the natural environment the presence of fish (a predator) can result in a greater toxicity of CPF to cladocerans.

Rose *et al.* (2002) found that interaction between algae and daphnia are important in determining toxicity of CPF to daphnia. Limited food significantly increased the toxicity of chlorpyrifos. The authors conclude that the effect of food concentration on toxicity appears to depend on the mechanism by which the chemical exerts its toxicity and on food-chemical interactions.

3.2.6 Bioaccumulation and biomagnification of chlorpyrifos

Deneer (1993) studied the uptake and elimination of CPF in the guppy at sublethal and lethal concentrations. He found that elimination through metabolic transformation was most important when dealing with relatively lipophilic substances (such as CPF), because for these substances the rate of elimination through passive diffusion is rather low. At high exposure levels, metabolic pathways may either become saturated or

inhibited, resulting in reduction of elimination rate (elimination rate coefficient for exposure concentration of 37 μg/L was 0.19 day^{-1}, almost three times less than at an exposure concentration of 0.9 μg/L, which was 0.51 day^{-1}).

However, metabolism probably does not play a very important role in the elimination of CPF in the isopod *Asellus aquaticus* (Montañés *et al.* 1995). Though the elimination rate coefficient at high exposure concentration was 5 times lower than at a low exposure concentration, the uptake coefficient was 10 times lower respectively. The study indicated that isopods bioaccumulate CPF at higher levels than fish, and are therefore suitable for sampling in freshwater biomonitoring programmes to assess the fate and effects of organophosphorus compounds.

Tilak *et al.* (2004) confirmed that after 8-d exposures at concentrations of 20% of the corresponding 96-h LC_{50}, CPF was accumulating in brain (0.11-0.38 mg/g) and liver (0.08-0.09 mg/g) of three species of Indian carp. The authors speculated that biomagnification is also possible as a result of bioconcentration.

Varo et *al.* (2000) conducted a bioconcentration study on a crustacean *Artemia parthenogenetica* and two species of fish and found that CPF had a high accumulation rate in both fish and the crustacean. They suggest that CPF presents a potential risk as it might have a biomagnification ability.

Table 3.2 Chlorpyrifos toxicity to different organisms, concentrations in μg/L unless otherwise specified, nc-nominal concentration, mc-measured concentration

	Chlorpyrifos formulation	Species	Effect measured	Parameter		Reference
				Name	Value	
	1	2	3	4	5	6
ALGAE						
1	Dursban® 4E	Cyanobacterium *Synechoccosus leopoliensis*	Significant culture growth rate difference: Non-limited growth P-limited growth	 Growth reduction Growth reduction	 >10000 >10000	Van Donk *et al., 1992*
2	Dursban®	Marine diatom *Skeletonema costatum*	Cell death	48-h EC_{50}	1200 nc	Walsh, *1983*
3	Dursban® 4E	Freshwater diatom *Cyclotella sp.*	Significant culture growth rate reduction: Non-limited growth P-limited growth	 Growth reduction Growth reduction	 >320 >100	Van Donk *et al., 1992*
4	Dursban® 4E	Green alga *Selenastrum capricornutum*	Significant culture growth rate reduction: Non-limited growth P-limited growth	 Growth reduction Growth stimulation	 >1000 >30	Van Donk *et al., 1992*
INVERTEBRATES						
5	Dursban®4E in tapwater	Oligochaete *Limnodrilus hoffmeisteri*	Movement & breakage	96-h EC_{10} semi-static	>36 mc	Van Wijngaarden *et al., 1993*
6	Dursban	Rotifer *Brachionus calyciflorus*	72-h reproduction asecxual sexual	NOEC LOEC Chronic value (geometric mean of NOEC and LOEC) NOEC LOEC Chronic value (geometric mean of NOEC and LOEC)	300 nc 400 nc 350 nc 200 nc 300 nc 240 nc	Snell & Carmona, *1995*

7	Chlorpyrifos	Worm *Branchiura sowerbyi*	Survival	96-h LC_5 96-h LC_{50} 96-h LC_{95}	3 66 100.4	Amma & Konar, *1996*
8	Chlorpyrifos solution in acetone	Gastropoda *Anisus vortex,* *Bithynia tentaculata,* *Lymnaea stagnalis*	No movement >24 h	96-h EC_{10}	>94 mc	Van Wijngaarden *et al., 1993*
9	Chlorpyrifos	Stonefly *P. badia*	Survival	24-h LC_{50}	4.2	Sanders & Cope, *1968*
10	Chlorpyrifos	Amphipod *Gammarus lacustris*	Survival	96-h LC_{50}	0.11	Sanders, *1969*
11	Chlorpyrifos	Mysid *Americamysis bahia*	Survival	96-h LC_{50}	0.04	Schimmel *et al., 1983*
12	Chlorpyrifos	Crustacean *Mysidopsis bahia*	Survival	96-h LC_{50}	0.035	Schimmel *et al., 1983*
13	Chlorpyrifos	Crayfish *Procambarus clarkii*	Survival	96-h LC_{50}	21.0	Cebrian *et al., 1992*
14	Chlorpyrifos in acetone	Cladoceran *Daphnia magna*	Survival	24-h LC_{50} 48-h LC_{50}	3.7 (2.5-5.9) nc 1.0 (1.0-1.1) nc	Kersting & van Wijngaarden, *1992*
15	Chlorpyrifos	Mysid *Neomysis mercedis*	Survival	96-h LC_{50}	0.15	CDFG, *1993*
16	Chlorpyrifos	Shrimp *Palaemonetes pugio*	Survival	96-h LC_{50}	0.44	Key & Fulton, *1993*
17	Dursban®4E	Cladoceran *Daphnia longispina*	Ability to maintain in suspension	48-h EC_{10} 96-h EC_{10} 48-h EC_{50} 96-h EC_{50}	0.2(0.2-0.3) mc 0.2 mc 0.3(0.3-0.3) mc 0.3 mc	Van Wijngaarden *et al., 1993*
18	Dursban®4E	Cladoceran *Daphnia longispina*	Survival	48-h LC_{10} 96-h LC_{10} 48-h LC_{50} 96-h LC_{50}	0.2(0.2-0.7) mc 0.2 mc 0.8(0.6-1.0) mc 0.3 mc	Van Wijngaarden *et al., 1993*
19	Dursban®4E	Cladoceran *Simocefalus vetulus*	Ability to maintain in suspension	48-h EC_{10} 96-h EC_{10} 48-h EC_{50} 96-h EC_{50}	0.3(0.2-0.4) mc 0.3(0.2-0.4) mc 0.4(0.4-0.5) mc 0.4(0.3-0.5) mc	Van Wijngaarden *et al., 1993*

20	Dursban®4E	Cladoceran *Simocefalus vetulus*	Survival	48-h LC_{10} 96-h LC_{10} 48-h LC_{50} 96-h LC_{50}	0.4(0.3-0.7) mc 0.3(0.2-0.5) mc 0.8(0.7-0.9) mc 0.5(0.4-0.6) mc	Van Wijngaarden *et al., 1993*
21	Chlorpyrifos solution in acetone	Isopoda *Asellus aquaticus*	Response to tactile stimulus	48-h EC_{10} 96-h EC_{10} 48-h EC_{50} 96-h EC_{50}	2.0(1.2-4.3) mc 1.4(1.8-3.0) mc 4.3(3.3-5.6) mc 2.7(2.1-3.6) mc	Van Wijngaarden *et al., 1993*
22	Dursban®4E	Isopoda *Proasellus coxalis*	Response to tactile stimulus	96-h EC_{10}	>20 mc	Van Wijngaarden *et al., 1993*
23	Dursban®4E	Amphipoda *Gammarus pulex*	Survival	48-h LC_{10} 96-h LC_{10} 48-h LC_{50} 96-h LC_{50}	0.03(0.01-0.07) mc 0.02(0.01-0.05) mc 0.08(0.05-0.14) mc 0.07(0.04-0.11) mc	Van Wijngaarden *et al., 1993*
24	Dursban®4E	Heteroptera *Corixa punctata*	Trembling of extremities	48-h EC_{10} 96-h EC_{10} 48-h EC_{50} 96-h EC_{50}	2.2(1.6-3.3) mc 1.0(0.6-2.0) mc 3.2(2.4-4.3) mc 1.7(1.1-2.5) mc	Van Wijngaarden *et al., 1993*
25	Dursban®4E	Heteroptera *Corixa punctata*	Survival	48-h LC_{10} 96-h LC_{10} 48-h LC_{50} 96-h LC_{50}	2.2(1.3-3.8) mc 1.0(0.7-1.6) mc 6.0(4.2-8.5) mc 2.0(1.5-2.6) mc	Van Wijngaarden *et al., 1993*
26	Dursban®4E	Ephemeroptera *Cloeon dipterum*	Response to tactile stimulus	48-h EC_{10} 96-h EC_{10} 48-h EC_{50} 96-h EC_{50}	0.3(0.2-0.4) mc 0.1(0.1-0.2) mc 0.4(0.3-0.4) mc 0.2(0.2-0.2) mc	Van Wijngaarden *et al., 1993*
27	Dursban®4E	Ephemeroptera *Cloeon dipterum*	Survival	48-h LC_{10} 96-h LC_{10} 48-h LC_{50} 96-h LC_{50}	0.3(0.2-0.9) mc 0.1(0.1-0.3) mc 1.0(0.8-1.4) mc 0.3(0.2-0.3) mc	Van Wijngaarden *et al., 1993*
28	Dursban®4E	Ephemeroptera *Caenis horaria*	Response to tactile stimulus	48-h EC_{10} 96-h EC_{10} 48-h EC_{50} 96-h EC_{50}	0.6(0.3-0.9) mc 0.3(0.3-0.6) mc 0.7(0.6-0.8) mc 0.5(0.4-0.6) mc	Van Wijngaarden *et al., 1993*

29	Dursban®4E	Ephemeroptera *Caenis horaria*	Survival	96-h LC_{10}	>3 mc	Van Wijngaarden *et al., 1993*
30	Dursban®4E	Diptera *Chaoborus obscuripes*	Response to tactile stimulus	48-h EC_{10} 96-h EC_{10} 48-h EC_{50} 96-h EC_{50}	0.6(0.4-1.2) mc 0.3(0.2-0.6) mc 1.4(1.1-1.7) mc 0.7(0.6-0.8) mc	Van Wijngaarden *et al., 1993*
31	Dursban®4E	Diptera *Chaoborus obscuripes*	Survival	96-h LC_{10} 96-h LC_{50}	2.5(1.7-7.5) mc 6.6(3.0-14.6) mc	Van Wijngaarden *et al., 1993*
32	Chlorpyrifos	Midge *Chironomus tentans*	Survival	10-d LC_{50}	0.07	Ankley *et al., 1994*
33	Chlorpyrifos	Amphipod *Hyalella azteca*	Survival	10-d LC_{50}	0.086	Phipps *et al., 1995*
34	Chlorpyrifos	Calanoid copepod *Diaptomus forbesi*	Survival	96-h LC_5 96-h LC_{50} 96-h LC_{95}	<0.1 3.6 8.8	Amma & Konar, *1996*
35	Chlorpyrifos	Cladoceran *Ceriodaphnia dubia*	Survival	96-h LC_{50}	0.06 (0.04-0.07)	Bailey *et al, 1996*
36	Dursban® 4E	Mayfly *Cloeon dipterum*	Immobility	Lab 48-h EC_{10} Lab 48-h EC_{50} Lab 96-h EC_{10} Lab 96-h EC_{50} Cage 48-h EC_{10} Cage 48-h EC_{50} Mesocosm EC_{10} Mesocosm EC_{50}	0.3(0.2-0.4) nc 0.4(0.3-0.4) nc 0.1(0.1-0.2) nc 0.2(0.2-0.2) nc 0.1(0.04-0.40) nc 0.4(0.21-0.60) nc 0.2(0.07-0.74) nc 0.3(0.17-0.50) nc	Van Wijngaarden *et al., 1996*
37	Dursban® 4E	Ephemeroptera *Caenis horaria*	Immobility	Lab 96-h EC_{10} Lab 96-h EC_{50} Mesocosm EC_{10} Mesocosm EC_{50}	0.3(0.3-0.6) nc 0.5(0.4-0.5) nc 0.3(0.13-0.54) nc 0.4(0.25-0.50) nc	Van Wijngaarden *et al., 1996*
38	Dursban® 4E	Crustacean *Asellus aquaticus*	Immobility	Lab 48-h EC_{10} Lab 48-h EC_{50} Lab 96-h EC_{10} Lab 96-h EC_{50} Cage 48-h EC_{10} Cage 48-h EC_{50}	2.0(1.2-4.3) nc 4.3(3.3-5.6) nc 1.8(1.4-3.0) nc 2.7(2.1-3.6) nc 1.2(0.56-3.71) nc 3.4(2.22-5.13) nc	Van Wijngaarden *et al., 1996*

39	Dursban® 4E	Crustacean *Gammarus pulex*	Immobility	Cage 48-h EC_{10} Cage 48-h EC_{50}	0.1(0.08-0.34) nc 0.3(0.24-0.45) nc	Van Wijngaarden *et al., 1996*
40	Dursban® 4E	Damselfly *Coenagrionidae spp.*	Immobility	Mesocosm EC_{10} Mesocosm EC_{50}	0.1(0.00-33.3) nc 0.5(0.02-12.8) nc	Van Wijngaarden *et al., 1996*
41	Dursban® 4E	Chironomid *Ablabesmia spp.*	Immobility	Mesocosm EC_{10} Mesocosm EC_{50}	2.7(1.08-7.00) nc 2.8(1.41-5.75) nc	Van Wijngaarden *et al., 1996*
42	Dursban® 4E	Midge *Chaoborus obscuripes*	Immobility	Lab 48-h EC_{10} Lab 48-h EC_{50} Lab 96-h EC_{10} Lab 96-h EC_{50} Cage 48-h EC_{10} Cage 48-h EC_{50} Mesocosm EC_{10} Mesocosm EC_{50}	0.6(0.4-1.2) nc 1.4(1.1-1.7) nc 0.3(0.2-0.6) nc 0.7(0.6-0.8) nc 0.4(0.05-1.96) nc 0.5(0.17-1.60) nc 0.4 0.4	Van Wijngaarden *et al., 1996*
43	Dursban® 4E	Caddisfly *Mystacides spp.*	Immobility	Mesocosm EC_{10} Mesocosm EC_{50}	0.01(0.00-1.98) nc 0.1(0.01-1.01) nc	Van Wijngaarden *et al., 1996*
44	Dursban® 4E	Cladoceran *Simocephalus vetulus*	Immobility	Lab 96-h EC_{10} Lab 96-h EC_{50} Mesocosm EC_{10} Mesocosm EC_{50}	0.3(0.2-0.4) nc 0.4(0.3-0.5) nc 0.3(0.00-23.9) nc 0.6(0.02-16.7) nc	Van Wijngaarden *et al., 1996*
45	Dursban®4E in demineralized water	Cladoceran *Daphnia pulex* (juvenile)	Survival	24-h LC_{50} 48-h LC_{50} 72-h LC_{50} 144-h LC_{50} 192-h LC_{50} 240-h LC_{50}	2.9 nc 0.25 nc 0.23 nc 0.17 nc 0.19 nc 0.17 nc	Van der Hoeven & Gerritsen, *1997*
46	Dursban®4E in demineralized water	Cladoceran *Daphnia pulex* (adult)	Survival	24-h LC_{50} 48-h LC_{50} 72-h LC_{50} 144-h LC_{50} 192-h LC_{50}	>1.6 nc >1.6 nc >1.6 nc 0.48 nc 0.33 nc	Van der Hoeven & Gerritsen, *1997*

47	Dursban®4E in demineralized water	Cladoceran *Daphnia pulex* (juvenile)	Immobilization	24-h EC_{50} 48-h EC_{50} 72-h EC_{50} 144-h EC_{50} 192-h EC_{50} 240-h EC_{50}	0.42 nc 0.21 nc 0.18 nc 0.17 nc 0.19 nc 0.17 nc	Van der Hoeven & Gerritsen, *1997*
48	Dursban®4E in demineralized water	Cladoceran *Daphnia pulex* (adult)	Immobilization	24-h EC_{50} 48-h EC_{50} 72-h EC_{50} 144-h EC_{50} 192-h EC_{50}	1.1 nc 0.80 nc 0.72 nc 0.43 nc 0.28 nc	Van der Hoeven & Gerritsen, *1997*
49	Technical grade chlorpyrifos	Cladoceran *Daphnia pulex* (juvenile)	Survival	24-h LC_{50} 48-h LC_{50}	4.9 nc 0.30 nc	Van der Hoeven & Gerritsen, *1997*
50	Technical grade chlorpyrifos	Cladoceran *Daphnia pulex* (juvenile)	Immobilization	24-h EC_{50} 48-h EC_{50}	0.42 nc 0.25 nc	Van der Hoeven & Gerritsen, *1997*
51	Chlorpyrifos	Amphipod *Hyalella azteca*	Survival	48-h LC_{50}	0.1	Moore *et al., 1998*
52	Chlorpyrifos	Midge *Chironomus tentans*	Survival	48-h LC_{50}	0.3	Moore *et al., 1998*
53	Lorsban™	Cladoceran *Daphnia magna*	Survival	48-h LC_{50}	06±0.04	Moore *et al., 1998*
54	Lorsban™	Amphipod *Hyalella azteca*	Survival	48-h LC_{50}	0.1±0.04	Moore *et al., 1998*
55	Lorsban™	Midge *Chironomus tentans*	Survival	48-h LC_{50}	0.3±0.07	Moore *et al., 1998*
56	Chlorpyrifos solution in acetone	Mysid *Neomysis interger*	Survival	96-h LC_{50} : semi-static flow-through (adults) flow-through (juvenile)	 0.15 (0.08-0.31) nc 0.13 (0.11-0.16) nc 0.19 (0.16-0.23) nc	Roast *et al., 1999*
57	Chlorpyrifos	Crustacean *Artemia parthenogenetica*	Survival: Nauplii Juveniles Adults	24-h LC_{50}	 >18000 nc 3900±900 nc 80±10 nc	Varo *et al., 2000*

58	Dursban™	Cladoceran *Daphnia magna*	Survival	48-h LC_{50}	0.42 nc	Schulz, *2001*
59	Dursban™	Amphipod *Paramelita nigroculus*	Survival	24-h LC_{50}	0.9 (0.3-1.6) nc	Schulz, *2001*
60	Chlorpyrifos in acetone	Brine shrimp *Artemia salina*	Survival	24-h LC_{50}	3190(1350-6340) nc	Varo *et al.*, 2002
61	Chlorpyrifos in acetone	Brine shrimp *Artemia parthenogenetica*	Survival	24-h LC_{50}	>18000 nc	Varo *et al., 2002*
62	Dursban XP	Cladoceran *Ceriodaphnia dubia*	Survival	48-h LC_{50}	0.056 (0.054-0.059) mc	Harmon *et al, 2003*
63	Dursban XP	Cladoceran *Daphnia ambigua*	Survival	48-h LC_{50}	0.035 (0.032-0.037) mc	Harmon *et al, 2003*
64	Technical grade chlorpyrifos in acetone	Cladoceran *Ceriodaphnia dubia*	Survival	48-h LC_{50}	0.05 (0.048-0.053)	El-Merhibi *et al., 2004*
65	Chlorpyrifos in ethanol	Cladoceran *Daphnia magna*	Immobilization AchE activity	48-h EC_{50} 48-h IC_{50}	3.14 (2.62-3.71) pM 1.40±0.06 pM	Printes & Callaghan, *2004*
AMPHIBIANS						
66	Dursban®4E	Newt *Triturus vulgaris*	Locomotional behaviour	96-h EC_{10}	>96 mc	Van Wijngaarden *et al., 1993*
67	Technical grade chlorpyrifos in acetone	Clawed frog *Xenopus laevis*	Survival Malformation AchE activity reduction	96-h LC_{50} 10-d LC_{50} 96-h EC_{50} 10-d EC_{50} 96-h EC_{50} 10-d EC_{50}	2410 (2135-2722) 511 92.5 35 ~25 ~50	El-Merhibi *et al., 2004*
FISH						
68	Dursban™	Rainbow trout *Oncorhynchus mykiss*	Survival	96-h LC_{50}	9 nc	Schulz, *2001*
69	Chlorpyrifos	Rainbow trout (1.4 g)	Survival	96-h LC_{50}	7.1	Macek *et al., 1969*

70	Chlorpyrifos	Fish *Morone saxalitis*	Survival	96-h LC_{50}	0.58	Korn & Earnest, *1974*
71	Chlorpyrifos	Fish *Fundulus heteroclitus*	Survival	96-h LC_{50}	4.7	Thirugnanam & Forgash, *1977*
72	Chlorpyrifos	Channel catfish (0.8 g)	Survival	96-h LC_{50}	280	Johnson & Finley, *1980*
73	Chlorpyrifos	Fish *Mugil cephalus*	Survival	96-h LC_{50}	5.4	Schimmel *et al., 1983*
74	Chlorpyrifos	Sheepshead minnows *Cyprinodon variegates* (juvenile)	Survival	96-h LC_{50} (flow-through system)	136 (113-153)	Clark *et al., 1985*
75	Chlorpyrifos	Atlantic silverside *Menidia menidia* (juvenile)	Survival	96-h LC_{50} (flow-through system)	1.7 (1.4-2.0)	Clark *et al., 1985*
76	Chlorpyrifos	Tidewater silverside *Menidia peninsulae* (juvenile)	Survival	96-h LC_{50} (flow-through system)	1.3 (1.0-1.7)	Clark *et al., 1985*
77	Chlorpyrifos	Inland silver side *Menidia beryllina* (juvenile)	Survival	96-h LC_{50} (flow-through system)	4.2 (3.4-5.4)	Clark *et al., 1985*
78	Chlorpyrifos	California grunion *Leuresthes tenuis* (juvenile)	Survival	96-h LC_{50} (flow-through system)	1.3 (1.0-1.7)	Clark *et al., 1985*
79	Chlorpyrifos	Gulf toadfish *Opsanus beta* (juvenile)	Survival	96-h LC_{50} (flow-through system)	520 (450-600)	Clark *et al., 1985*
80	Chlorpyrifos in acetone	Freshwater catfish *Heteropneustes fossilis*	Kidney histopathology	Shrinkage of glomerular tuft Vacuolation of blood cells in the glomerular tuft Dilation of the lumina of the renal tubules	72-h exposure to 2mg/L of CP	Srivastava *et al., 1990*

81	Chlorpyrifos in acetone	Eel *Anguilla anguilla*	Survival	24-h LC_{50} 48-h LC_{50} 72-h LC_{50} 96-h LC_{50}	1290 (1110-1670) 690 (560-900) 590 (480-710) 540 (420-650)	Ferrando *et al., 1991*
82	Dursban®4E	Gasterosteiformes *Gastrosteus aculeatus*	Survival	48-h LC_{10} 96-h LC_{10} 48-h LC_{50} 96-h LC_{50}	4.5(2.3-13.7) mc 3.8(2.0-9.2) mc 13.4(9.0-19.9) mc 8.5(6.2-11.9) mc	Van Wijngaarden *et al., 1993*
83	Chlorpyrifos solution in acetone	Gasterosteiformes *Pungitius pungitius*	Survival	48-h LC_{10} 96-h LC_{10} 48-h LC_{50} 96-h LC_{50}	2.3(1.3-5.5) mc 2.1(1.3-4.6) mc 5.7(4.4-7.5) mc 4.7(3.6-6.0) mc	Van Wijngaarden *et al., 1993*
84	Chlorpyrifos	Fish *Oreochromis mossambicus*	Survival	96-h LC_5 96-h LC_{50} 96-h LC_{95}	1 52 129	Amma & Konar, *1996*
85	Chlorpyrifos	Fish *Oreochromis mossambicus*	Respiratory rate	Opercular movement significant reduction	10	Amma & Konar, *1996*
86	Chlorpyrifos	Fish *Oreochromis mossambicus*	Feeding rate		<5	Amma & Konar, *1996*
87	Chlorpyrifos	Fish *Oreochromis mossambicus*	Behaviour	Lethargy Number of nests significant reduction	10 10	Amma & Konar, *1996*
88	Chlorpyrifos	Fish *Oreochromis mossambicus*	Growth significant reduction		<5	Amma & Konar, *1996*
89	Chlorpyrifos	Fish *Oreochromis mossambicus*	Reproduction	Condition factor Fecundity	10 10	Amma & Konar, *1996*

90	Chlorpyrifos	Fish *Oreochromis mossambicus*	Histopathology	Vacuolation and swelling of liver cells Nuclear swelling and disintegration Abnormal morphology of liver cells Abnormal morphology of uriniferous tubules Breakage of intestinal villi Breakage of gill lamellae Thickening of gill lamellae Disintegration of lamellar epithelium	10 10 20 16 20 5 16 20	Amma & Konar, *1996*
91	Lorsban™	Fathead minnow *Pimephales promelas*	Survival	48-h LC_{50}	162.7±13.7	Moore *et al., 1998*
92	Chlorpyrifos	Mosquitofish *Gambusia affinis*	Survival	48-h LC_{50} 72-h LC_{50} 96-h LC_{50}	520±60 nc 540±50 nc 520±50 nc	Varo *et al., 2000*
93	Chlorpyrifos	Fartet *Aphanius iberus*	Survival	48-h LC_{50} 72-h LC_{50}	38.6±7.2 nc 18.01±0.02 nc	Varo *et al., 2000*
94	Lorsban	Guppy *Poecilla reticulata*	Survival Malformations Paralysis Hemorrhage	96-h LC_{50} 14-d EC_{50} 14-d EC_{50} 14-d EC_{50}	7.17 1.0 between 0.5 & 1.0 between 1.0 & 2.0	De Silva & Samayawardhena, *2002*
95	Technical grade chlorpyrifos in acetone	Rainbowfish *Melanotaenia splendida splendida*	Survival: Eggs prior fertilizing Eggs after fertilizing 16-d old larvae adults	 96-h LC_{50} 96-h LC_{50} 96-h LC_{50} 96-h LC_{50}	 23 2019 117 396	Humphrey & Klumpp, *2003*
96	Chlorpyrifos	Tilapia *Oreochromis mossambicus*	Survival	96-h LC_{50}	25.97 (19.7-32.0)	Rao *et al., 2003*

97	Technical grade chlorpyrifos in acetone	Indian carp *Catla catla*	Survival: Static Flow-through 	 24-h LC_{50} 48-h LC_{50} 72-h LC_{50} 96-h LC_{50} 24-h LC_{50} 48-h LC_{50} 72-h LC_{50} 96-h LC_{50}	 510 460 420 350 460 380 350 300	Tilak *et al., 2004*
98	Technical grade chlorpyrifos in acetone	Indian carp *Labeo rohita*	Survival Static Flow-through 	 24-h LC_{50} 48-h LC_{50} 72-h LC_{50} 96-h LC_{50} 24-h LC_{50} 48-h LC_{50} 72-h LC_{50} 96-h LC_{50}	 740 660 560 470 580 480 400 300	Tilak *et al., 2004*
99	Technical grade chlorpyrifos in acetone	Indian carp *Cirrihinus mrigala*	Survival Static Flow-through 	 24-h LC_{50} 48-h LC_{50} 72-h LC_{50} 96-h LC_{50} 24-h LC_{50} 48-h LC_{50} 72-h LC_{50} 96-h LC_{50}	 940 840 760 650 850 450 660 550	Tilak *et al., 2004*
100	Lorsban	Mosquitofish *Gambusia yucatana*	Survival Muscle AchE activity	96-h LC_{50} 96-h EC_{50}	11 (8-17) 11 (8-14)	Rendon-von Osten *et al., 2005*

CHAPTER 4

MAINTENANCE OF TEST ORGANISMS

4.1 MAINTENANCE OF ALGAL CULTURES

SUMMARY

Three species of algae were used in various experiments and for maintenance of *D. carinata* cultures: *Chlorella vulgaris, Chlorella pyrenoidosa* and *Pseudokirchneriella subcapitata*. Batch cultures were used for maintenance of the algae, grown in flasks on a light-table. Sub-culturing was conducted once a week or as required. Two media were used for maintenance of these cultures: Tamiya (Vasser 1989) and Keating (1985). The recipes for media preparation are contained in Appendix 2.

4.1.1 Test species

Three species of unicellular freshwater green algae were used in the study: *Chlorella vulgaris* (Fig. 4.1.1) was obtained from North Carolina, USA, *Chlorella pyrenoidosa* (Fig. 4.1.2) from Southern Biological (Nunawading, Victoria, Australia) and *Pseudokirchneriella subcapitata* (former *Selenastrum capricornutum*) (Fig. 4.1.3) was obtained from Michael J. Barry (then Victoria University, St. Albans, Victoria, Australia). All three species are representatives of Australian freshwaters (Day *et al*. 1995), and two of three species were collected locally in Victoria, Australia.

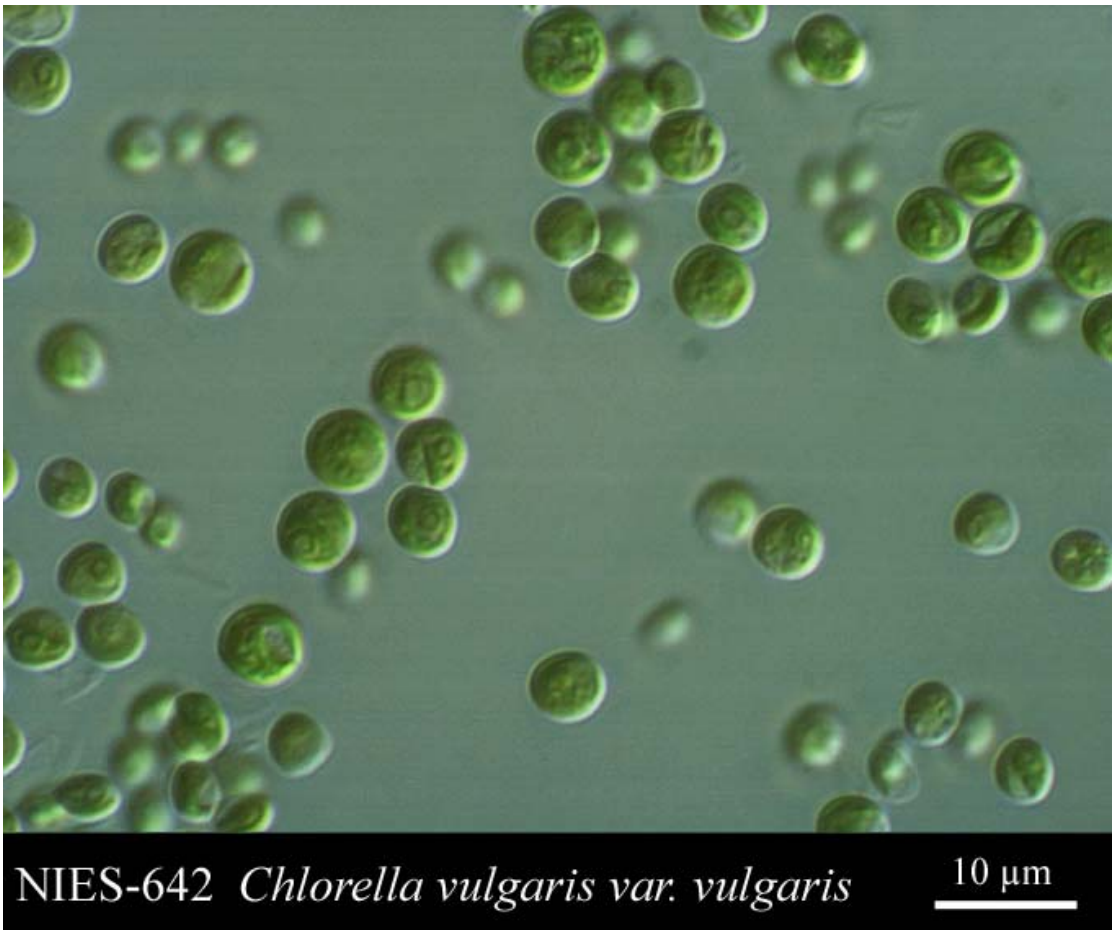

Figure 4.1.1 Cells of *Chlorella vulgaris.*

Image from http://shigen.lab.nig.ac.jp/algae/images/strainsimage/nies-0642.jpg.

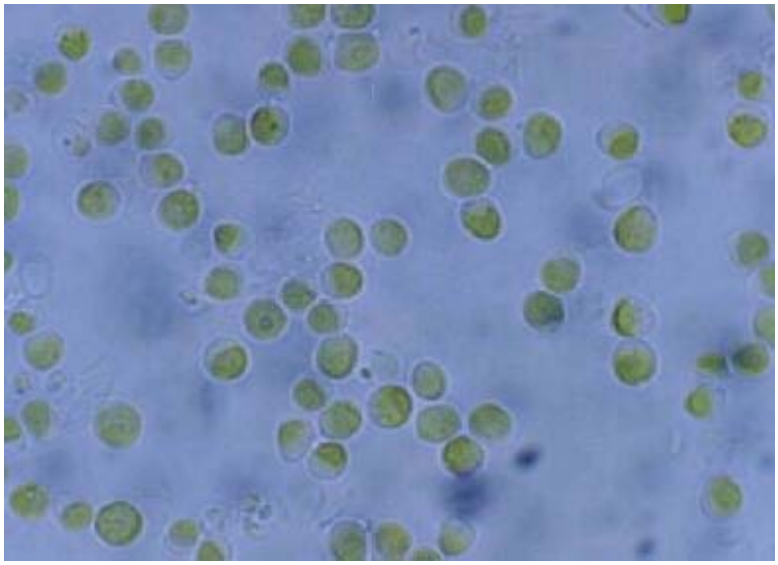

Figure 4.1.2 Cells of *Chlorella pyrenoidosa.* Image from

http://www1.ocn.ne.jp/~bio-soci/chlorella.htm.

Figure 4.1.3 Cells of *Pseudokirchneriella subcapitata*.

Image from http://shigen.lab.nig.ac.jp/algae/images/strainsimage/nies-0035.jpg.

4.1.2 Methods

Algae were cultured in cotton-stoppered 500-mL conical flasks with 300 mL of medium on a light table (luminosity 3000±10 lux, continuous (no photoperiod) measured with a flat collector, temperature 25±1^{o}C). Cultures were aerated continuously with filtered air. The setup for culturing algae is presented in Fig. 4.1.4.

The algal cultures were sub-cultured once a week. An aliquot of 10 mL of culture from the exponential phase of growth was taken and placed in a sterile medium in a laminar flow cabinet to minimize contamination. While there would likely always have been a small amount of bacteria present in the cultures, the purity of a single species culture was maintained throughout the study.

Chlorella sp were maintained in Tamiya medium (Vasser 1989), as this medium was well-suited for these species (see Appendix 1 for recipe). However later for the purpose of feeding *Daphnia*, *C. pyrenoidosa* was also cultured in Keating (1985) algal medium. For maintenance of *P. subcapitata* also Keating algal medium was used (see Appendix 1 for recipe).

When algae were used to feed animals, after culturing, the flasks were placed into a refrigerator for algae to settle overnight, then the supernatant was discarded and a dense culture of algae from the exponential stage of growth fed to *Daphnia* as required. When there was no requirement for algae for an extended period of time, cultures were kept in a refrigerator at 4^{o}C as dense culture or paste, and later reconstituted and sub-cultured as necessary.

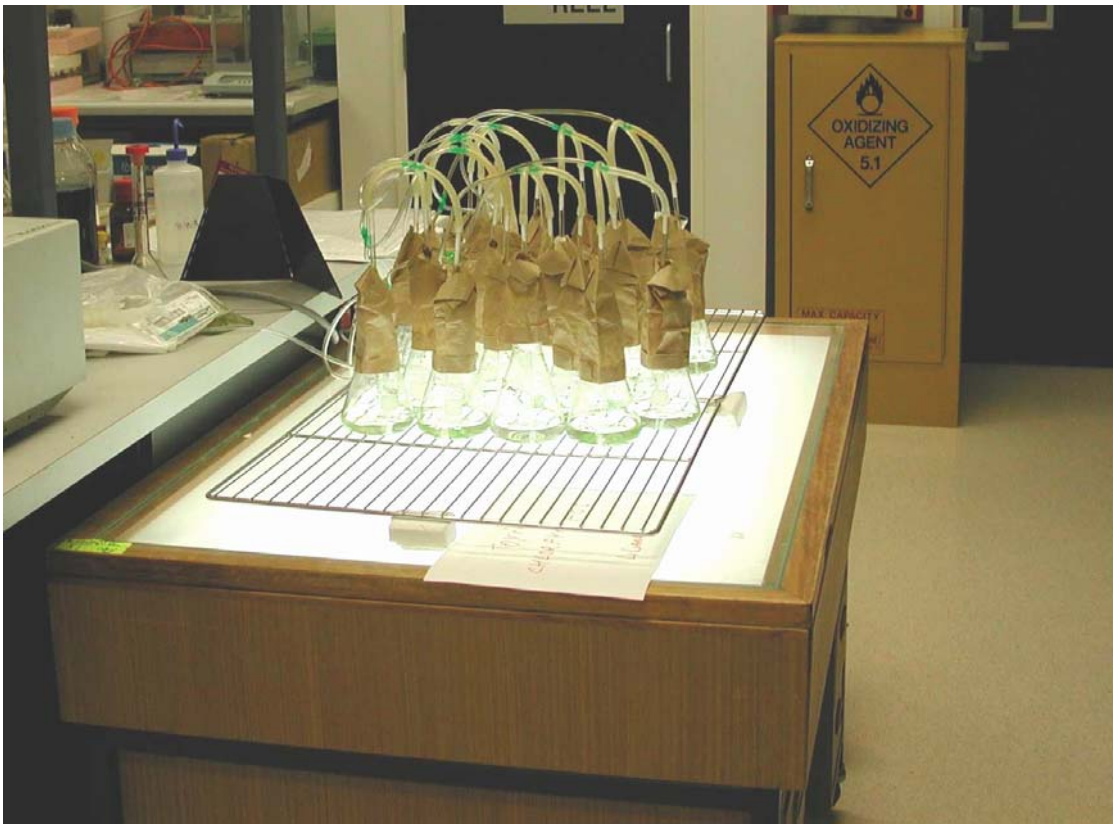

Figure 4.1.4 Setup for culturing algae on a light table.

4.2 MAINTENANCE OF *DAPHNIA CARINATA* CULTURE FOR USE IN TOXICITY TESTING

Published as:

Maintenance of *Daphnia carinata* culture for use in toxicity testing.

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Australasian Journal of Ecotoxicology (2004) **10:** 65-69.

SUMMARY

A growing concern for use of local species for toxicity testing leads to the need to create the toxicity data banks for these species. In order to have healthy animals to conduct the tests, procedures for maintenance of stock cultures of these species should be developed. The most widespread daphnid in Australia, *Daphnia carinata*, is considered to be one of the most suitable for toxicity testing of contaminants entering Australian freshwaters. However very little data is available on the culture requirements of the species. In this study different types of food were tested: *Chlorella vulgaris* cultured in two different media - Keating and Tamiya, *Chlorella pyrenoidosa* cultured in the same two media, and a suspension of trout pellets. Intrinsic rates of natural increase of individual cultures of *D. carinata* were determined from "life tables". The best food from among those tested were *C. pyrenoidosa* cultured in either Keating or Tamiya medium. Two different procedures of individual cultures are proposed for the maintenance of *D. carinata* for use in toxicity testing, based on tests conducted using different culture volumes.

4.2.1 Introduction

Cladocerans of *Daphnia* species have been widely used for many years as test organisms for toxicity testing under laboratory conditions. Different guidelines for testing of chemicals are used in different countries (US EPA, OECD Guidelines, National standards etc.), and *Daphnia magna* and *Daphnia pulex* are among the most frequently used species in the northern hemisphere. A considerable amount of toxicity data is available on these species together with requirements for their cultures (Lee *et al*. 1985, Elendt 1990a, 1990b, Elendt & Bias 1990). In Australia and South-East Asia the most abundant and widely distributed daphnid is *Daphnia carinata* (Benzie 1988). A growing concern for use of local species in ecotoxicology creates an agenda for establishing toxicity data for such species. In Australia *Ceriodaphnia dubia* is commonly used for toxicity testing (Rose *et al.* 2004, Warne and Schifko 1999, NSW EPA 1999, 2003a). However very little data is available not only on toxicity testing, but also on the culture and requirements of the common Australian cladoceran *D. carinata* (NSW EPA 2003b). In our study we tested several experimental procedures in order to choose the most suitable among them (in terms of medium, volume requirements and food type) for culturing *D. carinata* for use in toxicity testing.

4.2.2 Methods

Our experiments consisted of three parts:

1. Growth rates of algae in two culture media
2. Effects of different diets on daphnid survival and reproduction
3. Effects of culture volume on daphnid survival and reproduction

Experiment 1. **Growth rates of algae in two culture media**

Two species of freshwater green unicellular alga *Chlorella vulgaris* and *Chlorella pyrenoidosa* were cultured in order to determine their growth rates. Two different media were used for culturing each of the species: (a) Keating MS medium with low concentrations of nutrients and pH=8.0 (Keating 1985) and (b) Tamiya medium with high concentrations of nutrients and pH=5.0 (cited in Vasser 1989).

Algae were cultured axenically in cotton-stoppered 250-mL conical flasks on a light table (luminosity 3000±10 lux, continuous (no photoperiod), temperature 25±1^{o}C). Cultures were aerated continuously using aquarium air pumps. There were four replicates of each culture (16 total). Cell density was counted every six hours for 60 hours, using a haemocytometer. On each occasion four subsamples of 0.01 cm^3 were taken from each replicate and all algal cells in the grid were counted.

Growth rates of the cultures (μ) were calculated as

$$\mu=(\ln N_t - \ln N_{t-1})/\Delta t$$

where N_t is algal cell density at time t,

N_{t-1} is algal cell density at time of previous observation,

Δt is time between the observations.

Algae from the exponential phase of growth were then used as food for daphnids (along with trout pellets) in a feeding trial.

Experiment 2. **Effects of different diets on daphnid survival and reproduction.**

Female neonates of *D. carinata* (< 24 hours of age) were placed individually into 28-mL McCartney bottles with 25 mL of medium in each. The medium was carbon-

filtered tap water with 0.5 g/L of scientific grade sea salt "Coralife" (Coralife Scientific Grade Marine salt, Energy Savers Unlimited, Inc, Carson, CA USA) (nitrates and phosphates free). The temperature of the media was 25±1^{o}C, pH=7.0±0.1, luminosity was maintained at 500 lux at daytime, photoperiod 15 h day/ 9 h night. Twenty animals were tested for each type of food. The experiment was conducted for 21 days. Medium was replaced daily, daphnids were fed with algae or trout pellets once a day. Concentration of algae was 2×10^5 cells/mL when algae were added to the culture. The solution of trout pellets was prepared as follows (on recommendation from ARI, see acknowledgments): 20 g of trout pellets were ground and suspended in 150 mL of distilled water, then filtered 3 times using a fine strainer (pore size 140 μm). The suspension was then stored in the refrigerator for up to three weeks and used for the feeding trials. Daphnids were fed daily with 10 μL of the suspension. Survival and fecundity of females were recorded and the intrinsic rate of natural increase was computed using the Lotka formula (Lotka 1913):

$$\Sigma\, l_x m_x e^{-rx}=1,$$

where l_x is the proportion of individuals surviving to age x,

m_x is the age specific fecundity (number of females produced per surviving female at age x),

x is days

The type of food that produced the best results in terms of intrinsic rate of natural increase was then used as food in the test of different culture volumes.

Experiment 3. **Effects of culture volume on daphnid survival and reproduction**

Individual culture of *D. carinata* was proposed as an alternative to the use of cohorts for toxicity testing recommended by OECD (1996). In the chronic (21-day) toxicity test, according to the OECD guidelines, the volume of media provided per female should not be less than 40 mL (for *D. magna*), and it is recommended that the medium be replaced every other day (for a cohort of 10 animals). However, the issue of preference of individual culture over the use of cohorts of daphnids in toxicity testing has been widely discussed (Sims and van Dijk 1996). According to these researchers there is no difference in statistical power between the OECD-recommended procedure and testing using individual culture of daphnids. However, biological information obtained from individual culture is greater than can be obtained from cohort culture, since individual daphnids could not be identified in the cohort. Therefore two types of individual culture were investigated:

- Individual culture of *D. carinata* in 25 mL of medium with daily feeding and daily replacement of medium.
- Individual culture of *D. carinata* in 75 mL of medium with daily feeding and replacement of medium every alternate day.

Conditions and end-points were as in Experiment 2. The aim of these experiments was to check if both of these conditions satisfy the requirements for long-term toxicity testing using daphnia, i.e. not more than 20% mortality and not less than 25 offspring per female 14 days of age (OECD, 1996).

Statistics

Data derived from the experiments (algal growth rate, body length of females, time to the first brood, number of offspring per female) were analysed in paired comparisons

using analysis of variance. The mean value of intrinsic rate of natural increase and its error were determined using a jackknife approach as described by Taberner *et al.* (1993).

4.2.3 Results and discussion

Experiment 1. **Growth rates of algae in two culture media**

The highest growth rate was recorded for *C. pyrenoidosa* cultured in Keating medium - 0.129 h^{-1}. *C. pyrenoidosa* also had a higher growth rate in Tamiya medium (0.102 h^{-1}) than *C. vulgaris* - 0.068 h^{-1} (Table 4.2.1).

The higher growth rates obtained in Keating medium are the results of the following effect. *C. pyrenoidosa* stock culture was maintained in Tamiya medium and then taken for the experiment and placed in the two different media. Though the cell size of *C. pyrenoidosa* is reported to be 3-6 μm (Bellinger 1992), we observed cells up to 20 μm in diameter in Tamiya medium. It seemed that cells were not dividing, but accumulating biomass. When placed in Keating medium these cells multiplied by producing up to 8 new small (3-6 μm) cells at a time. This indicates that cell density is not always an accurate measure of growth. We noted that though growth rates of *C. vulgaris* were also higher when cultured in Keating medium than in Tamiya medium, the enlarged cells were not observed.

Table 4.2.1 Comparison of growth rates (μ, h^{-1}) of *C. vulgaris* and *C. pyrenoidosa* cultured in different growth media. Mean±SE (n=4).

C. pyrenoidosa (in Keating medium)	*C. pyrenoidosa* (in Tamiya medium)	*C. vulgaris* (in Keating medium)	*C. vulgaris* (in Tamiya medium)
0.129 ± 0.023^{a}	0.102 ± 0.027^{a}	0.075 ± 0.006^{b}	0.068 ± 0.003^{b}

Superscripts *a* and *b* indicate significant ($p<0.05$) differences between values.

Experiment 2. **Effect of different diets on daphnid survival and reproduction**

Survival and fecundity was higher for daphnids fed with *C. pyrenoidosa*, than those fed with *C. vulgaris* or trout pellets (Table 4.2.2, Fig. 4.2.1). In daphnids fed on *C. pyrenoidosa* (cultured in Tamiya medium) total mortality was lower than 20 % as recommended by the OECD (1996), while for those fed with the same algae cultured in Keating medium, mortality was only 10 %. After 21 days, survival of daphnids fed with *C. vulgaris* cultured in Keating medium was 35 % with production of very few offspring. There was 100 % mortality in those fed with *C. vulgaris* grown in Tamiya medium by the 13th day of the experiment, and they failed to reproduce. This could be a result of the antibiotic chlorellin, produced by *C. vulgaris* (Pratt and Fong 1940, Pratt *et al.* 1945). This antibiotic is reported to adversely affect the feeding rate of daphnids (Ryther 1954). As we can see from Fig. 4.2.1, feeding on *C. vulgaris* affects to a greater degree the juvenile stages of daphnids, compared to animals fed with *C. pyrenoidosa*, reducing their survival. The feeding trial with trout pellets produced some offspring, but all daphnids had died by the 18th day of the experiment. This confirms the work of previous researchers who have recommended that daphnids be fed with live algae rather than solely on trout pellets (Cowgill 1989, Sergy 1990), because trout pellets lack essential nutrients.

Tong et al. (1996) suggest that instead of the intrinsic rate of natural increase (r) calculated over a 21-day experiment, one can use 14-day values. They argue that the first three broods mostly contribute to this value. However, in our experiments, 14-day and 21-day values differ in some cases (*C. vilgaris* in Keating medium and trout pellets suspension, Table 4.2.2), and this leads to underestimation of r-values in 14-day computations. These errors can lead to crucial mistakes in modelling.

Table 4.2.2 Comparison of end points of individual culture of *D. carinata* fed with different types of food. Mean±SE (n=20).

Endpoint	Food provided				
	C. pyrenoidosa (in Tamiya medium)	*C. pyrenoidosa* (in Keating medium)	*C. vulgaris* (in Tamiya medium)	*C. vulgaris* (in Keating medium)	Trout pellets suspension
Time to the 1st brood (days)	8.1±0.2[a]	6.6±0.2[b]	--	11.7±1.6[c]	11.5±1.1[c]
Total number of offspring per female	69±4[a]	42±4[b]	_	7±1[c]	5±1[c]
Intrinsic rate of natural increase after 21 days (day^{-1})	0.279±0.012[a]	0.287±0.012[a]	NA	0.047±0.025[b]	0.024±0.038[b]
Intrinsic rate of natural increase after 14 days (day^{-1})	0.260±0.013[a]	0.274±0.013[a]	NA	-0.045±0.042[b]	-0.001±0.055[b]

Superscripts a, b and c indicate significant ($p<0.05$) differences between values for each endpoint on any given row.

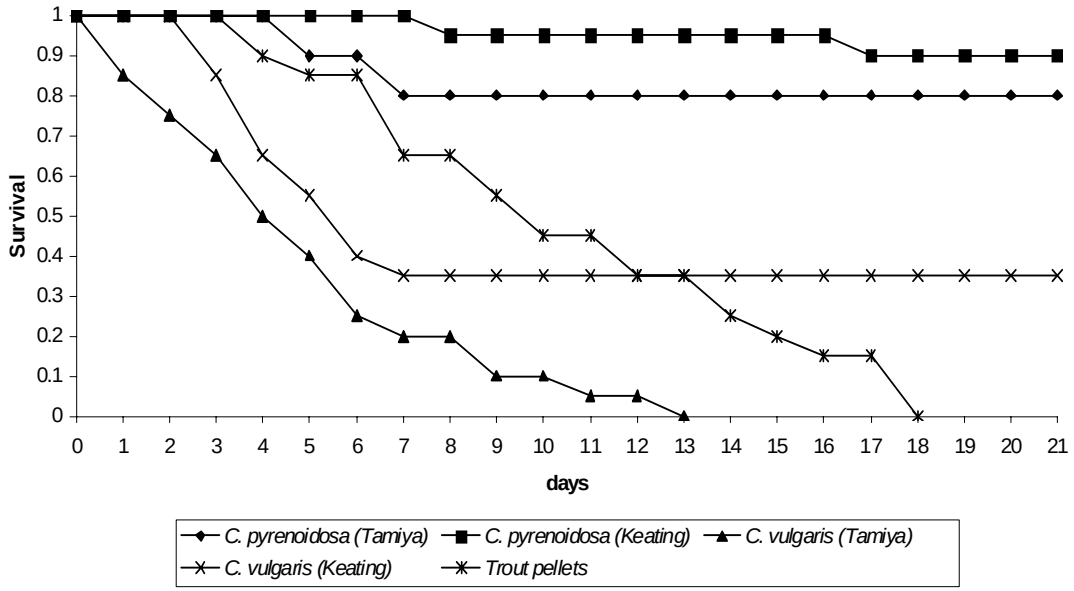

Figure 4.2.1 Survival of *D. carinata* fed with different types of food (experiment 2).

The time to the first brood is supposed to be the most significant contributing factor in *r*-value, however, it is not the only one determining it. Though the time to the first brood is significantly different for *C. pyrenoidosa* cases (columns 2 and 3 of Table 4.2.2), their *r*-values for both 14 and 21 days are not. Significantly different total number of offspring per female nullified the differences in the time to the first brood. As we showed, the length of the test is also important for *r*-value. When the length is increased from 14 to 21 days, this changes the *r*-value markedly in some cases - from negative (meaning that population is eliminated) to positive (indicating some growth) (Table 4.2.2, two last columns).

Though the *r*-value is highest with *C. pyrenoidosa* (Keating)-fed animals, the mean number of offspring produced per female was highest with *C. pyrenoidosa* (Tamiya)-fed animals (69 compared to 42 in the case of those fed *C. pyrenoidosa* (Keating)). It should be noted that the number of offspring was chosen as an endpoint (not the biomass produced by a female), because a certain number of offspring over 14 days is a requirement for long-term toxicity testing (OECD, 1996). Based on these results *C. pyrenoidosa* cultured in Tamiya medium was used as the food for daphnids in later experiments. In addition the recommended pH of the culture medium for *C. pyrenoidosa* is 5.0, therefore Tamiya medium is the preferred medium for culturing *C. pyrenoidosa* (Myers 1947).

Experiment 3. **Effects of culture volume on daphnid survival and reproduction**

Standard 21-day observations showed that there were no significant ($P>0.05$) differences between all calculated values (Table 4.2.3) for both experimental procedures.

However in the 75-mL beakers mortality was higher (10%), than in the 25-mL vials (0%). Our observation showed that the antennae of daphnids in beakers were clogged with algae, when they died. The test vessels could not be aerated (daphnids do not tolerate vigorous agitation), and algae settled on the bottom (after 24 h algal concentrations reduced from 2×10^5 to 2×10^4 cells/mL). On the days when the media were not replaced, new algae had to be mixed with those that settled. This could cause overfeeding (despite attempts to minimise this) and consequent clogging of daphnids' antennae, leading to reduction of the feeding rate.

Though 25 mL per female is less than the volume of medium recommended by OECD (1996), with daily replacement of medium it is possible to achieve virtually the same (if not better) results than with the conditions recommended by the OECD (1996). Moreover 40 mL is recommended for *D. magna*, which is larger than *D. carinata* and thus would produce more metabolites. It is possible, as demonstrated in this experiment, that the volume of medium used for individual culture be reduced. Also in toxicity tests with rapidly degrading chemicals it is preferable that the medium be replaced every day in order to maintain a constant concentration of the pollutant. In addition small vials are easier to handle, and they require less algae, water and chemicals, making toxicity testing less expensive. There are, however, some negative issues potentially associated with the use of smaller volumes, which should be considered when conducting a toxicity test. Changes in the surface-to- volume ratio of containers can change the rate of adsorption of chemicals on the walls of a container, thus affecting the amount of toxicant in the medium. Replacing the medium every day

Table 4.2.3 Comparison of end points for *D. carinata* cultured in different volumes of medium. Mean±SE (n=20). There were no significant differences between treatments.

End points	Volume of medium	
	25 mL	75 mL
Time to the 1st brood, days	8.2±0.2	7.8±0.3
Total number of offspring per female	60±4	56±3
Body length of females after 21 day, mm	3.72±0.04	3.61±0.07
Intrinsic rate of natural increase (over 21 days), day^{-1}	0.300±0.007	0.298±0.007
Intrinsic rate of natural increase (over 14 days), day^{-1}	0.283±0.009	0.282±0.007

can help alleviate this problem. Also the absorption rate depends on the chemical, and this should be taken into account while conducting a toxicity test.

4.2.4 Conclusions

This study has developed some improved conditions for the culture and maintenance of *D. carinata* for use in toxicity testing. It was shown that *C. pyrenoidosa* cultured in either Tamiya or Keating medium can be used successfully as food for *D. carinata*, while trout pellets are not recommended for prolonged use as the only food for *D. carinata*. The volume of culture medium can be reduced from the 40 mL per female recommended by the OECD (1996) to 25 mL per female for *D. carinata* without compromising the quality of daphnid culture (in terms of survival and reproduction), in order to conduct toxicity testing.

This paper also demonstrated that individual culture provides a significant number of different endpoints that cannot be measured in cohorts. We also recommend that when using *D. carinata* in individual culture, the number of animals be reduced from the OECD recommended 40 (4 cohorts of 10 animals) to 20 or 15.

CHAPTER 5

EFFECT OF GLYPHOSATE (TECHNICAL GRADE AND ROUNDUP BIACTIVE) AND CHLORPYRIFOS ON FRESHWATER ALGAE *CHLORELLA PYRENOIDOSA* AND *PSEUDOKIRCHNERIELLA SUBCAPITATA*

SUMMARY

A series of 72-h toxicity tests were conducted to determine the effects of two formulations of the herbicide glyphosate (technical grade and Roundup Biactive®) and the insecticide chlorpyrifos on the growth of two unicellular freshwater algae *Chlorella pyrenoidosa* and *Pseudokirchneriella subcapitata.* The EC_{50} values for all toxicants for both species of algae were determined. With glyphosate and Roundup Biactive® the 72-h EC_{50} values were: *C. pyrenoidosa* = 788 and 763 mg/L, and *P. subcapitata* = 429 and 397 mg/L, while hormesis was observed when *P. subcapitata* was exposed at concentrations equal to 7% and 4% of the EC_{50} respectively. No such effect was noted for *C. pyrenoidosa,* although it is possible that this effect may be present at very low concentrations, which were not tested in this study. For chlorpyrifos the 72-h EC_{50} was well above environmentally realistic concentrations for both algae (3736 for *C. pyrenoidosa* and 2060 μg/L for *P. subcapitata*). However at concentrations 0.3-5 μg/L (with a maximum at 0.06% of EC_{50}) hormesis was observed for both species, where growth rate exceeded that of control by as much as 20% for *C. pyrenoidosa* and 40% for *P. subcapitata*. *P. subcapitata* was more

sensitive to all toxicants tested, and we recommend it as a test species for pesticides in preference to *C. pyrenoidosa.*

5.1 Introduction

Application of pesticides in agriculture can create potentially hazardous situations in the aquatic environment due to run offs and sometimes the method of pesticide application (spray drift off or direct water application as in aquatic weed control (Hartley and Kidd 1990)). There is a need to study the effects of pesticides on water ecosystems because of their increased load in inland waters. Algae are the primary producers in the ecosystems. If they are affected by the pesticides, the balance within the ecosystem could change (Hanazato and Kasai 1994, McCormick and Cairns 1994). Algae respond fast to pollutants, thus providing a convenient early warning signal of disturbance and its possible causes (McCormick and Cairns 1994). Most ecotoxicological studies of effects of agrochemicals on algae in laboratory-based experiments measure various inhibitory effects, such as photosynthesis (Hernando *et al.* 1989), culture growth reduction (Maule and Wright 1984, Peterson *et al.* 1994; Ferrando *et al.* 1996a), macromolecular synthesis (Nyström and Blanck 1997) or accumulation (Ferrando *et al.* 1996b). Only few studies reported effects other than inhibitory without discussing them in detail (Gardner *et al.* 1997 effects of triclopyr and glyphosate on *Ankistrodesmus*). Wong and Chang (1988) found that at low concentrations 2,4-D and six organophosphorus insecticides stimulated photosynthesis of *Chlamydomonas reinhardtii*, and 2,4-D and fenitothion also stimulated algal growth and chlorophyll *a* synthesis. Based on these results, not only

adverse effects of pesticides (such as decrease of growth) on algae should be noted, but also the effects producing increased growth, as this could lead to algal blooms.

The present study investigated the effects of the herbicide glyphosate and the insecticide chlorpyrifos on growth of two unicellular freshwater algae *Chlorella pyrenoidosa* and *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) at a range of concentrations including those reported in aquatic systems.

5.2 Materials and methods

5.2.1 Maintenance of algal cultures.

C. pyrenoidosa and *P. subcapitata* require different conditions for growth in terms of media composition and pH. For *C. pyrenoidosa* Tamiya medium was used (Vasser 1989), with pH 5.0 as an optimum for this species (Myers 1947). For *P. subcapitata* Keating MS medium was used (Keating 1985) with pH 8.0 as optimum for this species. Both cultures were grown axenically on a light table. The algae were cultured in conical 500 mL flasks with 400 mL of media in them. The luminosity was continuous at 1865±38 lux, measured with a flat collector, temperature was 24±1°C, and continuous aeration with filtered air was provided. Once a week an aliquot of 10 mL was transferred to a new medium and subcultured. The rest of the cultures were put in the refrigerator to settle overnight. The settled cells were harvested for further use.

5.2.2 Test chemicals

5.2.2.1 Glyphosate

Two types of glyphosate – technical grade with concentration of 551 g/L of active ingredient, and Roundup Biactive with glyphosate concentration of 336 g/L were obtained from Monsanto (batch # K554 for technical grade glyphosate, and batch # 728408 for Roundup Biactive).

Glyphosate is a broad spectrum, non-selective systemic post-emergence herbicide. It inhibits the activity of 5-enolpyruvyl shikimic acid-3-phosphate (EPSP) synthase, an enzyme of the shikimic acid pathway (Duke 1988), affecting aromatic amino acid synthesis, and consequently, protein synthesis and growth in plants. It is considered non-persistent in the environment, because it is biodegraded by soil and water micro-organisms (Duke 1988). The minimum half-life observed in the aquatic environments was two weeks, while in static natural waters it was 7-10 weeks (Sáenz *et al.* 1997).

5.2.2.2 Chlorpyrifos

Chlorpyrifos Pestanal® (O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)-phosphorothioate) was obtained from Riedel-de-Haën Laborchemikalien GmbH (purity 99.6%).

Chlorpyrifos is a broad-spectrum organophosphorothioate insecticide that is used against a variety of insect and arthropod pests in agriculture, industry and residences (Barron and Woodburn 1995). Chlorpyrifos is an acetylcholinesterase inhibitor, and its toxicity to target species is well documented (see review by Barron and Woodburn 1995). Its toxicity can vary a million-fold across species depending on species differences in behaviour, feeding pattern and pharmacokinetics (Marshall and Roberts 1978).

Chlorpyrifos dissipates in water with a half-life from 16 to 72 days in laboratory experiments (hydrolysis) and 30-52 days (photolysis) (Racke 1993). However, in natural environments a half-life as short as 0.08-2.4 days in the water column has been reported (Racke 1993).

Chlorpyrifos is virtually insoluble in water, so a stock solution of 100 g/L was prepared in acetone. A series of successive dilutions in acetone (analytical grade) was used for preparation of the experimental concentrations of chlorpyrifos.

5.2.3 Experimental protocol

The algal growth test was performed according to OECD guidelines for testing of chemicals (OECD 1996). Three replicates of each treatment concentration and control(s) were used in the test. All tests were conducted in 250-mL cotton-plugged Erlenmeyer flasks with 100 mL of medium. The physical conditions were as described for algal culturing. In order to control any influence of the solvent used to dissolve chlorpyrifos, an acetone control was also used in the chlorpyrifos tests with the same concentration of acetone as found in the highest treatment.

All concentrations of chemicals were nominal, with concentrations of technical grade glyphosate and Roundup Biactive expressed as concentrations of active ingredient.

Cell density was counted at the beginning of experiments and after 72 hours, using a haemocytometer. On each occasion four sub samples of 0.01 cm^3 were taken from

each replicate and all algal cells in the grid of the haemocytometer (but not less than 400) were counted.

Growth rates of the cultures (μ) were calculated as

$$\mu=(\ln N_t - \ln N_0)/\Delta t$$

where N_t is algal cell density at time t,

N_0 is algal cell density at the beginning of the experiment,

Δt is time between the observations in days

Some experiments consisted of several series of tests. If the concentration of test chemical was too low to have any effect on the growth rate, another set of treatments with higher concentrations was used (Figs. 5.4, 5.5, 5.7). If several tests were performed, the combined curve of growth rate vs. concentration of the chemical was plotted. In this case the growth rate was calculated as proportion of control (Figs. 5.2, 5.4d, 5.6). Though the same conditions were used for each toxicity test, tests were not conducted simultaneously. Water quality parameters were measured at the start and at the end of the exposure, and they were the same and within the optimal range for the species. No reference toxicants were used in the tests.

5.2.4 Statistical analysis

The 72-h EC_{50} were determined by Probit analysis (SPSS). Comparison of treatments to control was performed pair wise using Student t-test (P=0.05).

5.3 Results

5.3.1 Glyphosate

Both technical grade glyphosate and Roundup Biactive had very low toxicity to both algae with the EC_{50} for *P. subcapitata* lower than for *C. pyrenoidosa* (Table 5.1). The values for EC_{50} for *C. pyrenoidosa* were in accordance with those previously reported (Anton *et al.* 1993). *P. subcapitata* had an increased growth (hormesis) around 20% of control at concentrations of 7% (around 30 mg/L) and 4% (16 mg/L) for technical grade glyphosate (Fig. 5.1) and Roundup Biactive (Fig. 5.2) respectively. No hormesis was observed for *C. pyrenoidosa* (Fig. 5.3, 5.4), however, concentrations below 30 mg/L of technical grade glyphosate were not tested.

5.3.2 Chlorpyrifos

Chlorpyrifos had a very low toxicity to both species of algae. The EC_{50} was 3736 (3100 – 4584) µg/L for *C. pyrenoidosa* and 2060 (1580 – 2816) µg/L for *P. subcapitata* (Table 5.1). Both species of algae had an increase in growth compared to control at concentrations of around 0.06% of 72-h EC_{50} (Fig. 5.5-5.8). This is around 1.8 µg/L for *C. pyrenoidosa* and 1.2 µg/L for *P. subcapitata.* Hormesis was significant in *C. pyrenoidosa* – around 20% relative to control (Fig. 5.6) and even more pronounced in *P. subcapitata* – over 40% (Fig. 5.8).

Acetone at concentrations used in our experiments did not have measurable negative or positive effects on the growth of both algae (Fig. 5.5-5.8).

Table 5.1 The 72-h EC_{50} values of three pesticides for two species of algae tested.

In brackets are 95% confidence intervals.

Pesticide tested	*Chlorella pyrenoidosa*	*Pseudokirchneriella subcapitata*
Chlorpyrifos (μg/L)	3736 (3100 – 4584) *	2060 (1580 – 2816) *
Glyphosate (technical grade) (mg/L)	788 (343 – 2545)	429 (229 – 970) *
Roundup Biactive (mg/L)	763 (436 – 1587)	397 (203 – 934) *

(*) indicates hormesis at low concentrations.

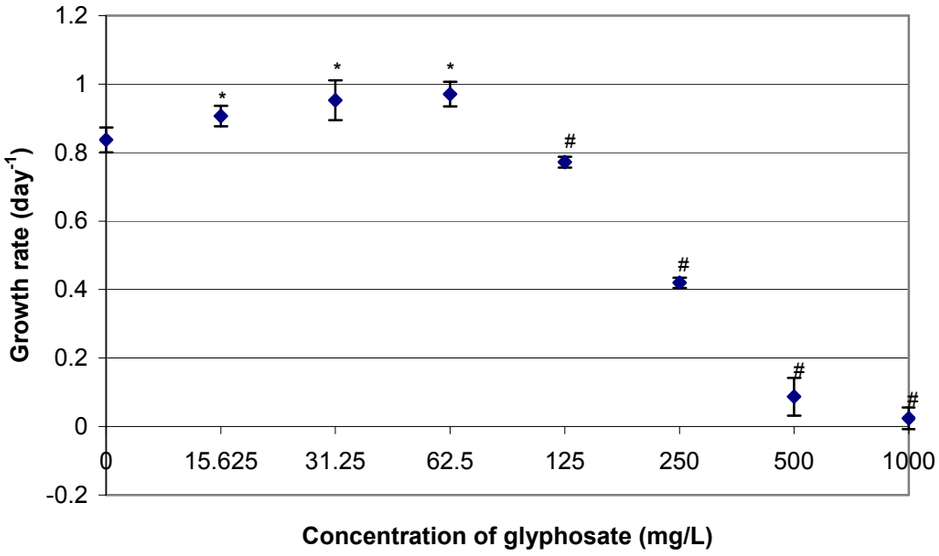

Figure 5.1 Growth rate of *P. subcapitata* exposed to different concentrations of glyphosate (technical grade). Mean±SE, N=3; * - significantly higher, than control, # - significantly lower, than control.

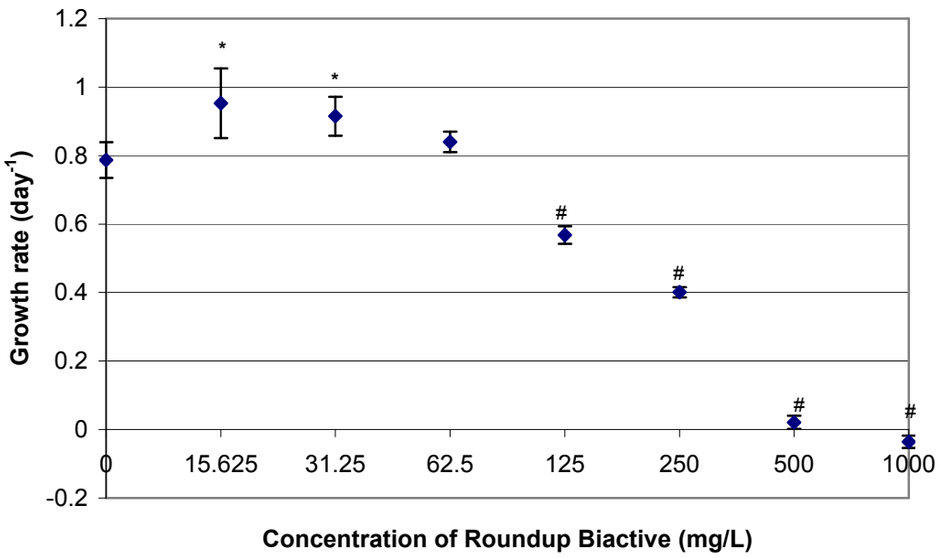

Figure 5.2 Growth rate of *P. subcapitata* exposed to different concentrations of glyphosate (as active ingredient in Roundup Biactive). * - significantly higher than control, # - significantly lower than control.

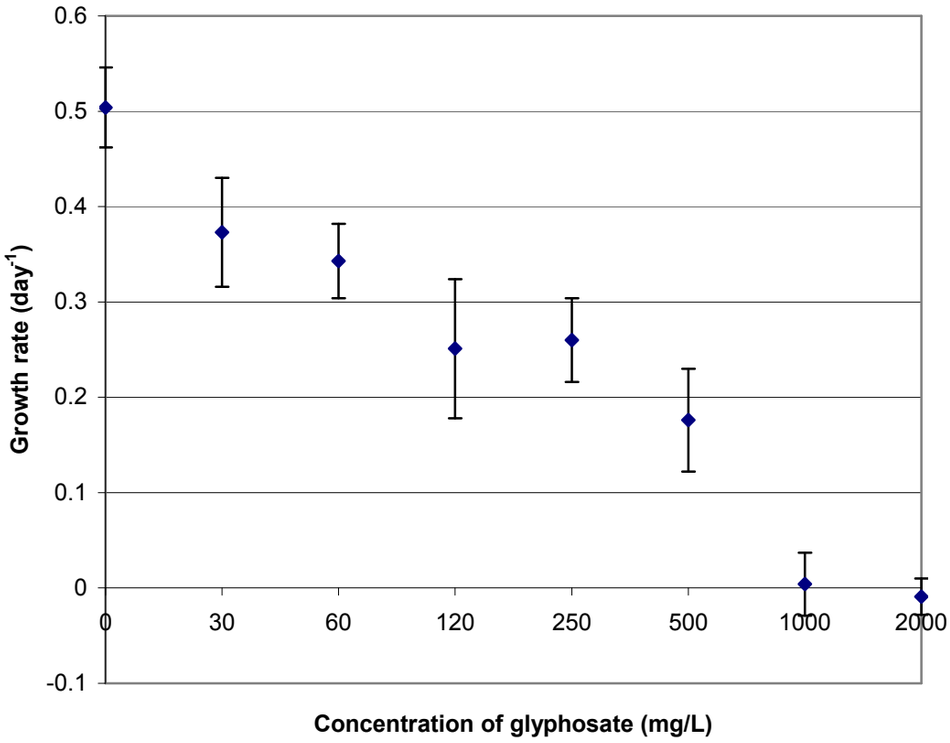

Figure 5.3 Growth rate of *C. pyrenoidosa* exposed to different concentrations of glyphosate (technical grade). Mean±SE, N=3.

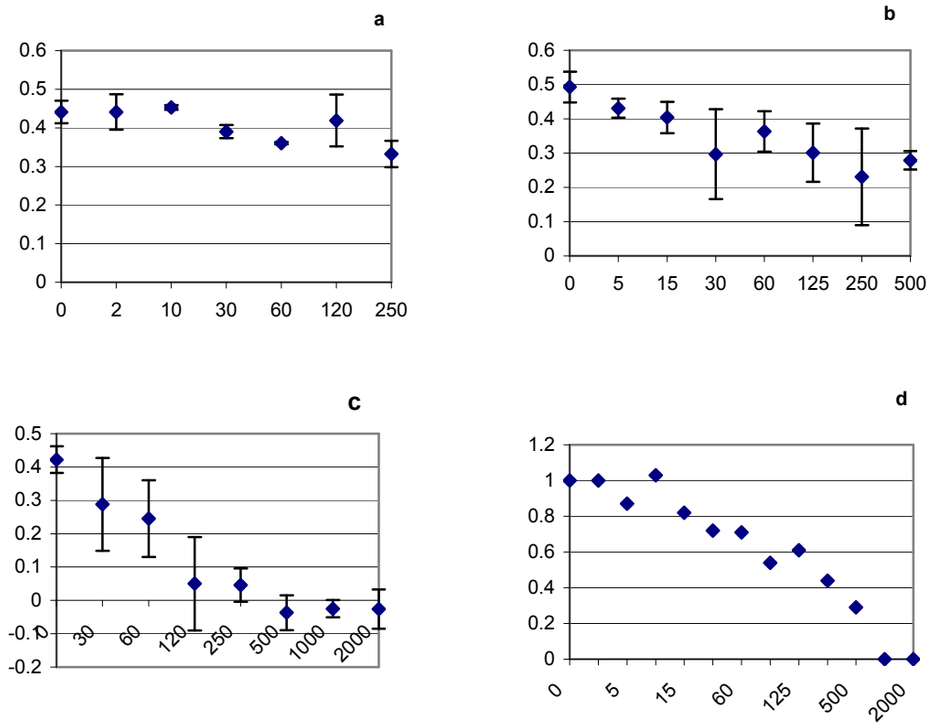

Figure 5.4 Growth rate of *C. pyrenoidosa* exposed to different concentrations of glyphosate (as active ingredient in Roundup Biactive). Mean±SE, N=3;

a – series 1, b – series 2, c – series 3, d – as proportion of control (all series combined). *X*-axis is concentration of Roundup Biactive (mg/L a.i.), *Y*-axis is growth rate (day^{-1}) (a-c) and (proportion of control) (d).

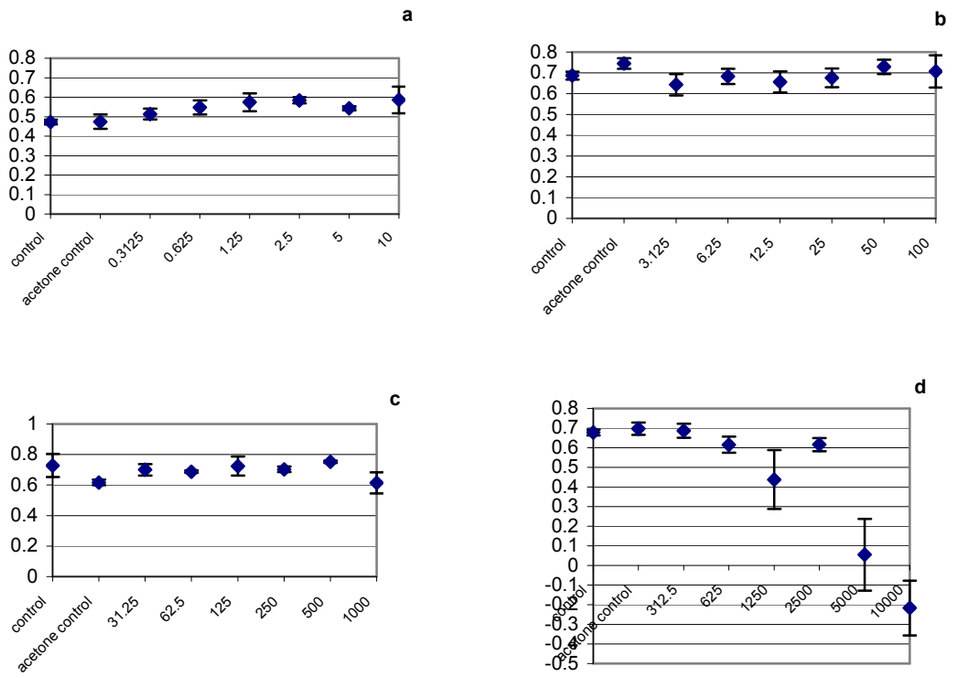

Figure 5.5 Growth rate of *C. pyrenoidosa* exposed to different concentrations of chlorpyrifos. Mean±SE, N=3; a - series 1, b – series 2, c – series 3, d – series 4. *X*-axis is concentration of chlorpyrifos (µg/L), *Y*-axis is growth rate (day^{-1}).

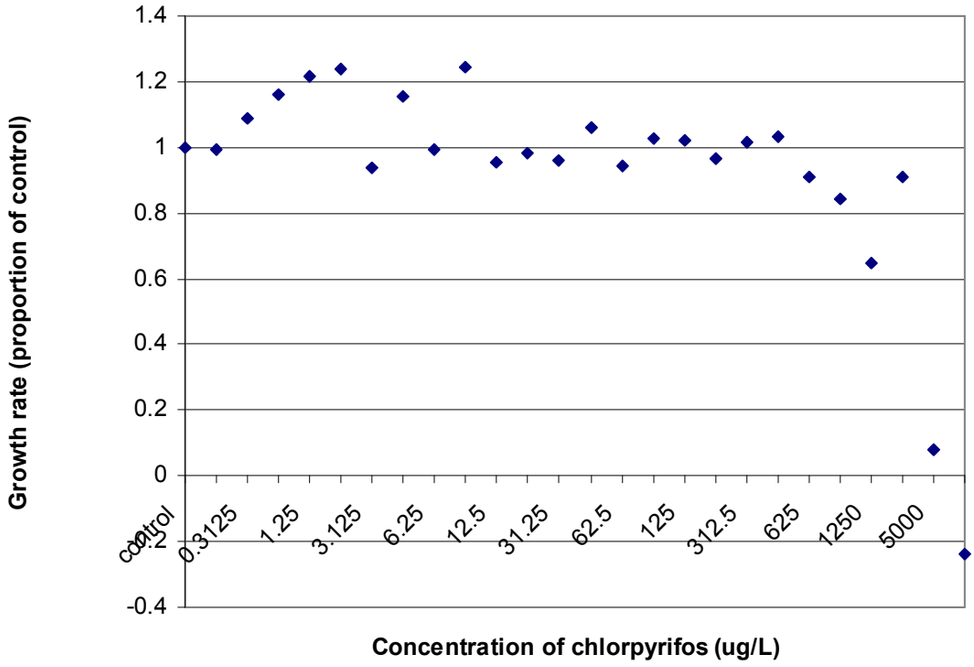

Figure 5.6 Growth rate of *C. pyrenoidosa* exposed to different concentrations of chlorpyrifos as proportion of control. All series combined.

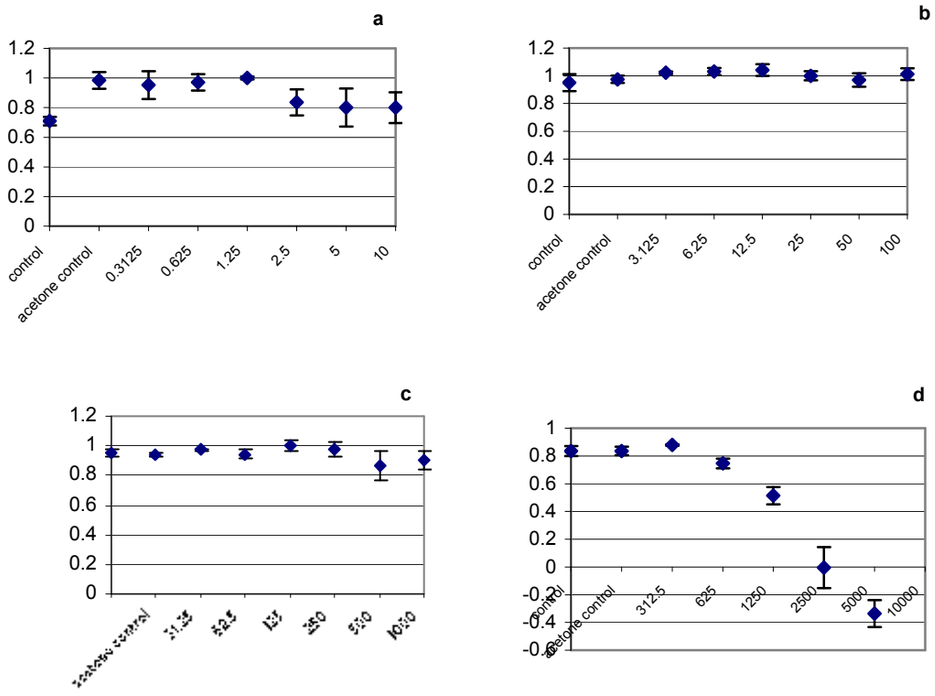

Figure 5.7 Growth rate of *P. subcapitata* exposed to different concentrations of chlorpyrifos. Mean±SE, N=3; a – series 1, b – series 2, c – series 3, d – series 4. *X*-axis is concentration of chlorpyrifos (µg/L), *Y*-axis is growth rate (day^{-1}).

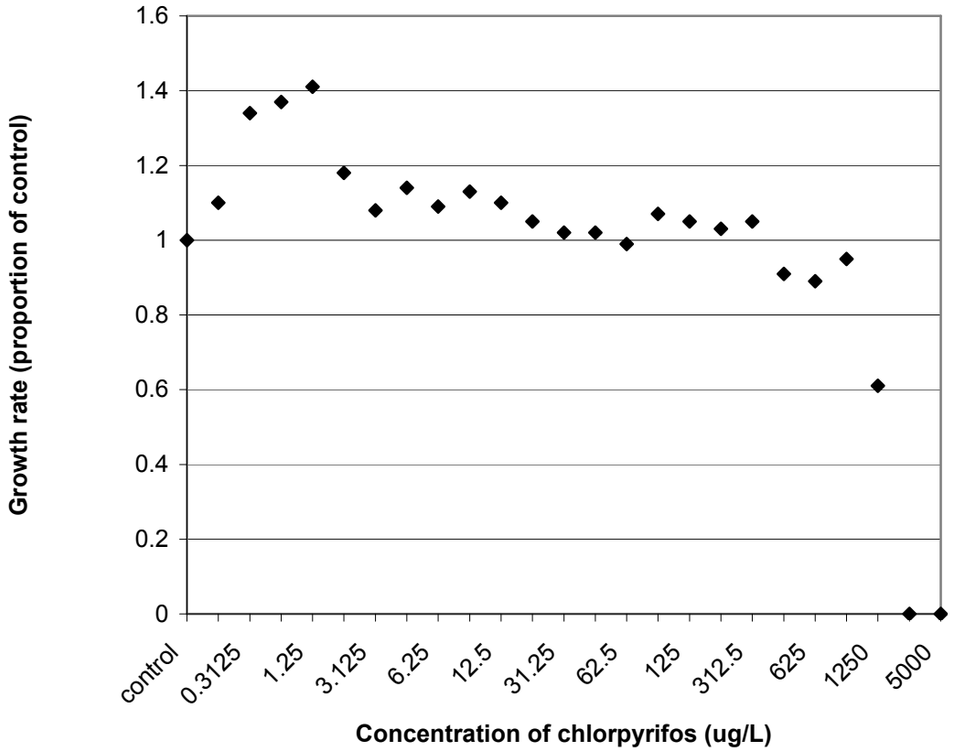

Figure 5.8 Growth rate of *P. subcapitata* exposed to different concentrations of chlorpyrifos as proportion of control. All series combined.

5.4 Discussion

P. subcapitata was more sensitive to all pesticides tested. All EC_{50} values for this alga were almost half of those for *C. pyrenoidosa* (Table 5.1). Glyphosate is generally considered to be practically non-toxic to algae. Peterson *et al.* (1994) found that among 2 species of green algae, 2 species of diatoms, 5 species of cyanobacteria and duckweed only diatoms and one cyanobacterium showed sensitivity to glyphosate. Maule and Write (1984) reported EC_{50} values for culture growth between 68 mg/L (*Chlorococcum hypnosporum*) and 590 mg/L (*Chlorella pyrenoidosa*). In the present study both formulations of glyphosate had a similar toxicity (Table 5.1). The surfactant present in the Roundup formulation of glyphosate is generally more toxic than the active ingredient to animals (Wan *et al.* 1989, Servizi *et al.* 1987). However from our results it appears not to have a negative effect on algae – the growth rates for both algae were not significantly different (Table 5.1). Sáenz *et al.* (1997) also found that 96-h EC_{50} of technical grade and glyphosate formulation Ron-do were not significantly different for two *Scenedesmus* species. However, EC_{50} values reported for *Selenastrum capricornutum* (now *P. subcapitata*) were as low as 2.6-8 mg/L of Roundup and 22-485 mg/L for glyphosate (Giesy *et al.* 2000). Some researchers reported that glyphosate was not toxic to microalgae, and in some cases even produced a stimulatory effect (Thomas *et al.* 1986), though the nature of this effect was not understood. Schaffer and Sebetich (2004) found that the primary productivity of four algal species assemblage increased by 161-168 % relative to control when treated with 0.125-12.5 mg/L of glyphosate. They suggested that this effect was due to release of nitrogen and phosphorus through the degradation of glyphosate, however, the observed stimulation could not be solely attributed to glyphosate

availability (Schaffer and Sebetich 2004). Shikha and Singh (2004) reported a twofold increase in Hill activity (photosynthetic electron transport) in *Anabaena doliolum* when exposed to 50-200 mg/L of glyphosate, and an even greater increase in the rate of oxygen evolution (2-305 times of that in control). Our results also demonstrated an increased growth in *P. subcapitata* cultures when exposed to both technical grade glyphosate and Roundup Biactive at a concentration of around 5% of the EC_{50}.

Information on the effects of pesticides on the ecology of algae is scarce (Gardner *et al.* 1997). Judging from the data available regarding, for instance, glyphosate toxicity to algae (Hess 1980, Christy *et al.* 1981, Maule and Wright 1984, Sáenz *et al.* 1997, Gardner *et al.* 1997, Pipe 1992) one can conclude that a great variation exists in EC_{50} values for different species of algae, ranging from 2 to 590 mg/L of active ingredient. Barron and Woodburn (1995) reported that algal species exhibit large species differences in sensitivity to chlorpyrifos (>100 fold). Ma and Liang (2001) tested 12 herbicides using *C. pyrenoidosa* and *Scenedesmus obliqus* and found differences in sensitivities of these species, which could vary more than tenfold for some herbicides. This means that in the environment when a multiple species algal community is affected by a pesticide, the biodiversity of this community can be greatly affected as a result of different responses of single species to a particular pesticide, leading to a change in a community structure. Butcher *et al.* (1977) observed a replacement of the predominant algal species *Mougeotia* sp. with *Chlorella* sp. in artificial pond communities exposed to chlorpyrifos. The exposures also resulted in greater algal abundance and more persistent algal blooms compared to control ponds. Differences in the EC_{50} of chlorpyrifos for the two algae in our study also add weight to the hypothesis that changes in algal community structure can result from exposure to

pesticides. This in turn can cause shifts in the consumer communities because of their preferences for a particular food type. Zalizniak and Nugegoda (2004) showed that the cladoceran *Daphnia carinata* performed better when fed with *C. pyrenoidosa* than with *Chlorella vulgaris*. Yet our further studies of daphnid cultures have shown that the cladocerans grew better when fed with *P. subcapitata* than with either *Chlorella* species (personal observations). If *P. subcapitata* (being a more sensitive species compared to *C. pyrenoidosa*) were negatively affected in a natural ecosystem, *D. carinata* (and possibly other cladocerans species) would also be negatively affected, changing the balance in species abundances and community structure.

Our results confirmed that chlorpyrifos had a very low toxicity to both species of algae studied, with EC_{50} values (Table 5.1) much higher than those observed in the environment (the highest reported in the literature is 3.7 μg/L (Wood and Stark 2002)). Approximately the same order EC_{50} values were reported for freshwater (7.8 mg/L for *C. vulgaris*, Nikolenko and Amirkhanov 1993; 5 mg/L for *Anabaena* sp., Lal *et al.* 1987) and marine algae (1.2 mg/L for *Skeletonema costatum,* Walsh 1983). Concentrations of more than several microgram per litre are very rarely observed in the environment and for a short time only. In the present study increased algal culture growth was observed at concentrations consistent with that found in the environment (Braun and Frank 1980, Holladay *et al.* 1996). Hormesis was significant in both algae at low chlorpyrifos exposure concentrations (Fig. 5.6 and 5.8). The same effect was earlier observed by van Donk *et al.* (1992) in a P-limited medium, where chlorpyrifos acted as an additional source of phosphorus. In our study, however, phosphorus was not limiting, and the mechanism of this effect is not clear. Birmingham and Colman (1977) reported an increase of up to 20% in growth of freshwater algae *Anabaena*

flos-aquae and *Clamydomonas reinhardtii* exposed to chlorpyrifos, which may have been a result of the algae taking up chlorpyrifos. Zalizniak and Nugegoda (2006) reported that *Daphnia carinata* performed better in the presence of *P. subcapitata* than without it. Rose *et al.* (2002) also found that low food levels caused a significant increase in the toxicity of chlorpyrifos to *Ceriodaphnia* cf. *dubia.* This suggests that there could have been an uptake of chlorpyrifos by algae, reducing the available concentration of the insecticide to daphnids. A similar effect was reported by Karen *et al.* (1998) with the freshwater microphyte *Elodea densa* accumulating chlorpyrifos from the water column. In a microcosm study Van den Brink *et al.* (1995) reported an increase in the algal cell numbers in microcosms four weeks after treating with chlorpyrifos for a number of species when compared with controls. Though the differences were not always statistically significant, 7 species out of 17 showed an increase in biomass compared to control.

Hormesis in algal cultures has important environmental implications. With exposures to environmentally realistic low concentrations of pesticides (when hormesis usually occurs) increased growth can result in algal blooms. In conjunction with the negative effects of the same concentrations of pesticide on algal consumers, the structure of an ecosystem may be greatly affected. Hanazato and Kasai (1994) studied the effects of another organophosphorus insecticide, fenthion, on experimental pond communities and reported that fenthion induced an increase in the density of rotifers and phytoplankton, while suppressing the cladoceran populations. They concluded that this was a secondary effect of the chemical, however, as we have demonstrated in this study with chlorpyrifos, exposure to organophosphates can directly result in a significant increase in the production of some algal species.

5.5 Conclusions

The pesticides glyphosate and chlorpyrifos are generally considered to be non-toxic to microalgae, at least at environmentally realistic concentrations. However, our study showed an increased growth in algal cultures exposed to low concentrations of these agrochemicals. This indicates the potential for algal blooms, if and when low concentrations of these pesticides reach the aquatic environment. We suggest that hormesis should be taken into account when considering the effects of low concentrations of agrochemicals. More research is required in this area, as previous studies have mainly examined toxic effects of pesticides at higher concentrations, disregarding such effects as hormesis, which can lead to significant environmental consequences.

CHAPTER 6

EFFECT OF CHLORPYRIFOS ON THREE SUCCESSIVE GENERATIONS OF *DAPHNIA CARINATA*

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Effect of sublethal concentrations of chlorpyrifos on three successive generations of *Daphnia carinata.*

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SUMMARY

Effects of sublethal concentrations of chlorpyrifos (ranging from 0.005 µg/L ('0.01 LC_{50}') to 0.500 µg/L ('1 LC_{50}')) on population characteristics of individual cultures of *Daphnia carinata* were investigated over 21 days with subsequent testing of the two next generations. The endpoints for the first and second generations observed were: survival, fecundity, time to first brood, size of the females after 21-day exposure and number of offspring per female. The results were incorporated into the computation of the intrinsic rate of natural increase for daphnids in each of the treatments. Exposure to chlorpyrifos affected survival and fecundity of animals in the first generation. In the second generation the most affected endpoint was time to the first brood with an indication of hormesis. Acute LC_{50} tests were then conducted using animals of the third generation from each of the exposures in individual tests. Despite the absence of a negative effect of chlorpyrifos in the second generation, results of testing the third generation showed constant significant decline in LC_{50} values from control daphnids through to '0.1 LC_{50}' pre-exposed daphnids

('0.1 LC_{50}', or 0.05 µg/L being the highest concentration in which animals survived exposure to the toxicant in the second generation).

6.1 Introduction

The water flea *Daphnia* (Cladocera) is one of the most abundant zooplankton taxa in Australia and, indeed, in the whole Southeastern Asian region (Benzie 1988). *Daphnia carinata* is a native Australian species, which is widespread throughout the continent. Being a primary consumer and, in turn, a food source for secondary consumers (i.e. predators) this species plays an important role in aquatic food webs. Weakening this trophic link can threaten the stability of a freshwater ecosystem as a whole, leading to algal blooms and reduction in the populations of the predator species.

The agricultural use of insecticides may result in the contamination of the aquatic environment, in which arthropods are the most vulnerable non-target species. The insecticides affect crustaceans, since they are closely related to insects, in the same way as the target species.

Chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) (CPF) is a non-systemic insecticide with contact, stomach and respiratory action and is a cholinesterase inhibitor. Organophosphorus insecticides replaced organochlorines, and are today widely used on a variety of crops to control a great number of insect pests in soil, foliage and stored products. It is also used to eliminate mosquitoes (adults and larvae). According to data presented in Barron and Woodburn (1995) and Wijgaarden *et al*. (1993) representatives of crustaceans, insects and fish can be considered particularly sensitive to

CPF. Direct application to water (as in case of elimination of mosquitoes) and indirect entry (as agricultural runoff) can cause concentrations of CPF in water bodies as high as 3.7 μg/L (Wood and Stark 2002), leaving it for 10 days afterwards at concentrations above 0.5 μg/L. This lower concentration corresponds to the 48-h LC_{50} for *D. carinata* (our data), which is similar to reported values for *D. longispina* – 0.8 μg/L (Van Wijngaarden *et al*. 1993). For other cladoceran species even lower LC_{50} has been reported - 0.25 μg/L for *D. pulex* (Van der Hoeven and Gerritsen 1997). Generally, 48-h LC_{50} for daphnids is reported to be 0.1-0.5 μg/L (Barron and Woodburn 1995). However, not many studies are conducted on the chronic (long term) toxicity of CPF to cladoceran species. Some researchers state that CPF "does not result in significant sub-lethal responses" (eg Naddy *et al*. 2000). However, according to Pesticide Action Network (PAN), toxic effects of CPF to zooplankton include accumulation, behaviour and development problems, effect on cells, enzyme, feeding behaviour, growth and reproduction (www.pesticideinfo.org, see references therein).

Our aims were to investigate the sublethal effects of CPF on *D. carinata* over multi generational exposure and to determine if they changed with time.

6.2 Materials and methods

6.2.1 Preparation of media.

For experiments and maintenance of *D. carinata* culture M4 medium (Elendt and Bias 1990) was used with slight modifications. Instead of the vitamins listed in the original recipe we used the commercially available Reptivite™ (ZOO MED, San Luis, OBISPO, CA 93401), prepared as follows: 1 g of Reptivite powder was dissolved in 100 mL of

MilliQ water, then filtered using 20 μm sterile filter, made up to 100 mL with MilliQ water and kept in a refrigerator. One mL of this vitamin solution was added to 5 L of medium. Water quality parameters of this medium are: total hardness – 2.5 mmol/L, alkalinity – 0.9 mmol/L, conductivity – 610 μS/cm, pH – 8.2±0.2. The medium was aerated for at least 24 hours before use.

6.2.2 Feeding.

Freshwater green unicellular algae *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) was used as food for daphnids. Algae were cultured in 0.5 L conical flasks with 0.4 L Keating algal medium (Keating 1985) on a light table (continuous luminosity 2800±100 lux, temperature 23±1^{o}C), and continuous aeration with filtered air was provided. Once a week the culture was harvested and put in the refrigerator for 2 days to settle. Supernatant was discarded and the algal suspension was added during cladoceran medium replacement to achieve 3.5×10^{5} cells/mL in the culture medium.

6.2.3 Maintenance of daphnid culture.

Several females of *D. carinata* were obtained from the Arthur Rylah Institute, Department of Natural Resources and Environment, Heidelberg, Victoria, Australia. The stock culture was kept in 2-L glass jars with M4 medium. Half the medium was replaced twice a week. When preparing the experiment, 50 adult females with broods were placed in 40-mL glass vials with M4 medium (2 in each vial). Medium was replaced every second day; offspring were removed daily. The neonates from the first two days were discarded, because there

could be males in these broods. Offspring starting from the third day were used in the experiments.[1]

6.2.4 Preparation and analysis of chemical solution.

Chlorpyrifos Pestanal® (99.6% purity) was obtained from Riedel-de-Haën Laborchemikalien GmbH. CPF is virtually insoluble in water, so a stock solution in acetone was prepared with a concentration of 100 g/L. A series of successive dilutions in acetone (analytical grade) was used for preparation of the experimental concentrations of CPF. Because of the very low nominal concentrations of CPF used in the study and small volume of test solutions, routine analysis of CPF could not be made. However, the analysis of the highest concentration tested (0.5 µg/L) was performed by the Australian Government Analytical Laboratories (AGAL). Methods are based on USEPA 3510C (for sample prep) and detection is based on USEPA8141 (OP pesticides). An aliquot of water was extracted using dichloromethane. The combined extract was filtered through sodium sulphate then concentrated. Extracts were then exchanged into hexane. If required, the extract was cleaned up by Gel Permeation Chromatography (GPC) and/or sulfur removal. The final extract was analysed by GC-ECD and GC-NPD. Extraction was made 48 hours after solution preparation. For the nominal concentration of 0.5 µg/L the measured concentration was 0.44 µg/L with the recovery of 97%.

[1] When the adult females are taken for breeding in the individual culture, it is not known what conditions they were grown before. If they already contained eggs, those eggs can hatch into males or females depending on prior conditions. Breeding in the individual culture usually produces females, if all conditions are optimal for a breeding female. To ensure that all offspring are females, breeding females are allowed to breed in the individual culture for some time (2-3 broods), hence only the third brood is taken for exposure.

6.2.5 Experimental protocol.

Animals (< 24 hours old) were pooled to reduce the risk of choosing a particularly sensitive (or tolerant) clone for the experiment (see Zalizniak and Nugegoda 2004).

A 48-h acute static toxicity test was performed to determine the LC_{50} for CPF according to OECD guidelines (OECD 1996). Three replicates of 10 animals in 100 mL of medium were used for each of the concentrations and control. Concentrations tested were in the range of 0.2 – 3 µg/L (nominal concentrations). An acetone control with the same concentration of acetone as for the highest treatment was also prepared (100 µL/L). Death of an animal was confirmed only after microscopical examination of its heartbeat. The 48-h LC_{50} for CPF for *D. carinata* was 0.512±0.062 µg/L. Based on this value the following concentrations of CPF were used for investigating the long-term toxicity to *D. carinata*: 0.005, 0.025, 0.05, 0.25 and 0.5 µg/L ('0.01 LC_{50}', '0.05 LC_{50}', '0.1 LC_{50}', '0.5 LC_{50}' and '1 LC_{50}' respectively). The variable factor in dilution series for CPF concentrations was used to cover a broader concentration range. The concentrations of CPF are expressed in terms of proportion of the LC_{50} value because LC_{50} values for a particular species vary depending on the conditions, testing laboratories etc. We suggest that expression of concentration this way is more useful for comparison across different studies.

The experiment consisted of three parts:

1. *Long-term (21 days) toxicity of CPF to individual culture of D. carinata.*

Individual culture of *D. carinata* was chosen as an alternative to the OECD (1996) approved procedure for ecotoxicological experiments. In chronic toxicity testing, according to OECD guidelines, the volume of media per female should not be less than 40 ml (for *D. magna*), and medium replacement every other day is recommended (this is for a cohort of 10 animals). However there is no difference in statistical power between the OECD recommended procedure (4 cohorts of 10 animals) and individual culture (10 animals) testing (Sims and van Dijk 1995). However, individual culture is more informative, especially in terms of attributing offspring to a particular animal. The number of contacts in a cohort can also influence test results, especially when numbers of individuals in cohorts are unequal due to mortality. There is also a question whether to reduce the volume of test media per cohort, if mortality occurs. If not - the dose of chemical per animal can be potentially greater, if yes - there are discrepancies between replicates. These issues do not arise when individual culture is used, see also Zalizniak and Nugegoda (2004).

Fifteen juveniles <24 h old were placed individually in 25-mL McArtney bottles with M4 medium. Animals were fed daily with green algae *P. subcapitata* so that the concentration of algae was 3.5×10^5 cells/mL in the exposure solutions. The cladoceran were transferred daily to fresh media, offspring were removed and counted. The measured endpoints were: survival, number of offspring per female, time to the first brood, and size of the females after 21-day exposure (measured under dissecting microscope with an eye-piece micrometer, precision 0.05 mm). We did not use total biomass produced per female as an endpoint, because it was not feasible in this experiment. Survival and fecundity data were used to compute an intrinsic rate of natural increase (r) as determined by Lotka (1913):

$$\Sigma \quad l_x m_x e^{-rx} = 1,$$

where l_x is the proportion of individuals surviving to age x,

m_x is the age specific fecundity (number of females produced per surviving female at age x),

x is days.

2. *Long-term (21 days) toxicity of CPF to the second generation of D. carinata.*

When the animals in the parent generation started to reproduce, 15 offspring were taken from each treatment concentration and both controls, and placed in the corresponding concentrations of CPF. There was no concentration of 0.5 μg/L ('1 LC_{50}'), because all the animals in the parent generation died at this concentration before they started to reproduce. Offspring were usually taken on the third day from the onset of reproduction. On the first two days there were not enough animals to start the second-generation test, because just one or two females were reproducing at some treatment concentrations. The same experimental protocol and endpoints were used for the second-generation test as for the parent-generation.

3. *Post-experiment 48-h LC_{50} tests to determine the change in sensitivity to CPF in the third generation of daphnids.*

When the second generation of *D. carinata* produced enough offspring, a 48-h LC_{50} test (static) was conducted for each treatment and controls. We required not less than 120 animals from each exposure concentrations to conduct this test, and usually this high number was only obtained on the 5^{th} or 6^{th} day from the onset of reproduction. There was

no LC_{50} test for the concentration of 0.25 μg/L ('0.5LC_{50}'), because there were not enough offspring to conduct the test. This test was conducted according to OECD guideline for the testing of chemicals (OECD 1996). There was a variation from the first 48-h LC_{50} test. There were 4 replicates of 5 animals for each tested concentration and controls (versus 3×10 in the original test). We had enough animals only to conduct this variation of test. However this is allowed by the guidelines, and both versions are acceptable. Concentrations from 0.1 to 0.8 μg/L were used. The volume of a replicate was 25 mL. The 48-h LC_{50} values were determined separately for each group of third generation daphnids from parents pre-exposed to CPF ('0.01 LC_{50}', '0.05 LC_{50}' and '0.1LC_{50}') and plotted against these concentrations.

6.2.6 Statistics

Data derived from the experiments (body length of females, time to the first brood, number of offspring per female) were analysed using analysis of variance (SPSS). The LC_{50} values were determined using PROBIT analysis (SPSS). Because there were no significant differences between control and acetone control, except in the survival of the parent generation, the controls were combined for analysis of all other parameters and all treatments were compared to this "combined" control. A one-way ANOVA followed by Dunnet multiple comparisons was performed, but the results were inconclusive. However when each treatment was compared pair wise to control using Student t-test for unequal variances the differences were evident at P=0.05. The mean value of intrinsic rate of natural increase and its standard error were determined using a jackknife approach as described by Taberner *et al.* (1993). Standard error was used throughout the results.

6.3 Results

The results for the parent generation of *D. carinata* are presented in Fig. 6.1 and Fig. 6.2. All daphnids at CPF concentrations of '0.5LC_{50}' died by day 13 of the exposure, and at '1 LC_{50}'– by day 7, before the onset of reproduction. The survival at day 21 in all of the treatments including the acetone control was lower than in control, mostly affecting older animals (Fig. 6.1). Cumulative survival at the end of the 21-day exposure was between 73 and 80 % for all surviving treatments. There were significant differences between combined control and surviving treatments for the number of offspring per female (Fig. 6.2a) and size of animals at the end of exposure for concentration '0.05LC_{50}' (Fig. 6.2b). All treatments showed lower fecundity and size values than combined control. It appears that low concentrations of CPF (up to 10% of LC_{50}) adversely affect individual characteristics of *D. carinata* in the parent generation. The intrinsic rate of natural increase (*r*) was also adversely affected at three out of four concentrations tested.

A slightly different picture emerges for the second-generation toxicity test. Though the survival patterns seemed similar to the parent generation test, there were some differences (Fig. 6.3). The survival curve for the concentration of '0.5LC_{50}' for the parent generation yielded a distinctive sigmoid shape, while for the second generation it was practically linear. However maximum longevity was similar in both generations (13 and 12 days). There is a marginal increase in survival for concentrations '0.01LC_{50}' and '0.05LC_{50}' compared to the control value of 80% and then a drop to 60% survival at a concentration of '0.1LC_{50}' (Fig. 6.4d). The survival at this concentration is the lowest, however the number of offspring per female was

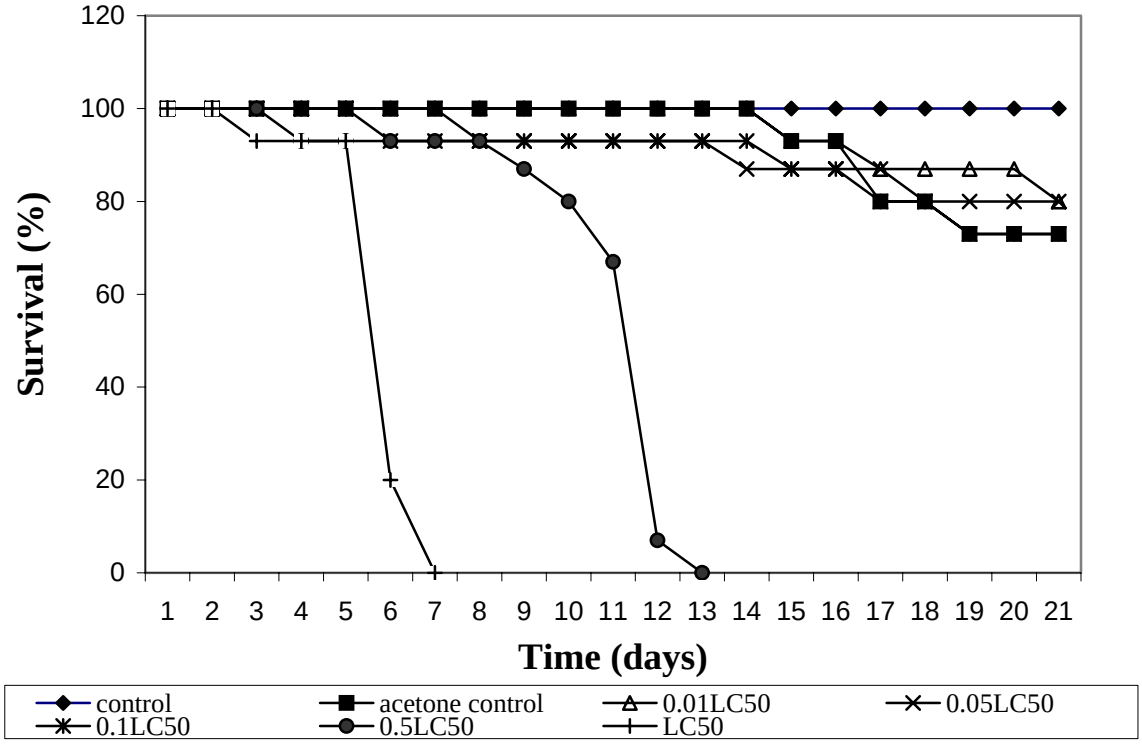

Figure 6.1 Survival of the parent generation of *D. carinata* at different nominal concentrations of CPF.

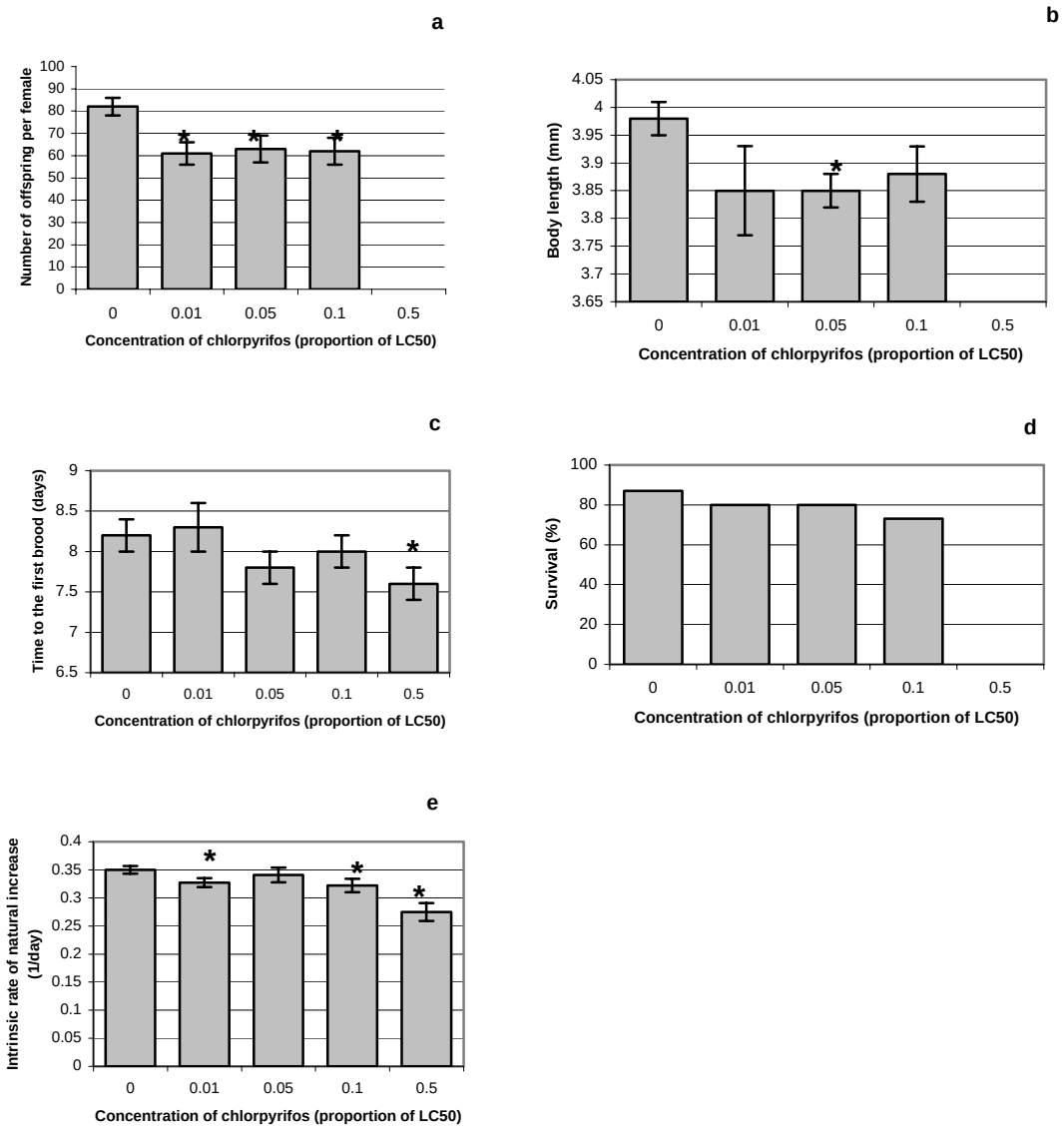

Figure 6.2 Population and individual characteristics of individual culture of *D. carinata* (parent generation) exposed to different nominal concentrations of chlorpyrifos for 21 days. Mean±SE, N=15;

a – Number of offspring per female

b – Body size of females after 21-day exposure

c – Time to the first brood

d – Cumulative survival of animals after 21-day exposure

e – Intrinsic rate of natural increase

An asterisk indicates values significantly different from combined control.

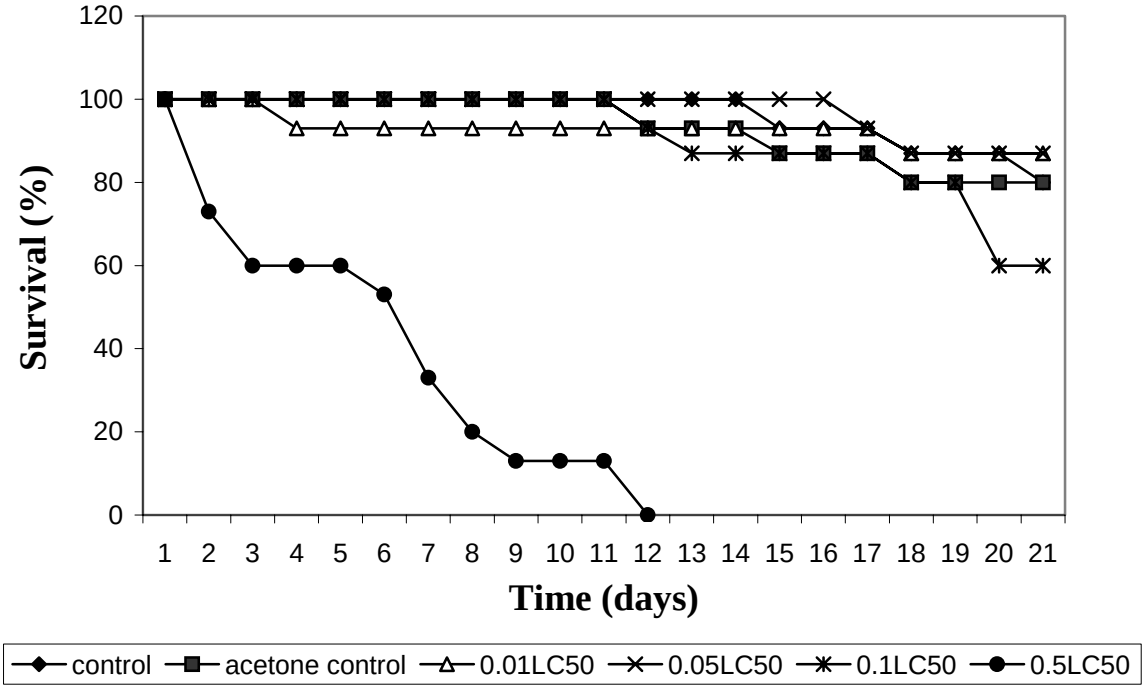

Figure 6.3 Survival of the second generation of *D. carinata* at different nominal concentrations of CPF.

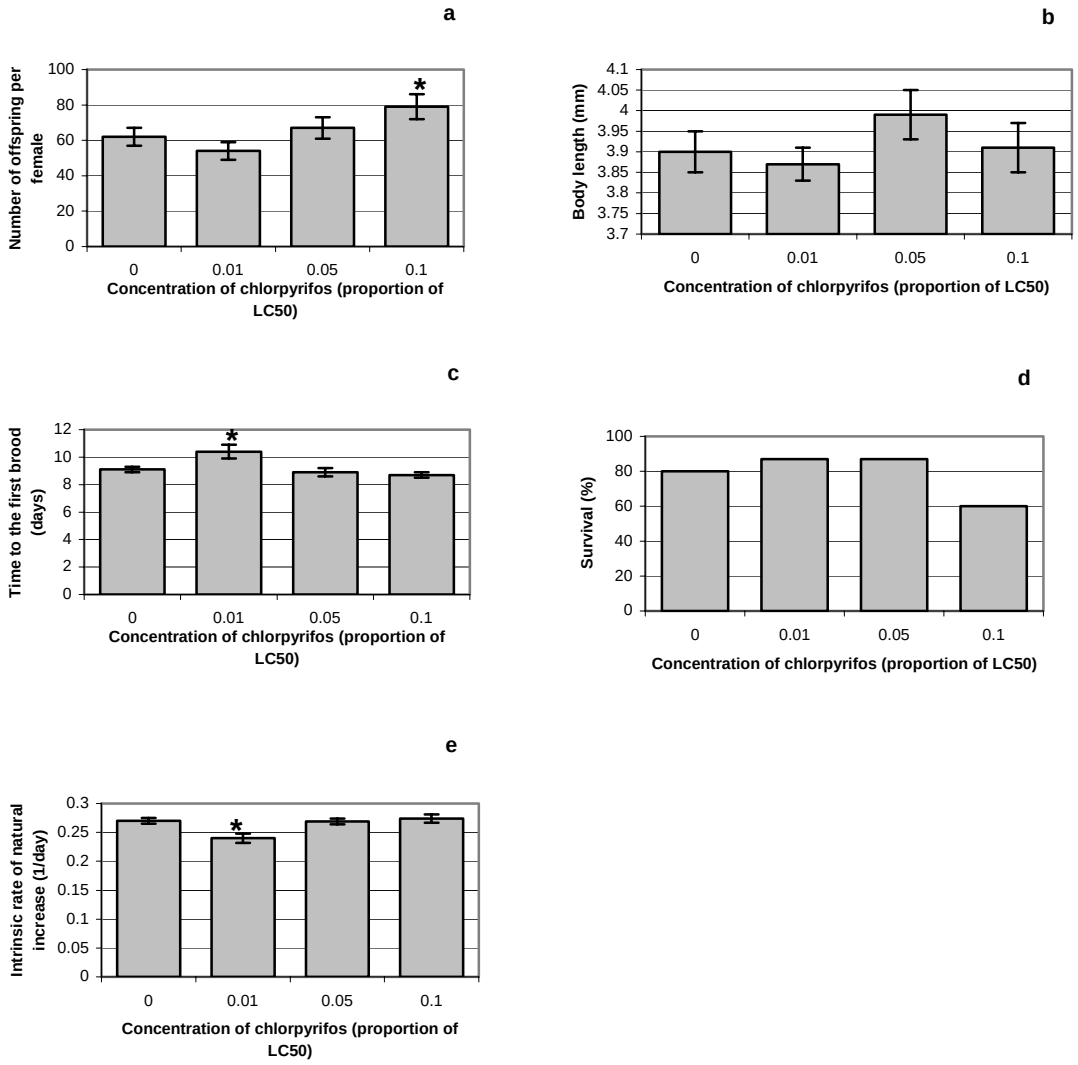

Figure 6.4 Population and individual characteristics of individual culture of *D. carinata* (second generation) exposed to different nominal concentrations of chlorpyrifos for 21 days, following exposure of the parent generation. Mean±SE, N=15;

a – Number of offspring per female

b – Body size of females after 21-day exposure

c – Time to the first brood

d – Cumulative survival of animals after 21-day exposure

e – Intrinsic rate of natural increase

An asterisk indicates values significantly different from combined control.

statistically significantly higher at this concentration (79±7 compared to 62±5 for combined control, Fig. 6.4a), and time to the first brood was shorter (8.7±0.2 compared to 9.1±0.2 for combined control, Fig. 6.4c), indicating improved performance. Combination of these parameters resulted in a slightly greater (though not significantly different) intrinsic rate of natural increase for concentration '$0.1LC_{50}$' (0.274±0.007 day^{-1} against 0.270±0.005 day^{-1} for combined control). At the same time animals at the concentration of '$0.01LC_{50}$' had almost all their characteristics (except survival) lower than in control (Fig. 6.4). This resulted in a significantly (10%) lower intrinsic rate of natural increase for this concentration (0.240±0.008 day^{-1}).

To determine if there was a change in sensitivity to CPF after exposure of two generations of daphnids to this insecticide, the offspring of the second generation were collected and tested. The 48-hour LC_{50} values were determined for each exposure concentration, and are shown in Fig. 6.5. There were no significant changes in sensitivity for the exposure concentration of '$0.01LC_{50}$' and both controls. Their 48-h LC_{50} values were not significantly different from the initial value of 0.512±0.062 µg/L (Fig. 6.5). However, the sensitivity was significantly greater for the CPF exposure concentrations of '$0.05LC_{50}$' and '$0.1\ LC_{50}$'. The 48-h LC_{50} values for these exposure concentrations were 0.235±0.032 and 0.280±0.046 µg/L respectively (Fig. 6.5).

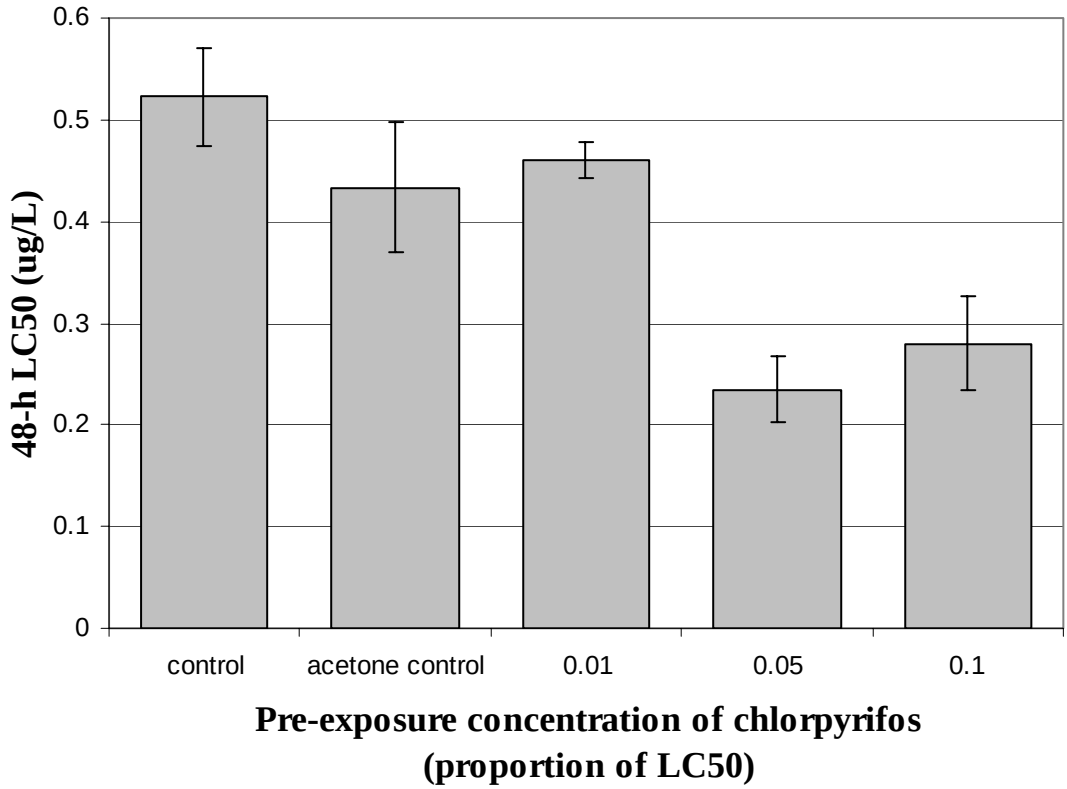

Figure 6.5 The 48-h LC_{50} values for the third generation of *D. carinata* taken from different nominal second-generation exposure concentrations of chlorpyrifos. Mean±SE, N=4.

6.4 Discussion

The results of the toxicity testing for the first generation showed that at a long-term exposure to CPF concentration of 0.5 µg/L (48-h LC_{50}), the survival is greater than in the acute toxicity testing. The LT_{50} (time from the start of the experiment at which 50% of test animals die in a given concentration of a toxicant) for this concentration was 48 hours for acute exposure; in chronic exposure at the same concentration the LT_{50} was 5.5 days. The acute toxicity testing was conducted without feeding the animals. Probably the presence of algae in the chronic exposure reduces the amount of CPF available for daphnids through the water route of exposure. There is no agreement between researchers on this issue. Naddy and Klaine (2001) reported that the presence of food increased the toxicity of CPF to *D. magna* in a pulse-exposure experiment. Acute toxicity testing at the same laboratory yielded similar results (Baladi 1998, cited in Naddy and Klaine 2001). However Rose e*t al.* (2002) in their study with different food concentrations found that limited food significantly increased the toxicity of CPF to daphnids. Kooijman and Metz (1984) indicated that the toxicity of chemicals, which directly affect survival, such as CPF, is aggravated by food limitation. Our results support the findings of Rose *et al.* (2002) and Kooijman and Metz (1984). The possible explanation for this effect is, that algae metabolise CPF as a source of phosphorus, reducing its content in the solution. Van Donk *et al.* (1992) observed a growth stimulating effect on *Selenastrum capricornutum* (now *P. subcapitata*) treated with Dursban®. A similar effect was reported with the culture of other green alga, *Chlamydomonas reinhardtii* treated with CPF (Birminghan and Colman, 1976). Butcher *et al*. (1977) stated that the occurrence of algal blooms after applications of CPF may be due to an increase in phosphorus, as a result of

degradation of the compound. The stimulating effect was observed only for green algae.

As was expected, the survival was clearly affected in the parent generation of *Daphnia*. CPF, being a cholinesterase inhibitor, leads to a paralysis of animals and their consequent death. At a CPF concentration of '$1LC_{50}$' all animals died before they started to reproduce (Fig. 6.1). Though the animals at a CPF concentration of '0.5 LC_{50}' produced enough offspring for the second-generation exposure, all animals at this concentration in the parent generation died by the 13^{th} day (Fig. 6.1). Second-generation animals at a CPF concentration of '0.5 LC_{50}' did not produce any offspring. Their survival curve (Fig. 6.3) was different to that of the parent generation (Fig. 6.1). This indicates, that exposure of parents to this concentration of CPF affects the response of the second generation, making younger animals more susceptible to the toxicant at this concentration. It indicates that the animals in the second generation were affected by CPF from their early life stages, contrary to the parent generation, where the effect of CPF on survival was not evident till the animals reached maturity (Fig. 6.1). In all other concentrations, exposure of the parent generation did not show a significant difference in terms of the survival of animals (Fig. 6.1). It should be noted, that animals for the second-generation test were not taken from the third brood (as per OECD 1996), but on the third day of reproduction (which could have been either the first or second brood). Contrary to the guidelines recommendations (OECD 1996) requiring that the first two broods be discarded, and only the third one used for the toxicity testing, Klein (2000) found no differences between broods including the first when conducting acute toxicity testing with potassium dichromate. Considering

this, and in order to minimise the test duration, the second-generation test was begun when we had enough offspring to start all treatments simultaneously.

Cumulative survival (after 21-d exposure) in the second generation was similar to their parent's, except at concentration of '0.1 LC_{50}', in which only 60% of animals survived compared to 80% in the parental generation (Fig. 6.3). This indicates that continuous exposure to low (0.05 μg/L) concentrations of CPF greatly reduces the survival of animals in the second generation. A further study on changes in sensitivity to CPF (a 48-h acute toxicity test with the third generation of daphnia), showed, that even exposure concentrations as low as '0.05 LC_{50}' or 0.025 μg/L greatly affects the acute sensitivity of animals. The LC_{50} value for animals taken from this exposure concentration for acute testing was less than half the LC_{50} for control animals (Fig. 6.5).

Among other endpoints for the parent generation of daphnids (Fig. 6.2), only 'number of offspring per female' was significantly reduced in all treatments compared to control (Fig. 6.2a). All other endpoints proved insensitive to the effect of CPF, at least in the sense that no clear concentration-response relationships were observed. Fernandez-Casalderrey *et al.* (1995) obtained a similar result for another organophosphate insecticide methylparathion.

An indication of hormesis can be noted in the second generation (Fig. 6.4). A significant increase in the number of offspring per female at '0.1 LC_{50}' compared to control, combined with reduction in time to the first brood indicated that animals experienced a stimulating effect of CPF (Fig. 6.4a, c). Though a hormesis effect has

been known for a long time and reported for a number of toxicants (Stebbing 1982 and references therein, Hadjinicolaou and LaRoche 1988, Stevenson *et al.* 1995) especially heavy metals (Bodar *et al.* 1990, Calabrese and Baldwin 1993, 1997), there are just a few reports on hormesis for organophosphates and cladocerans (Stark and Vargas 2003). The mechanism of this effect in our case is unknown and requires further investigation. Probably, a prolonged exposure to CPF promotes *Daphnia's* r-strategy (an increased fecundity combined with a low survival rate). A hormesis effect should not be considered beneficial in this case, especially since the third-generation test showed an increased sensitivity of daphnids to CPF. Rose *et al.* (2004) observed an increased tolerance of *C. dubia* to sublethal concentrations of 3,4-dichloraniline, when exposed for four generations (the end point observed was a number of offspring). They attribute this increase to physiological acclimation of *C. dubia* to this toxicant. However the number of offspring alone cannot be considered enough to make conclusions about sensitivity to a particular toxicant. Other endpoints should also be analysed, since as evidenced by our results different endpoints prove valuable in analysing effects of toxicants on multiple generation.

The lowest concentration tested in our study, i.e. '0.01 LC_{50}' (0.005 μg/L), yielded the lowest number of offspring per female in the second generation (though not significantly different from control) and significantly increased time to the first brood, which in combination produced the lowest *r*-value. This indicates, that even very low (and environmentally realistic) concentrations of CPF can affect populations of cladocerans. Some countries (for instance Canada) have water quality guidelines for the protection of freshwater aquatic life of CPF below this concentration i.e. 0.0035 μg/L (www.pesticideinfo.org). However, the US National Water Quality Criteria

recommend as freshwater quality criteria continuous exposure to 0.04 μg/L (www.pesticideinfo.org), which is ten times the concentration producing a negative effect in our study.

The intrinsic rate of natural increase (*r*) proved to be an insensitive parameter in our study. It combines fecundity, time to maturation and survival, and any of these parameters can affect its value. In this study low survival at a given concentration neutralized the hormesis effect of fecundity and maturation time. Investigating the effects of cadmium and copper on *D. pulex* Meyer *et al.* (1987) concluded that *r*-value was not adequate to fully determine changes in population dynamics. Our results support their conclusion. In order to understand mechanisms and effects of toxicants on a separate organism, observation of all population parameters is recommended. However when modelling an effect on a population and their recovery after exposure to toxicant, the intrinsic rate of natural increase alone could be appropriate.

6.5 Conclusions

CPF affects not only survival of *D. carinata*, but also their reproduction, including number of offspring per female and time to the first brood. While in the parent-generation exposure to low concentrations of CPF negatively affected reproduction, in the second-generation exposure, a hormesis effect was observed for all reproductive parameters investigated. From the results of our study we recommend that the intrinsic rate of natural increase be used only for population studies. If the mechanisms and effects of a toxicant are to be analysed, all reproductive parameters should be considered, since if even one is affected, a toxicant concentration cannot be

considered ‘safe’. Our study demonstrated a negative effect of CPF on reproduction of *D. carinata* during prolonged exposure to 0.005 μg/L, therefore even such a low concentration cannot be considered safe.

CHAPTER 7

EFFECT OF GLYPHOSATE AND ROUNDUP BIACTIVE ON *DAPHNIA CARINATA* IN MULTIPLE GENERATION TESTS

When the first experiments of the project using *D. carinata* were conducted, the sea salt medium (SSM) was used (Barry 1999). It consisted of deionised water with 0.4 g/L of sea salt (Coralife, scientific grade, Energy Savers Unlimited, Inc, Carson, CA, USA) and 0.1 g/L of $CaCl_2$ (pH=6.9±0.1, EC=900 µS/cm). It was noted while conducting experiments and maintaining culture that control animals quite often would not reproduce, or their fecundity was low. Therefore an alternative medium was sought and for further experiments M4 medium was adopted (Elendt and Bias 1990). However, when hormesis was observed in a number of experiments with daphnids in M4 medium as well, it was decided to compare these results with the earlier experiments in SSM.

7.1 COMPARISON OF TOXICITY RESULTS IN TWO DIFFERENT MEDIA

Submitted as:

Effects of two formulations of a herbicide glyphosate on *Daphnia carinata* in multiple-generation toxicity tests.

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Submitted to Environmental Toxicology and Chemistry, July 2006

SUMMARY

The long-term toxicity of glyphosate (technical grade and formulation Roundup Biactive) to three successive generations of *Daphnia carinata* was investigated. Survival, fecundity, time to the first brood, size of animals and the intrinsic rate of natural increase were measured as sublethal endpoints for two generations, each in a 21-d exposure. The third generation was subjected to a 48-h acute toxicity test to evaluate their sensitivity to glyphosate-based compounds. Glyphosate was tested in two different media: sea salt solution and M4 medium specially designed for daphnids, while Roundup Biactive was tested in M4 medium. Results indicated that glyphosate and Roundup Biactive had relatively low toxicity to *D. carinata.* Hormesis was evident in sea salt medium exposures in the first and second generations of daphnids with technical grade glyphosate. When exposed to glyphosate and Roundup Biactive in M4 medium animals showed no indication of hormesis. We hypothesize that glyphosate may have compensated for the lack of microelements in the sea salt medium, and possible mechanisms are discussed.

7.1.1 Introduction

Since their discovery in the 1970s, glyphosate-based products have been the most widely used herbicides for control of a broad range of weeds (Hartley and Kidd 1990). They are extensively used in orchards, vineyards, conifer plantations, and many plantation crops throughout the world.

Glyphosate is a broad spectrum, non-selective systemic post-emergence herbicide. It inhibits the activity of 5-enolpyruvyl shikimic acid-3-phosphate (EPSP) synthase, an enzyme of the shikimic acid pathway (Duke 1988), affecting aromatic amino acid synthesis, and consequently, protein synthesis and growth in plants. It is considered non-persistent in the environment, because it is biodegraded by soil and water micro-organisms (Duke 1988). The minimum half-life observed in the aquatic environments was two weeks, while in static natural waters it was 7-10 weeks (Sáenz *et al.* 1997).

Roundup Biactive is a formulation of glyphosate (41 % as isopropylamine salt) and surfactant (10-20 %) with the same mode of action as glyphosate. This formulation was designed after Roundup ® (earlier formulation of glyphosate) was found to be quite toxic to aquatic organisms. Wan *et al.* (1989) found 96-h LC_{50} for four fish species to be 11-33 mg/L of Roundup ®. Mann and Bidwell (1999) determined 48-h LC_{50} for several species of tadpoles for Roundup ® (containing MON 2139 surfactant) and Roundup Biactive ® (surfactant MON 77920). In acid equivalent (a.e.) the toxicity was 2.9-11.6 mg/L (depending on the species) for Roundup ®, and 328-494 mg/L for Roundup Biactive ® -

two orders of magnitude less than Roundup ® (Mann and Bidwell 1999). Manufacture Monsanto claims that Roundup Biactive ® can be safely applied directly to control weeds in channels, drains, streams, rivers, dams etc (http://www.roundupaustralia.info/).

Glyphosate product sales are currently worth approximately US$1,200 million annually and represent about 60% of global non-selective herbicide sales (Agrow 1995). In arable agriculture in the UK, glyphosate was the 12th most extensively used pesticide active ingredient; the 5th most extensively used herbicide by weight with 251 tonnes being used; and the 38th most widely applied herbicide, being applied over 334,529 ha annually in 1994 (MAFF 1995). In the US nearly 8,500 tonnes was being used on 5-8 million hectares annually in the years leading up to 1991 (USEPA 1993). In Canada, Vision® (containing 356 g/L of glyphosate as an active ingredient) is a major forest management herbicide, representing 81% of all herbicides sprayed on the forests. Because of the aerial method of application it can enter aquatic systems. Once in there, its half-life can vary from several days to ten weeks depending on the pH of the water (Trotter *et al.* 1990). The Canadian Water Quality Guidelines recommend the IMAC (Interim Maximum Accepted Concentration) for protection of aquatic life to be 65 μg/L. However, on occasion, glyphosate concentrations were found to be up to 270 μg/L in some Canadian water bodies (Morgan and Kiceniuk 1992).

In Australia the recommended maximum level of glyphosate to ensure protection of 99 and 80% of aquatic freshwater life is 0.37 and 3.6 mg/L respectively (ANZECC and ARMCANZ 2000). Though glyphosate is considered to be non-persistent, it can last in

the aquatic environment for a considerable time (up to ten weeks) and thus has the potential to affect non-target species with a short life cycle, such as cladocerans. Based on the scientific data available on glyphosate toxicity to aquatic animals, major organizations (USEPA 1993, WHO 1994) conclude that glyphosate and its formulation Roundup can be used with minimal risk to the environment. Glyphosate is considered to be non-toxic to animals, since they lack the metabolic pathway along which the chemical reacts, as the shickimate pathway is found only in plants (Giesy *et al.* 2000). However some researchers suggest that though glyphosate is not toxic to animals, it still can affect them at concentrations found in the environment. For example, Morgan and Kiceniuk (1992) observed that at the highest tested concentration of 0.1 mg/L, rainbow trout demonstrated a higher frequency of aggressive behaviour; authors reported that this effect was observed at concentrations much lower than those found in some water bodies after spray application (Trotter *et al.* 1990). When calculated from the recommended application rate (WHO, 1994), glyphosate concentrations can reach 3.9 mg/L of a.i. in surface waters. Tate *et al.* (1997) found that exposure of three successive generations of a freshwater snail *Pseudosuccinea columella* to glyphosate affected the animals' reproduction and development in the third generation at environmentally realistic concentrations.

Investigating only one generation of animals may not be adequate to conclude if a chemical with low toxicity such as glyphosate has any long-term effect. We investigated the effects of sub-lethal concentrations of technical grade glyphosate in two different

media and the glyphosate formulation Roundup Biactive in one medium on three successive generations of *Daphnia carinata* (Cladocera, Crustacea).

7.1.2 Materials and methods

7.1.2.1 Culture maintenance of daphnids

Detailed description of *D. carinata* culture maintenance and feeding is described in Zalizniak and Nugegoda (2006), and only briefly outlined here. Daphnids were maintained individually in their corresponding test medium - sea salt medium (SSM) for the tests in SSM (Barry 1999), and in M4 medium (Elendt and Bias 1990) for M4 tests, and fed with green alga *Psedokirchneriella subcapitata* (formerly *Raphidocelis subcapitata* formerly *Selenastrum capricornutum*), which was cultured in Keating MS medium (Keating 1985).

7.1.2.2 Test chemicals

Two types of glyphosate – technical grade with a concentration of 551 g/L of active ingredient, and Roundup Biactive with a glyphosate concentration of 336 g/L were obtained from Monsanto (batch # K554 for technical grade glyphosate, and batch # 728408 for Roundup Biactive).

All concentrations in this study are nominal, expressed as concentrations of the active ingredient.

7.1.2.3 Media preparation

Two types of media were used for this study: SSM (Barry 1999) for technical grade glyphosate (Gly) testing and M4 (pH=8.2±0.2, EC=610 µS/cm) (Elendt and Bias 1990) for Gly and Roundup Biactive (RB) testing. SSM is constituted of deionised water with 0.4 g/L of sea salt (Coralife, scientific grade) and 0.1 g/L of $CaCl_2$ (pH=6.9±0.1, EC=900 µS/cm). Both media were vigorously aerated with filtered air for 24 h before use.

7.1.2.4 Experimental protocol

Initially 48-h LC_{50} values for both herbicides and media types were determined using the OECD recommended procedure (OECD 1996). For Gly in SSM, the 48-h LC_{50} was 150 mg/L (95% CI could not be calculated). In M4 the values were: for Gly - 341(323-365 [95% CI]) mg/L and for RB – 98 mg/L (95% CI could not be calculated). Based on these results, concentrations of Gly and RB were chosen for the long-term toxicity testing in two successive generations of daphnids (in terms of proportion of the corresponding LC_{50}): '0.01 LC_{50}', '0.05 LC_{50}', '0.1 LC_{50}', '0.5 LC_{50}' and '1 LC_{50}'. For Gly testing in SSM the concentrations corresponded to: 0 (control), 1.5, 7.5, 15, 75 and 150 mg/L; for testing in M4 they corresponded to: for Gly: 0 (control), 3.5, 17.5, 35, 175, and 350 mg/L; and for RB: 0 (control), 1, 5, 10, 50 and 100 mg/L. The variable factor in dilution series for Gly concentrations was used to cover a broader concentration range. Concentrations units were chosen and expressed as proportion of LC_{50} to evaluate the relative toxicity and for ease of comparison of the different results (Zalizniak and Nugegoda 2006).

Individual culture of *D. carinata* was chosen as an alternative to the OECD (1996) procedure for toxicity experiments (see Zalizniak and Nugegoda (2004) for details). 15 juvenile females per treatment/control (age <24 hours) were placed individually in 25-mL McCartney bottles and exposed for 21 days. Mortality and reproduction parameters were recorded daily and daphnids transferred to new treatment media with algae (3.5×10^5 cells/cm^3), which were prepared daily, just before use. At the end of exposure, body length of surviving females (from the top of a crest to the base of a tail-spine) was also measured with an eyepiece micrometer under the microscope to the nearest 0.05 mm.

The same protocol was applied to experiments with the second generation of *D. carinata*. On the third or fourth day of reproduction the offspring from the first generation were taken for second-generation testing. Depending on the individual females' start of reproduction, the first-, second- and third-brood offspring were combined without distinguishing between broods. Thus offspring on the day, when females produced enough young to start the second-generation test simultaneously in all treatments, were taken for testing. Though it is common practice to take the second or third brood only for experiments, Klein (2000) found no differences between the sensitivity of different broods to a reference toxicant potassium dichromate. Thus in order to minimize experiment duration a mixture of several brood offspring of the age <24 h were used in our experiments. The same end-points were observed as for the first generation.

Survival and fecundity values were calculated in all experiments and used in the computation of the intrinsic rate of natural increase *r*, which is determined from the formula (Lotka 1913):

$$\Sigma\, l_x m_x e^{-rx} = 1,$$

where l_x is the proportion of individuals surviving to age *x*,

m_x is the age specific fecundity (number of females produced per surviving female at age *x*),

x is days.

The second-generation offspring (effectively – the third generation of daphnids) were tested using the acute 48-h test protocol (Gly concentration range 0 - 500 mg/L, RB – 0-250 mg/L) in order to determine if the third generation of daphnids had an altered sensitivity to the toxicant. This test was conducted according to OECD guideline for testing of chemicals (OECD 1996). Volume of treatment solution was 25 mL for 5 animals. The LC_{50} values were determined separately for each pre-exposure concentration of Gly in each medium and RB and plotted against these pre-exposure concentrations.

7.1.2.5 Statistics

Data were analyzed using analysis of variance (SPSS). The LC_{50} values were determined using PROBIT analysis (SPSS 11.0 for Windows). The data were checked for normality and homogeneity for Probit analysis. A one-way ANOVA followed by Dunnett multiple comparisons were performed, but the results were inconclusive. However when each

treatment was compared pair wise to control using Student t-test for unequal variances the differences were evident at P=0.05. The mean value of the intrinsic rate of natural increase and its standard error were determined using a jackknife approach as described by Taberner *et al.* (1993). Standard error was used throughout the results unless otherwise specified.

7.1.3 Results

7.1.3.1 Sea salt medium

Glyphosate (technical grade)

First-generation daphnids showed improved performance for all endpoints apart from survival in all treatment concentrations except '1 LC_{50}' (Fig. 7.1.1). Though survival in this treatment was only 7% after 21-d exposure (Fig. 7.1.1d), time to the first brood was significantly shorter (Fig. 7.1.1c) and number of offspring per female was the same as in control (Fig. 7.1.1a) resulting in the same *r*-value as in control (Fig. 7.1.1e). Animals surviving after 21-d exposure were also larger in size in all treatments compared with control (Fig. 7.1.1b).

There were inadequate numbers of offspring produced in the first generation in the '1 LC_{50}' and this treatment was omitted in the second-generation test. Animals in the second generation also demonstrated slight improvement in all endpoints for survival, however

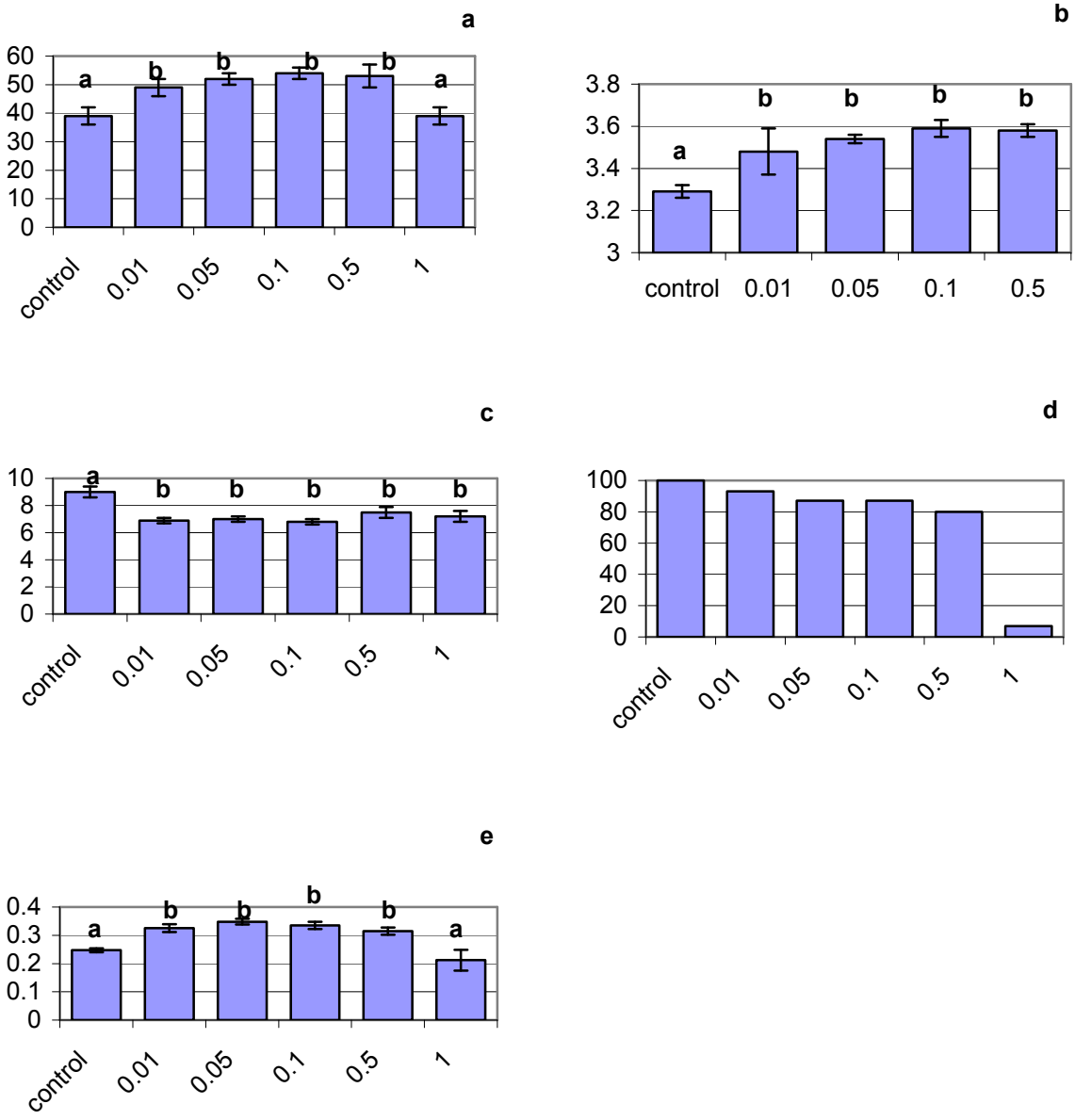

Figure 7.1.1 Performance of the first generation of *D. carinata* exposed to different concentrations of Gly in SSM. a – number of offspring per female, b – body length (mm), c – time to the first brood (days), d – cumulative survival (%), e – intrinsic rate of natural increase (day^{-1}) (Mean±SE, N=15). *X*-axis is concentration of Gly as proportion of LC_{50}. Values denoted by the same letter (alphabet) are not significantly different ($P>0.05$).

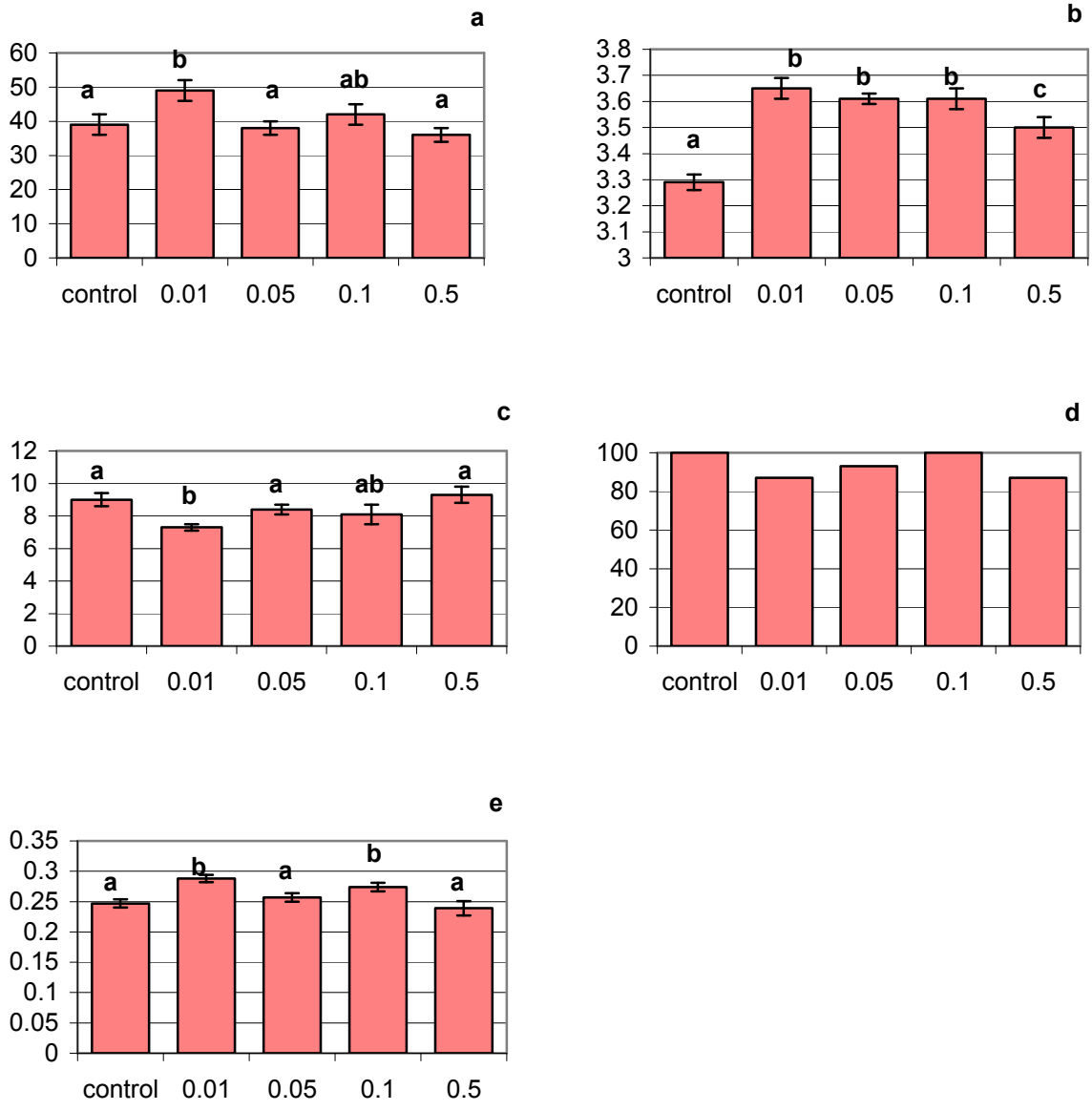

Figure 7.1.2 Performance of the second generation of *D. carinata* exposed to different concentrations of Gly in SSM. a – number of offspring per female, b – body length (mm), c – time to the first brood (days), d – cumulative survival (%), e – intrinsic rate of natural increase (day^{-1}) (Mean±SE, N=15). *X*-axis is concentration of Gly as proportion of LC_{50}. Values denoted by the same letter (alphabet) are not significantly different (P>0.05).

in contrast to the first generation, these were mainly in the lowest concentration tested i.e. '0.01 LC_{50}'. Number of offspring per female was significantly higher (Fig. 7.1.2a) and time to the first brood significantly shorter (Fig. 7.1.2c) in this treatment compared with control, resulting in a higher *r*-value (Fig. 7.1.2e). All other treatments were the same as control or improved only some endpoints (*r*-value for '0.1 LC_{50}' was also significantly higher than in control, and all animals grew bigger than in control (Fig. 7.1.2b)).

The third generation animals showed a trend for increased sensitivity to Gly with increased concentration of exposure (Fig. 7.1.3). When third-generation daphnids from each exposure concentration were tested in a 48-h acute exposure (concentration of Gly ranged from 0 to 250 mg/L), their corresponding 48-h LC_{50} showed a gradual decrease with increase in exposure concentration of the first- and second-generation animals (termed pre-exposure concentration (Fig. 7.1.3) to distinguish from the acute test exposures). However due to great variations between replicates the differences in LC_{50} values for daphnids from each pre-exposure concentration were not statistically different. The LC_{50} value for the 3rd generation control was also not statistically different from that of the original test.

7.1.3.2 M4 medium

Glyphosate (technical grade)

Gly negatively affected the first generation animals only at the concentration '0.5 LC_{50}' where survival and number of offspring per female were lower than in control, thus

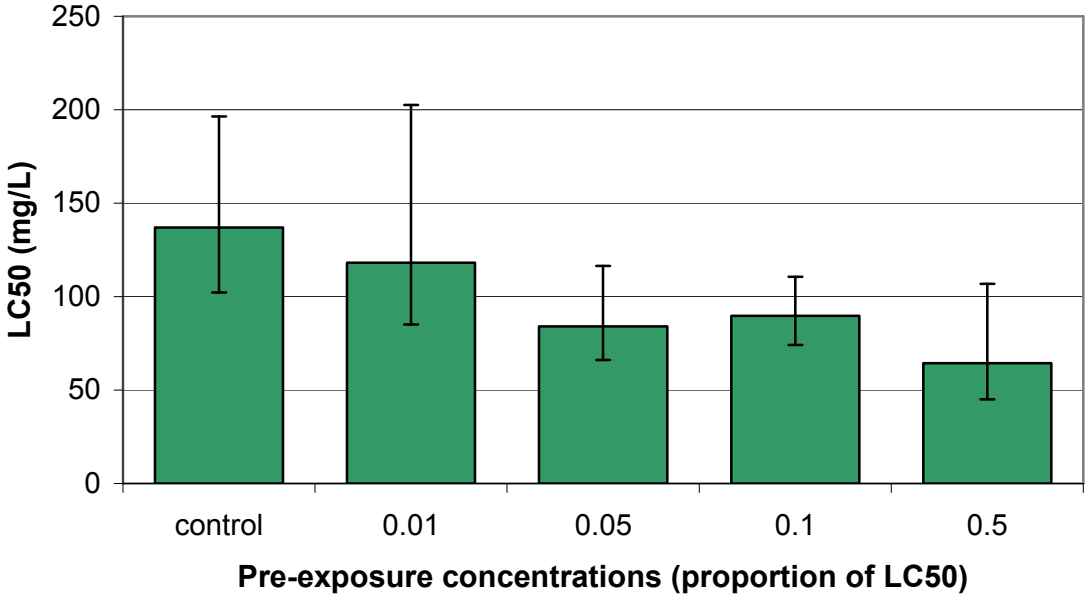

Figure 7.1.3 48-h LC_{50} values for the third generation of *D. carinata* pre-exposed to different concentrations of Gly in SSM. Error bars represent 95 % CI.

Table 7.1.1 Response of the first generation of *D.carinata* exposed to different concentrations of Gly in M4 medium. (Mean±SE, N=15). Here and in all tables the same superscript denotes values that are not significantly different from each other (P>0.05).

	Exposure concentration				
Endpoint	**Control**	**0.01 LC_{50}**	**0.05 LC_{50}**	**0.1 LC_{50}**	**0.5 LC_{50}**
Time to the 1st brood (days)	9.3^{a}±0.4	8.8^{a}±0.2	9.4^{ab}±0.3	10.6^{b}±0.6	9.4^{ab}±0.5
Body length at day 21 (mm)	4.17^{a}±0.08	4.10^{a}±0.06	4.12^{a}±0.05	4.08^{b}±0.08	3.9^{ab}±0.06
Number of offspring per female	89^{a}±7	84^{a}±8	75^{a}±7	77^{a}±8	49^{b}±6
Cumulative survival (%)	87	80	73	60	53
Intrinsic rate of natural increase, (day^{-1})	0.318^{a}±0.013	0.317^{a}±0.012	0.310^{a}±0.012	0.280^{ab}±0.016	0.235^{b}±0.020

measured in terms of survival of daphnids increased with increase of concentration.

Second generation daphnids showed an increased sensitivity to Gly (Table 7.1.2). The number of offspring per female was significantly lower than in control even at the lowest concentration tested '0.01 LC_{50}', and that and a longer time to the first brood, with the increased mortality compared to control, resulted in a significantly lower *r*-value. Since there was not enough offspring produced in the first-generation '0.5 LC_{50}' exposure, the second-generation exposure in this concentration could not be conducted.

The third generation of daphnids from different pre-exposure concentrations did not appear to show differences in sensitivity towards Gly - the 48-h LC_{50} for all treatments were not different (Fig. 7.1.4). However, because the LC_{50} for even those pre-exposed as controls was lower (179 mg/L) than the initial LC_{50} (341 mg/L), we cannot draw a valid conclusion from these results.

Roundup Biactive

In the first generation no concentration of RB showed increased toxicity compared to the control, except '0.01 LC_{50}' time to the first brood, which was significantly lower than the rest. However when different treatments were compared with each other, '0.5

Table 7.1.2 Response of the second generation of *D.carinata* exposed to different concentrations of Gly in M4 medium. (Mean±SE, N=15).

	Exposure concentration				
Endpoint	**Control**	**0.01 LC_{50}**	**0.05 LC_{50}**	**0.1 LC_{50}**	**0.5 LC_{50}**
Time to the 1st brood (days)	8.8^{a}±0.3	9.4^{b}±0.2	8.9^{a}±0.3	9.8^{b}±0.4	NA
Body length on day 21 (mm)	4.02±0.10	3.89±0.05	3.97±0.04	3.95±0.01	NA
Number of offspring per female	86^{a}±8	62^{b}±4	57^{b}±8	63^{b}±9	NA
Cumulative survival (%)	87	80	60	60	NA
Intrinsic rate of natural increase (day^{-1})	0.336^{a}±0.011	0.264^{b}±0.012	0.261^{b}±0.015	0.243^{b}±0.019	NA

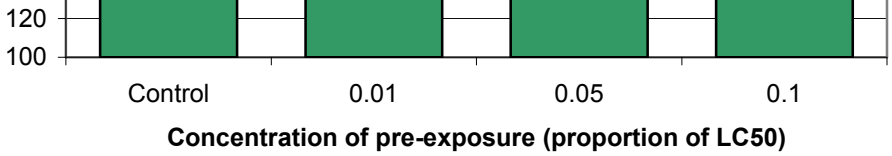

Figure 7.1.4 48-h LC_{50} values for the third generation of *D. carinata* pre-exposed to different concentrations of Gly in M4 medium. Error bars represent 95 % CI.

Table 7.1.3 Response of the first generation of *D. carinata* exposed to different concentrations of RB in M4 medium. (Mean±SE, N=15).

	Exposure concentration				
Endpoint	**Control**	**0.01 LC_{50}**	**0.05 LC_{50}**	**0.1 LC_{50}**	**0.5 LC_{50}**
Time to the 1st brood (days)	9.3^{a}±0.4	8.2^{b}±0.1	8.8^{a}±0.3	8.7^{a}±0.3	9.1^{a}±0.3
Body length at day 21 (mm)	4.17^{abc}±0.08	4.10^{ac}±0.10	4.23^{ab}±0.04	4.30^{b}±0.05	4.12^{c}±0.05
Number of offspring per female	89±7	90±8	100±11	99±9	83±7
Cumulative survival (%)	87	73	79	87	67
Intrinsic rate of natural increase, (day^{-1})	0.318^{ab}±0.013	0.335^{a}±0.008	0.334^{a}±0.010	0.330^{a}±0.010	0.303^{b}±0.009

LC_{50}' showed increased integrated toxicity (as *r*) compared with all treatments except control (Table 7.1.3). Also females were smaller in this treatment compared to '0.05 LC_{50}' and '0.1 LC_{50}'. At concentration '0.01 LC_{50}' time to the first brood was significantly lower that in all other treatments including control. Overall, almost all endpoints were seemingly improved at intermediate concentrations, with control and the highest concentration falling slightly (and not statistically significant) behind.

In the second-generation test there were no differences between endpoints in control and all treatments values (Table 7.1.4), but again, as in the first-generation test, the performance of animals at '0.5 LC_{50}' concentration was consistently worse than of those at '0.01 LC_{50}' with size, time to the first brood and *r*-value being significantly different.

The third generation did not show any change in sensitivity to RB (Fig. 7.1.5), with all LC_{50} values from different pre-exposures not different from each other or the initial value of 98 mg/L.

7.1.4 Discussion

7.1.4.1 Enhanced performance and effect of growth medium

In the experiment in SSM, first-generation daphnids in all sublethal concentrations and second-generation animals in some sublethal concentrations of Gly showed improved performance, i.e. hormesis (Fig. 7.1.1 and 7.1.2). Similarly Tate *et al.* (1997) found anomalies in the development of third-generation *Pseudosuccinea columella* snails following

Table 7.1.4 Response of the second generation of *D.carinata* exposed to different concentrations of RB in M4 medium. (Mean±SE, N=15).

	Exposure concentration				
Endpoint	**Control**	**0.01 LC_{50}**	**0.05 LC_{50}**	**0.1 LC_{50}**	**0.5 LC_{50}**
Time to the 1st brood (days)	8.8^{ab}±0.3	8.6^{a}±0.2	8.6^{ab}±0.3	8.7^{ab}±0.3	9.3^{b}±0.4
Body length on day 21 (mm)	4.02^{b}±0.10	4.12^{a}±0.04	4.01^{b}±0.06	4.04^{b}±0.03	4.03^{b}±0.04
Number of offspring per female	86±8	90±5	100±5	88±6	90±8
Cumulative survival (%)	87	87	80	80	73
Intrinsic rate of natural increase (day^{-1})	0.336^{ab}±0.011	0.338^{a}±0.009	0.333^{ab}±0.011	0.326^{ab}±0.010	0.316^{b}±0.010

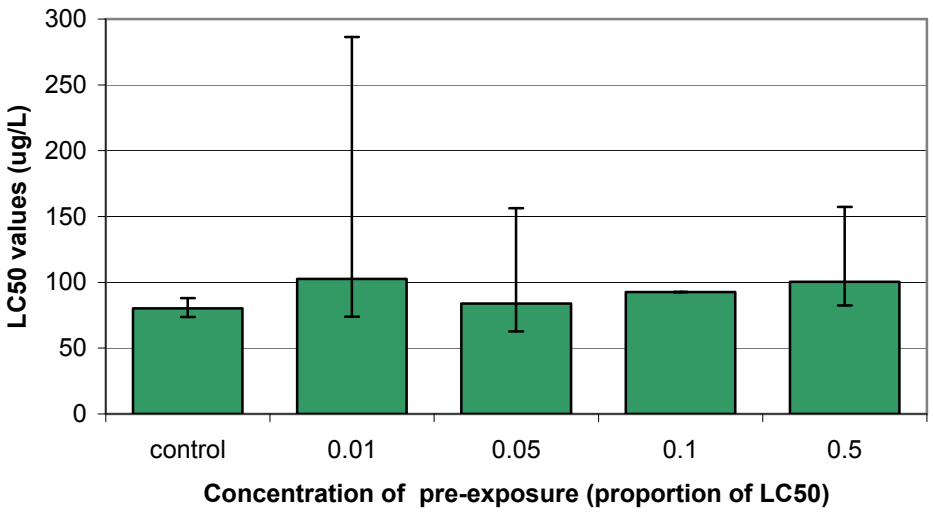

Figure 7.1.5 48-h LC_{50} values for the third generation of *D. carinata* pre-exposed to different concentrations of RB in M4 medium. Error bars represent 95 % CI.

continuous exposure to sublethal concentrations of Gly. The third-generation snails developed faster in the Gly concentration 1 mg/L than in control and other treatments. Tate *et al.* (2000) showed that concentrations of five free amino acids were higher in Gly treated animals than in control. This confirmed the findings of Thompson (1989) that the presence of sublethal concentrations of Gly stimulated significant biochemical and physiological responses (including egg laying capacity) in this snail species. Tate *et al.* (1997) hypothesized that Gly may serve as an energy source for these snails, though the mechanism of this effect remains unclear. Xi and Feng (2004) reported that in Gly concentrations ranging from 4 to 8 mg/L population growth rates of a rotifer *Brachionus calyciflorus* were significantly higher than in control, while a concentration of 2 mg/L did not have this effect. The population strategy of rotifers was also influenced by the presence of Gly – the females produced more resting eggs in Gly treatments than in control. Wojtaszek *et al.* (2004) reported that at 1.43 mg/L (Expected Environmental Concentration [EEC]) of Vision ® (active ingredient Gly) mean growth rates and maximum sizes of larvae of two amphibian species were the same or greater than in the control. These findings all suggest that there may be a general trend of improved performance in different aquatic animal species at low sublethal concentrations of Gly.

However, when daphnids in our study were exposed to Gly in a different type of medium (M4) with careful consideration of the presence of different microelements (such as selenium, a certain amount of which is essential to daphnids reproduction), a different set of results was obtained (Tables 7.1.1 and 7.1.2). Hormesis was not present in any generation in the tests with Gly conducted in M4 medium. This indicated that medium

composition could influence the toxicity of Gly. Two possible explanations can be proposed. If the concentrations of some of the essential microelements in the SSM are lower than that required for normal animal performance (e.g. selenium is essential in daphnids' reproduction), and knowing that Gly creates complexes with metals, it is possible that improved performance in SSM with Gly relative to control is due to the binding of essential elements to Gly and their easier delivery in this form to the animals (possibly with food). This hypothesis would explain the absence of hormesis in M4 medium, where there were no deficiencies in microelements. To check this hypothesis another experiment was conducted on the toxicity of cadmium to *D. carinata* with and without the presence of glyphosate (RB), the results of which are presented elsewhere (Zalizniak and Nugegoda 2006a). From this experiment we concluded that Gly was inducing an improved performance in daphnids, possibly by binding Cd and reducing its toxicity, however an improved performance was also observed in M4 medium with addition of Gly compared with Gly-free treatment in Cd-free treatments, which does not explicitly explain the results. Further studies are required to clarify the mechanism of improved performance of daphnids in the presence of Gly.

Wan et al. (1989) found that the toxicity of glyphosate and its formulations depends on the type of dilution water used. Overall they found that variation of 96-h LC_{50} values for MON 0818, MON 8709 (surfactants used in Gly formulations) and Roundup® for young salmonids was in the same order of magnitude irrespective of water types. For glyphosate these values could vary by an order of magnitude depending on water type, with water hardness and pH being the most contributing factors. Roundup®, MON 8709 and MON

0818 were more toxic to young salmonids in hard than they are in soft waters, while the reverse was true for glyphosate (Wan et al., 1989). Folmar et al. (1979) also reported that increased temperature and pH both result in an increased toxicity of glyphosate to rainbow trout. In our experiments both media were of intermediate hardness types (50-90 mg/L of total Ca^{2+} and Mg^{2+}), and their pH values ranged from 8.2 in control to 7.7 in 35 mg/L of Gly and 7.1 in 50 mg/L of RB, which are in the optimum range for this species and should not influence the results in this respect. All researchers who encountered this effect agree that the mechanism of hormesis resulting from exposure to Gly is unclear and requires further investigation.

7.1.4.2 Toxicity of glyphosate formulations: active ingredient vs. surfactant

In our experiments RB showed greater than 3 times the toxicity of Gly when tested in M4 medium (48-h LC_{50} for RB and Gly were 98 and 341 mg/L of a.i. respectively). A number of researchers (Wan *et al.* 1989, Servizi *et al.* 1987) indicated that the surfactants in Roundup (MON8709 or MON 0818 (part of MON 8709)) are more toxic to aquatic flora and fauna than the active ingredient glyphosate. Mitchell *et al.* (1987) compared toxicity values for Rodeo herbicide (active ingredient Gly) alone and for Rodeo herbicide with X-77 surfactant as recommended for application by the manufacturer Monsanto. They found that 96-h LC_{50} for rainbow trout exposed to Rodeo/X-77 mixture was about 4 times lower than when exposed to Rodeo without the surfactant (130 mg/L of active ingredient and 580 mg/L respectively). Henry *et al.* (1994) found the 48-h LC_{50} value for *D. magna* to be 218 mg/L when exposed to Rodeo herbicide. They also found that the surfactant X-77 used in some glyphosate formulations was about 100 times more toxic to *D. magna* than Rodeo (48-h LC_{50} is 2 mg/L for X-77). In general X-77 Spreader® was

83-136 times more toxic than Rodeo when tested using different species of animals (Henry *et al.* 1994). Similarly Folmar *et al.* (1979) found that glyphosate contributed only a small percentage of the toxicity of Roundup® and that the surfactant in the formulation was the primary toxic agent. Mann and Bidwell (1999) determined the acute toxicity of technical grade glyphosate acid, glyphosate isopropylamine, and three glyphosate formulations to adults of one species and tadpoles of four species of southwestern Australian frogs in 48-h static/renewal tests. They found that among those tested Roundup® herbicide was the most toxic for the tadpoles (between 2.9 and 11.6 mg/L glyphosate acid equivalent [a.e.]). Touchdown® herbicide was slightly less toxic (from 9.0 to 16.1 mg/L a.e.). All other formulations and technical grade glyphosate were practically non-toxic. These authors concluded that the surfactants in test formulations were the major contributing factor to their toxicity, and they need to be studied further.

7.1.4.3 General comments

Glyphosate-based herbicides are considered to be safe to the environment (aquatic included). However they can, as we have demonstrated and discussed, sublethally affect aquatic fauna at environmentally realistic concentrations. Folmar *et al.* (1979) also found that solutions of Roundup aged for up to 7 days in reconstituted water did not change toxicity to midge larvae, rainbow trout, or bluegills. These authors concluded that the chemical can accumulate to dangerous levels if there are repeated applications within short time intervals.

Glyphosate binds directly to sediment (Comes *et al.* 1976, Goldsborough and Beck 1989). It is expected therefore that in the water column its concentration will be reduced (Kilbride and Paveglio, 2001), resulting in lower toxicity to animals. However, Hartman and Martin (1984) demonstrated that the presence of suspended sediment in water significantly increased the acute toxicity of Roundup to *Daphnia pulex* (48-h EC_{50} for *Daphnia* was 3.2 mg/L with suspended sediment and 7.9 mg/L without it). Binding of glyphosate to suspended sediment probably results in its increased toxicity to filter feeders like cladocerans, and possibly grazers and benthos dwellers would also be affected, because they consume benthic sediments with their food.

Though glyphosate is relatively short-lived (half life up to 10 weeks depending on water quality), and natural populations of animals are unlikely to experience continuous exposure, glyphosate applications often coincide with the onset of the breeding season (e.g. in amphibians) thus exposing animals to an annual glyphosate pulse. Mann and Bidwell (1999) argued that this can have a cumulative effect, which can be expressed only after several generations (and can be inhibitory or stimulatory in a case of glyphosate exposure). Though in our experiments with M4 medium daphnids did not show increased sensitivity to continuous exposure with Gly and RB, in SSM there was an indication of increased sensitivity to Gly in the third-generation tests. Possibly such cumulative effects could be more pronounced in environmental communities of animals with a distinct seasonal breeding pattern, such as some insects, snails, amphibians or fish.

Improved performance, though seemingly beneficial to an animal, can be in fact detrimental to communities. We observed (Zalizniak and Nugegoda submitted a) that two species of algae showed a significantly increased growth (up to 40% greater than control) when exposed to chlorpyrifos at concentrations lethal to *D. carinata* (Zalizniak and Nugegoda 2006). In our study with *Pseudokirchneriella subcapitata,* it had higher growth rates at concentrations 15.6-62.5 mg/L of a.i. than in control, when exposed to both Gly and RB. At this concentration of Gly and RB daphnids are negatively affected (Tables 7.1.1-7.1.4) at least in terms of survival even in well-balanced M4 medium. This indicates that the balance in the community can be disrupted. Higher algal growth by itself in the presence of Gly can produce algal blooms, and when at the same concentrations the animals feeding on algae are negatively affected by the toxicant, it enhances the chance of the bloom even further.

In addition exposure to concentrations of a toxicant stimulating growth can result in promoting pest species and increased danger to humans. For example increased egg-laying capacity of *P. columella* snails (Thompson, 1989) can result in the increased abundance of this animal, which is the intermediate hosts for the liver fluke *Fasciola hepatica.* This in turn can lead to the increase of incidences of the infection in humans (Tate *et al.* 2000).

7.1.5 Conclusions

Low environmentally realistic concentrations of glyphosate-based herbicides can sublethally affect *Daphnia carinata*. Water quality (in terms of the culture medium) modified the toxicity of glyphosate, and in some cases results in an improved performance of the animals exposed to low concentrations for several generations. The mechanism of the observed hormesis is unclear and requires further investigation. Improved performance (especially fecundity) of animal species exposed to low concentrations of glyphosate-based products should be taken into account, as it may cause changes in community composition due to trophic interactions and competition between species with a different response to the toxicant. This in turn could promote pest organisms.

7.2 INVESTIGATION OF THE MODIFYING EFFECT OF GLYPHOSATE ON METAL TOXICITY TO *DAPHNIA CARINATA*

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Roundup Biactive modifies cadmium toxicity to *Daphnia carinata.*

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SUMMARY

The modifying effect of glyphosate on the toxicity of cadmium to *Daphnia carinata* was studied in long-term (21 days) exposures in two generations of cladoceran. It was found that low concentration of glyphosate (in the form of Roundup Biactive [RB]) reduces toxicity of Cd, and the performance of daphnia is enhanced in terms of animal size, survival, fecundity, and the intrinsic rate of natural increase in both generations of animals exposed in the presence of glyphosate. However when the third generation was tested for their sensitivity to Cd in the 48-h LC_{50} experiments there was no difference between RB-free and RB-spiked treatments in pair wise comparisons, indicating that no adaptive mechanisms were involved in the enhancement.

7.2.1 Introduction

In previous investigations (Zalizniak and Nugegoda submitted b), we found that technical grade glyphosate (Gly) in low concentrations (around 5-10% of its 48-h

LC_{50}) improved population parameters including number of offspring per female and time to the first brood, of *Daphnia carinata* in sea salt medium (SSM from Barry 1999), but not in the balanced M4 medium specially designed for daphnids (Elendt and Bias 1990). It is known that Gly complexes metals (Subramanian and Hoggard 1988, Wang *et al.* 2004) by binding them to one of its three chemical groups - amine, carboxylate and phosphonate (Pearson 1963). Artificial sea salt could lack an available form of some essential elements, for example, selenium, which has previously been identified as the most likely cause of daphnids' long-term poor reproduction performance in our earlier experiments in long-term culture. Because we did not observe an improved performance in the balanced M4 medium, we speculate that Gly in SSM facilitates the delivery of essential elements, resulting in better performance of the animals in low concentration Gly treatments compared with control. To test this hypothesis we investigated the effects of cadmium, which is highly toxic to daphnids, alone and with the addition of a low concentration (5 mg/L) of a Gly-based herbicide Roundup® Biactive (RB).

7.2.2 Materials and methods

7.2.2.1 Culture maintenance of daphnids

A detailed description of *D. carinata* culture maintenance and feeding is provided in Zalizniak and Nugegoda (2006), and only briefly outlined here. Daphnids were maintained individually in M4 medium (Elendt and Bias 1990), and fed with green alga *Pseudokirchneriella subcapitata* (formerly *Raphidocelis subcapitata* formerly *Selenastrum capricornutum*), which was cultured in Keating MS medium (Keating 1985).

7.2.2.2 Test chemicals

Roundup® Biactive with a Gly concentration of 336 g/L was obtained from Monsanto (batch # 728408). All concentrations are nominal, expressed as concentrations of the active ingredient.

Cadmium chloride $CdCl_2 \cdot 2.5H_2O$ (Sigma) was used for Cd exposures. To ensure that Cd concentration was maintained at nominal level, stock solution concentration was occasionally measured with a Flame Atomic Absorption Spectrophotometer. It was found to be within 5% of the nominal (10 mg/L) for the duration of the experiment, therefore nominal concentrations were used throughout the study.

7.2.2.3 Experimental protocol

Initially the 48-h LC_{50} for Cd was determined in acute toxicity tests (OECD 1996) with four replicates of 5 animals (20 total) in each exposure concentration (volume per 5 animals was 25 mL). Concentrations of Cd used were 50, 100, 250, 500, 600, 700, 800, 900, 1000, 1100, 1200 and 1300 µg/L. The LC_{50} was determined to be 899 (95% CI 851-947) µg/L of Cd. Based on this result, five concentrations of Cd (in terms of proportion of the 48-h LC_{50}) were chosen for the long-term (21 days) toxicity tests using two successive generations of daphnids: '0.01 LC_{50}' (or 9 µg/L), '0.05 LC_{50}' (45 µg/L), '0.1 LC_{50}' (90 µg/L), '0.5 LC_{50}' (450 µg/L) and '1 LC_{50}' (900 µg/L). To test our hypothesis the concentration of RB where daphnids demonstrated an improved performance in our previous experiments – 5 mg/L (Zalizniak and Nugegoda submitted b) was added to another set of Cd exposures and also tested

using two successive generations of *D. carinata.* Concentrations units were chosen and expressed as proportion of the LC_{50} of Cd to evaluate the relative toxicity and for ease of comparison of the different results (Zalizniak and Nugegoda 2006).

Individual culture of *D. carinata* was chosen as an alternative to the OECD (1996) procedure (which requires testing of cohorts) for toxicity experiments (see Zalizniak and Nugegoda (2004) for details). 15 juvenile females per treatment/controls (age <24 hours) were placed individually in 25-ml McCartney bottles and exposed for 21 days. Mortality and reproduction parameters were recorded daily and daphnids transferred to new treatment media with algae (3.5×10^5 cells/mL), which were prepared daily, just before use. At the end of exposure, body length of surviving females (from the top of the crest to the base of a tail-spine) was also measured with an eyepiece micrometer under the microscope to the nearest 0.05 mm.

The same protocol was applied to experiments with the second generation of *D. carinata*. On the third or fourth day of reproduction the offspring from the first generation were taken for second-generation testing. Depending on the individual females' start of reproduction, the first-, second- and third-brood offspring were combined without distinguishing between broods. Offspring for the second-generation test were taken on the day when females produced enough young to start testing simultaneously in all treatments. Though it is a common practice to take the second or third brood only for experiments, Klein (2000) found no differences between the sensitivity of different broods to a reference toxicant potassium dichromate. Thus, in order to minimize the duration of the experiment, a mixture of several brood offspring of the age <24 h were used in our experiments. Offspring

from daphnids exposed to 0.01 LC_{50} (Cd) in the first generation were exposed to 0.01 LC_{50} (Cd) in the second generation and so on for each treatment. The same end-points were observed as for the first generation.

Survival and fecundity values were calculated in all experiments and used in the computation of the intrinsic rate of natural increase *r*, which is determined from the formula (Lotka 1913):

$$\Sigma\, l_x m_x e^{-rx} = 1,$$

where l_x is the proportion of individuals surviving to age *x*,

m_x is the age specific fecundity (number of females produced per surviving female at age *x*),

x is days.

The second-generation offspring (effectively – the third generation of daphnids) from all exposures were tested using the acute 48-h test protocol to determine if their sensitivity to Cd changed because of exposure of their parents and to compare the results from two sets – Cd exposure only and with addition of RB. Cd concentration range in this 48-h exposure was 500-1000 µg/L. This test was conducted according to OECD guideline for testing of chemicals (OECD 1996). The volume of treatment solution was 25 ml for 5 animals. The LC_{50} values were determined separately for each pre-exposure concentration of Cd only and Cd+RB and plotted against these pre-exposure concentrations.

All tests were conducted at room temperature (21±1^{o}C), photoperiod 16 hours day:8 hours night. Water quality parameters for M4 medium are: total hardness 2.5 mmol/L,

alkalinity 0.9 mmol/L, conductivity 610 µS/cm, pH=8.2±0.1. The M4 medium is buffered, so the addition of acidic RB did not change the pH of exposure solutions.

7.2.2.4 Statistics

Data were analysed using analysis of variance with the SPSS® 11.0 computer package. The pairwise comparisons of the values with and without RB in long-term experiments were performed using t-test assuming unequal variances (SPSS®). The LC_{50} values were determined using PROBIT analysis (SPSS®). The mean value of the intrinsic rate of natural increase and its standard error were determined using a jackknife approach as described by Taberner *et al.* (1993). Standard error was used throughout the results unless otherwise specified.

7.2.3 Results

7.2.3.1 First generation sublethal toxicity testing

In first generation '1 LC_{50}' exposures for both Cd and Cd+RB all daphnids died by day 6 before they started to reproduce. In '0.5 LC_{50}' Cd only exposure animals died by day 14 without reproducing, and in Cd+RB by day 16 after producing a few offspring. Since death limited the data for these exposure concentrations, the results for these are not presented. Combined results for the first generation of *D. carinata* are presented in Table 7.2.1. The control ('0' Cd treatment) animals performed better when a small amount of RB was added. Though the survival and time to the first brood were not significantly different, the animals were bigger and the number of

offspring was greater in RB spiked control, the latter resulting in a higher *r*-value (Table 7.2.1). The results for '0.01 LC_{50}' and '0.05 LC_{50}' did not show much difference in pairwise comparisons, except that in '0.05 LC_{50}' survival and size of animals were greater in RB spiked treatments compared to those in RB-free. However, it did not affect their overall performance (the *r*-values are not statistically significantly different, Table 7.2.1). Surprisingly, the results for '0.1 LC_{50}' are the same as for '0' treatment with even greater differences between RB-spiked and RB-free exposures ($P<0.00001$).

7.2.3.2 Second generation sublethal toxicity testing

The second-generation results followed similar trend as the first one. The control animals performed better in RB-spiked exposure in terms of size, number of offspring per female and time to the first brood, resulting in a higher r-value (Table 7.2.2). Unlike the first generation, the second generation did not show any differences between RB-free and RB-spiked in '0.1 LC_{50}' exposures for observed endpoints, however, the resulting difference for r-value was significant in pairwise comparisons (Table 7.2.2). At concentration '0.05 LC_{50}' (Table 7.2.2) all endpoints were significantly 'better' in RB-spiked exposure, indicating that animals of the second generation respond at a lower concentration of cadmium if the media is spiked with RB.

Table 7.2.1 Response of the first generation of *D. carinata* exposed for 21 days to different concentrations of Cd only and with addition of 5 mg/L of RB. (Mean±SE, N=15). Here and in all tables, different superscripts denotes values that are significantly different from each other (P<0.05) in a pair wise comparison (without RB-with RB).

Endpoint	Cd exposure concentration (proportion of LC_{50})							
	0	**0+RB**	**0.01**	**0.01+RB**	**0.05**	**0.05+RB**	**0.1**	**0.1+RB**
Time to the 1st brood (days)	8.3±0.2	8.4±0.2	8.3±0.2	8.3±0.2	8.3±0.2	8.1±0.1	8.5±0.2	8.3±0.2
Body length at day 21 (mm)	4.00^{a}±0.14	4.30^{b}±0.05	4.26±0.04	4.19±0.06	3.95^{a}±0.04	4.13^{b}±0.03	3.55^{a}±0.16	3.86^{b}±0.05
Number of offspring per female	78^{a}±5	104^{b}±7	75±8	76±7	63±5	76±5	31^{a}±4	65^{b}±4
Cumulative survival (%)	80	80	87	87	53	80	67	73
Intrinsic rate of natural increase, (day^{-1})	0.311^{a}±0.009	0.330^{b}±0.006	0.311±0.007	0.317±0.008	0.307±0.008	0.318±0.006	0.226^{a}±0.013	0.308^{b}±0.007

Table 7.2.2 Response of the second generation of *D. carinata* exposed for 21 days to different concentrations of Cd only and with addition of 5 mg/L of RB. (Mean±SE, N=15).

Endpoint	Cd exposure concentration (proportion of LC_{50})							
	0	**0+RB**	**0.01**	**0.01+RB**	**0.05**	**0.05+RB**	**0.1**	**0.1+RB**
Time to the 1st brood (days)	9.3[a]±0.3	8.3[b]±0.2	8.6±0.2	8.7±0.3	9.2[a]±0.3	8.3[b]±0.2	9.0±0.3	9.6±0.6
Body length at day 21 (mm)	4.03[a]±0.03	4.13[b]±0.03	4.01±0.04	4.08±0.05	3.93[a]±0.03	4.02[b]±0.04	3.73±0.03	3.64±0.13
Number of offspring per female	80[a]±4	98[b]±4	68±6	76±6	63[a]±5	78[b]±4	33±4	41±6
Cumulative survival (%)	87	93	87	93	80	100	93	80
Intrinsic rate of natural increase, (day^{-1})	0.293[a]±0.010	0.317[b]±0.008	0.289±0.009	0.304±0.007	0.270[a]±0.011	0.312[b]±0.006	0.217[a]±0.011	0.271[b]±0.009

7.2.3.3 Third generation acute testing

There were no differences between 48-h LC_{50} in RB-spiked and RB-free Cd exposures (Table 7.2.3). Though the values were higher for Cd only pre-exposed animals than for animals pre-exposed to RB-spiked Cd treatments (except in '0.1 LC_{50}'), the differences were insignificant in all cases (tested with Fisher-Bonferroni adjustments).

7.2.4 Discussion

Based on the results of chronic sublethal exposures of two generations of *D. carinata* to RB-spiked and RB-free Cd treatments we conclude that addition of a small amount of RB (5 mg/L) reduces the toxicity of Cd to *D. carinata* (Table 7.2.1 and 7.2.2) contrary to our original hypothesis. In first generation exposures both '0' Cd treatment animals and those in '0.1 LC_{50}' (and in some cases in '0.05 LC_{50}') showed improved performance in RB-spiked Cd treatments compared with RB-free. Surprisingly, the lowest Cd treatment of '0.01 LC_{50}' did not show any improvement in RB-spiked exposures – values for all endpoints were practically identical in pairwise comparisons. The greatest differences were observed in '0.1 LC_{50}' for number of offspring per female (P=0.000004) resulting in a large difference in the intrinsic rate of natural increase, suggesting that the number of offspring was the main factor contributing to the difference in *r*-value.

Similar to the first generation, the second-generation animals also demonstrated hormesis in '0' Cd treatment (all endpoints). However, contrary to the first, the improved performance in RB-spiked treatments shifted towards a lower Cd

Table 7.2.3 Third-generation 48-h LC_{50} (μg Cd/L) for animals, whose parents and grandparents were exposed to different concentrations of Cd and Cd+RB. Mean with 95% CI in brackets, N=4.

Pre-exposure concentration (proportion of the initial Cd LC_{50})	**Cd only**	**Cd+RB**
0	595 (537-639)	577 (512-621)
0.01	552 (481-598)	546 (483-587)
0.05	593 (376-753)	542 (396-638)
0.1	401 (256-499)	408 (221-525)

exposure of '0.05 LC_{50}' (Table 7.2.2). All endpoints indicated presence of hormesis at '0.05 LC_{50}' Cd concentration in RB-spiked treatments compared with RB-free. It is worth noting that the differences were not as great as in the first generation. Our results suggest that with the increase of time (generation) of exposure, hormesis becomes less pronounced, however it still can be detected, though at a lower exposure concentration. Judging from this trend it is possible that with further exposure (for several more generations) hormesis could be eliminated altogether. This is perhaps reflected in the results of the third-generation toxicity testing. The 3rd generation RB-free and RB-spiked treatments did not show any differences in 48-h LC_{50} at any given Cd concentration (Table 7.2.3), indicating that at least in terms of survival there was no hormesis present. This might indicate that whatever advantage was given to the first and second generations by Gly addition did not last till the third generation. However this hypothesis requires further testing.

Tsui *et al.* (2005) studied the change in acute (48-h) toxicity of several heavy metals to *Ceriodaphnia dubia* in the presence of Gly. They found that the toxicity of Cd (as well as 6 other metals) was reduced in the presence of 2.88 mg/L of Gly (for Cd the reduction was 48% of the initial Cd toxicity). Gly clearly lowered the availability of Cd to daphnia. The authors explained this as due to the formation of insoluble metal complexes of Gly in hard waters and thus their reduced bioavailability. This was also confirmed in our long-term study, when the toxicity of Cd (provided in solution as Cd^{2+}) was lower in the presence of RB for some endpoints and Cd concentrations. However it does not explain the enhanced performance of daphnids when no Cd was added but RB was (control). Tsui *et al.* (2005) also reported that Se toxicity did not change with Gly addition, and mortality was 100% in both exposures with or without

Gly present. Because they used relatively high concentrations of Se (1.6 mg/L) to cause 100% mortality, it is not known if the result would be the same at low concentrations of Se. At low concentration of Se exposure it might be that with Gly present, the toxicity of Se could be altered. Alternately if Gly enhances the uptake of some essential elements when they are low/deficient in the solution, Gly may be beneficial to daphnids. This could be one possible explanation for the enhanced performance of daphnids in the presence of Gly without Cd in our experiments. Tsui *et al.* (2005) also measured Ag and Hg accumulation in *C. dubia* after 4-h exposure to metals with and without the addition of Gly. They found that with 100nM of Gly present there was a decrease in both metals in the solution, however uptake by the animals was only detected for Hg exposure, but not for Ag. Our results on reduction in Cd toxicity in the presence of Gly indicate that Cd uptake was possibly reduced in the presence of Gly similar to Ag. This suggests that different metals react differently with Gly, and need to be studied individually for changes in their toxicity in the presence of Gly.

The effect of Gly can have environmental implications when metal pollution is also an issue. Some metals (such as Cd) can have their availability reduced, but uptake of some (Hg) increases. Mercury uptake into aquatic organisms (fish in particular) is already at undesirably high levels, and this in turn can affect their consumers' health, including humans. Gly can also bind to the substrate, and its accumulation with later release can potentially increase Gly load in the environment. Though Gly is a herbicide, it is nevertheless toxic to some aquatic animals at environmentally realistic concentrations (Relyea 2005, Relyea *et al*. 2005).

CHAPTER 8

HORMESIS: FACTS, MODEL AND DISCUSSION

8.1 A DEFINITION AND BRIEF HISTORY OF HORMESIS

Hormesis (earlier known as Arndt-Schultz law) is defined in the On-line Medical Dictionary (http://cancerweb.ncl.ac.uk) as an effect where a toxic substance acts like a stimulant in small doses, but as an inhibitor in large doses. This effect was observed through the ages with small doses of poisons, and Paracelsus wrote in the 16th century that many substances, which are toxic, may be beneficial in small amounts (Stebbing 1982).

The classical and simplest case of a dose-response (or concentration-response) relationship is depicted in Fig. 8.1, where up to a certain level (threshold) there is no adverse effect, after which there is a direct correlation between dose (concentration) and inhibition effect of the toxicant till the death of the organism occurs. When hormesis is present, the shape of the dose-response curve changes to a so-called β-curve (Fig 8.2) (Stebbing 1982, Calabrese and Baldwin 1993, 1997), where the response (e.g. growth) to the toxicant at low doses is greater than that of control.

Calabrese and Baldwin (1998a) analysed earlier reports on hormesis in a comprehensive summary. They indicated that among the reviewed studies of a broad range of chemical classes the most prevalent biological endpoints were growth responses (see also Stebbing 1982), followed by metabolic effects, longevity (see also

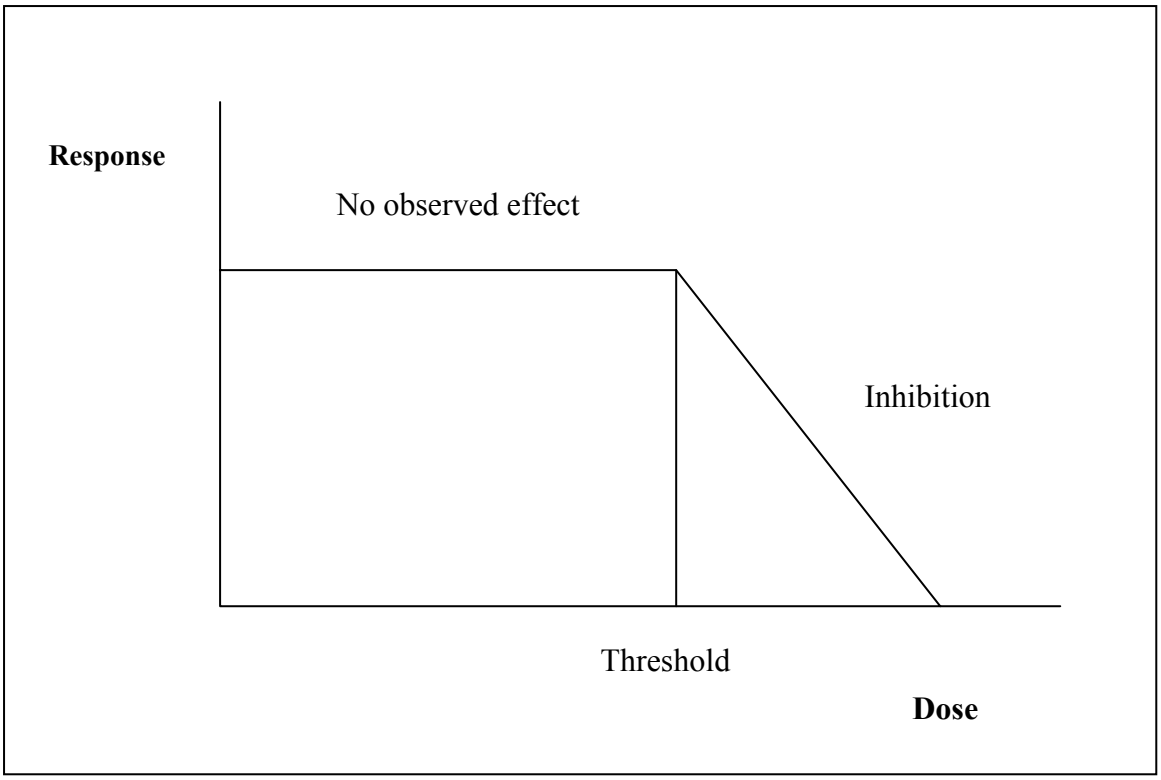

Figure 8.1 General case of a dose-response relationship of a toxic (non-carcinogen) agent.

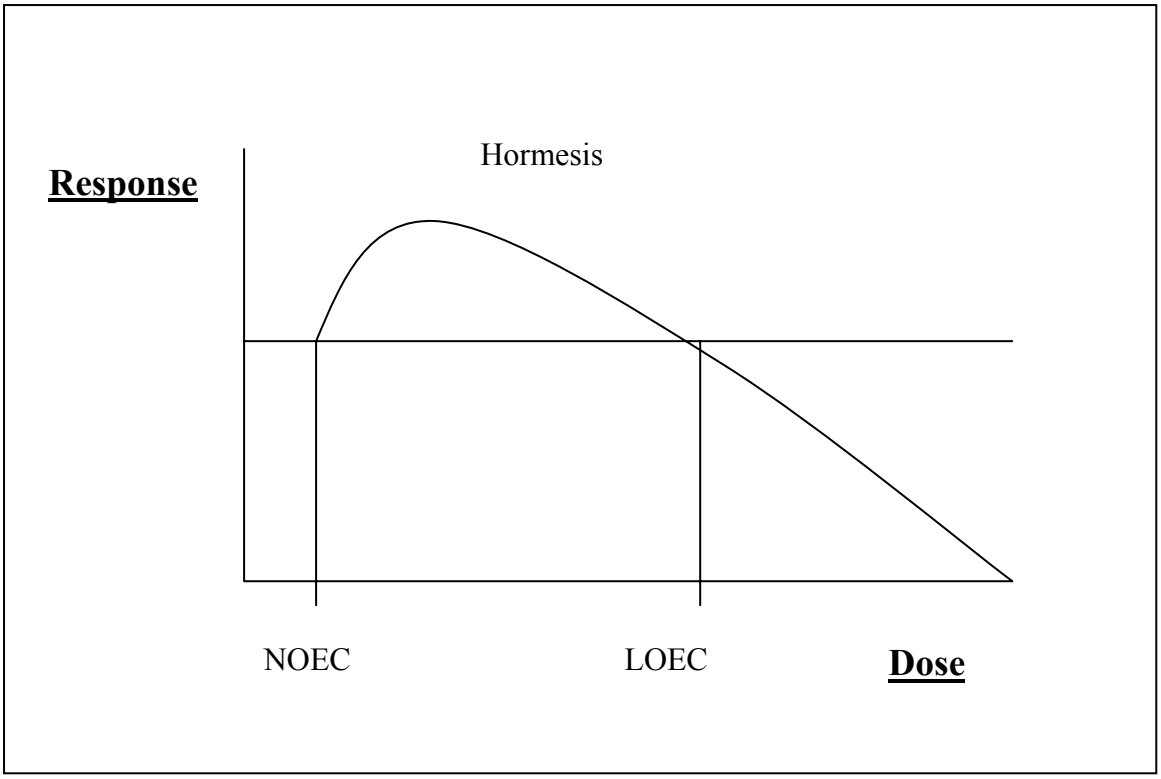

Figure 8.2 The β dose-response curve showing hormesis.

Boxenbaum 1994), reproductive responses, and survival. As a result of their analysis they concluded that hormesis is a widely observed phenomenon (starting with Schultz experimenting with yeasts in 1888, followed by Hueppe in 1896 with bacteria, and then later studies in the 20th century), yet it is not always reported for various reasons. Such reasons include:

1. inappropriate study design, where only a few concentrations are tested, and therefore definite conclusions regarding presence of hormesis cannot be made;
2. general perception of unimportance of beneficial (hormetic) effects in toxicological studies, where usually adverse effects are noted and discussed; and
3. lack of an explicit mechanistic explanation of hormesis.

8.2 PROPOSED MECHANISMS OF HORMESIS

The evolution of thinking how and why hormesis works took place mostly in the 20th century. After the initial discovery of the stimulating effects of then new antibiotics (Miller at al. 1945, Luckey 1956) and synthetic pesticides at low doses (concentrations) (Luckey 1968), there were numerous confirmations of hormesis with yet other new products (see Stebbing 1982), or even with substances previously thought to be without a toxicity threshold, such as some carcinogens (Portier and Ye 1998, Cohen 1995, Andersen and Conolly 1998). In each case, especially in earlier studies, the explanation of a hormesis mechanism (as in this thesis Chapters 5, 6 and 7) was given, or at least attempted, with respect to each particular toxicant and its biochemical effects on a tested organism. With the growing number of reported instances of hormesis for a wide range of biological endpoints and with the wide

range of chemicals representing different chemical groups, there emerged a tendency to generalize this phenomenon (Hayes 1975). Stebbing (1998) proposed a hypothesis that "hormesis is the cumulative consequence of transient and sustained over-corrections by rate-regulating control mechanisms to low levels of inhibitory challenge." However, he acknowledges (since he was only discussing growth hormesis), that it is not clear how this can explain other types of hormesis. Calabrese (1999) experimentally confirmed that growth hormesis represents an overcompensation to a disruption in homeostasis.

8.3 DISCUSSION OF THE RESULTS OF THE CURRENT PROJECT WITH RESPECT TO HORMESIS

The initial objective of the current project was to investigate the effects of low sublethal concentrations of commonly used pesticides, mostly their toxicity at low levels on non-target organisms. However, when results were examined, an interesting trend emerged, where at low concentrations, practically no adverse effect producing concentrations, of Gly and CPF, the investigated organisms improved their performance, i.e. hormesis was observed. First it was interpreted as a one off result, but with the continuation of the study, more evidence was collected that this was a rather common response of the organisms to the low concentrations of chemicals in question. Summarising the results of the current project with respect to observed hormesis I conclude that:

1. When exposed to CPF both species of algae exhibited hormesis at environmentally realistic concentrations (around 0.5-1% of EC_{50}).

2. When exposed to Gly and RB the alga *P. subcapitata* exhibited hormesis at environmentally realistic concentrations (around 4-7% of EC_{50}).
3. When exposed to CPF, daphnids showed hormesis for time to the first brood (both first and second generations), and number of offspring per female (second generation) at low, environmentally realistic concentrations, however the third generation demonstrated an increase in sensitivity to CPF with the increase of the pre-exposure pesticide concentration, i.e. the 48-h LC_{50} of the animals from the higher pre-exposure concentration was lower than those from the lower pre-exposure concentration.
4. When tested with Gly in different types of media there was hormesis present in daphnids cultured in sea salt water but there was no indication of hormesis in M4 medium, suggesting that improved performance in exposures to this chemical is dependent on media type. Similarly no hormesis was observed initially in daphnids cultures exposed to RB in M4 medium.
5. When *Daphnia* were exposed to low concentration of cadmium in the presence of RB in M4 medium, the toxicity of Cd to at least one endpoint was reduced in almost all Cd treatments. Also *Daphnia* showed improved performance in RB Cd-free treatments compared to control, contrary to the results of the previous experiments. Clearly these contradictory results require more studies on the subject of RB-induced hormesis.

In almost all experiments improved performance in algal and *Daphnia* cultures was observed. This suggests (together with numerous examples in literature) that this effect has a common underlying cause, suggestive of a defence response to the toxicant exposure. However, as the experiment with Cd and RB indicated, some

chemicals can reduce the toxicity of another, while simultaneously having a stimulatory effect on the organism exposed to them (see Chapter 7.2)

Hormesis may be regarded as a beneficial effect for an organism; after all it often results in a larger size and the ability to produce more offspring, as observed in this project and other studies. However, when subsequent generations were exposed to the same concentrations of chemicals, the advantage is reduced (as in the study of *Daphnia* and Gly), or even eliminated completely. For example in the case of *Daphnia* exposed to CPF, the third generation animals, whose parents and grandparents were also exposed to CPF, showed increased sensitivity towards CPF, i.e. greater toxicity observed in pre-exposed animals. The same concentration, which produced hormesis in the first and second generations, produced greater toxicity in the third (see Chapter 6).

There was no hormesis observed in acute short-term exposures to the chemicals studied here, however it is quite common to have hormesis present in such exposures (Calabrese and Baldwin 1997). Van Ewijk and Hoekstra (1993) and later Enserink and Van der Hoeven (1993) discussed the importance of inclusion of hormetic data into calculation of LC_{50} values. Usually the LC_{50} is calculated ignoring or discarding data that do not fit into a logistic model. Ignoring data gives lower LC_{50} (overestimation of toxic effect), and discarding - much higher values (underestimation of toxicity), while a proposed linear logistic model gives an intermediate, more realistic LC_{50} (Enserink and Van der Hoeven 1993). This new approach is important in setting goals for environmental concentrations of toxicants, as it changes the LC_{50} values, and consequently, calculated trigger values for the acute and chronic

environmental exposures based on LC_{50}. However, hormesis at sublethal concentrations should not be ignored. The results of the current project suggest that population characteristics can be affected at low environmentally realistic concentrations of at least some toxicants. Though these effects are not considered adverse for the organisms tested, they can tip the balance of a community, for example, potentially producing algal blooms, or create a surge in a population numbers of species – disease carriers (mosquitoes, snails). Chapman (1998) argued for the consideration of multiple working hypotheses to explain such complex phenomena as hormesis and essentiality of chemicals, and warns about the danger of favouring one particular theory.

Agricultural pesticide use remains high, and resurgence of pest insects and secondary pests outbreaks are commonly observed following insecticide application, and lately more attention is given to the hormesis effect in target organisms in pesticide applications (Morse 1998). In order to compensate for hormesis in such cases, a greater dose of a pesticide would be required to be effective in eliminating a pest organism, leading to the increased burden of this pesticide in the environment. This increased burden may coincide with the concentrations that produce hormesis in non-target organisms. Bailer and Oris (1998) argue, that if hazard identifications are carried out at low levels of this hazard (for example, in field settings), only stimulatory effects of a chemical might be observed resulting in a false negative error. This in turn can create additional difficulties in deriving trigger values for chemicals and setting goals for water quality criteria. Lately there was a call to incorporate hormesis in routine testing of hazards (Bailer and Oris 1998) in order to provide for its regulatory implications (Calabrese and Baldwin 1998b, Foran 1998) as more data

on hormesis becomes available. Because hormesis in one generation is not necessarily carried on to the next generations, but in fact can be reversed (as the current project indicated), more laboratory studies are needed on multiple-generation long-term toxicity assessments. There should however, also be a careful comparison of field and laboratory data. The laboratory exposures are usually drastic, while organisms in the wild are under constant stress, hence the differences between datasets (Chapman, 1998). Relying exclusively on laboratory data in setting environmental goals must be avoided; but to underestimate the importance of finely tuned laboratory experiment is equally dangerous, as they provide information otherwise unavailable from field observations only.

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APPENDICES

APPENDIX 1

PESTICIDES INFORMATION

Glyphosate

Chemical name: N-(phosphonomethyl)glycine

Chemical family: organophosphorus

Molecular formula: $C_3H_8NO_5P$

Molecular weight: 169.08

Corrosiveness: corrosive to iron and galvanised steel

Solubility: in water at 25^{o}C, 12 g/L. The alkali metal and amine salts are readily soluble in water. Insoluble in most organic solvents.

Mode of action: Non-selective systemic herbicide, absorbed by the foliage, with rapid translocation throughout the plant. Glyphosate inhibits the enzyme 5-enolpyruvyl shikimate-3-phosphate synthase thereby preventing the biosynthesis of aromatic amino acids. The shikimate pathway is the biosynthetic rout to the aromatic amino acids tryptophan, tyrosine and phenylalanine as well as a large number of secondary metabolites such as flavonoids, anthocyanins, auxins and alkaloids. Glyphosate is a post-emergence herbicide and has been shown to be translocated in the plant's phloem to meristematic tissue, underground storage organs and stem apices. It has no pre-emergence activity. Glyphosate forms comptex with metal ions. Metal ion-complexing capacity of glyphosate:metal ions strongly influence glyphosate absorption and translocation and vice versa; however it is not known if glyphosate has a significant effect on

intracellular distribution and availability of metal ions. Glyphosate also stimulates the formation of total nitrogen (mineralization of N).

Uses: Control of a great variety of annual, biannual, and perennial grasses, sedges, broad-leaved weeds, and woody shrubs. Used in fruit orchards, vineyards, conifer plantations, and many plantation crops (e.g. coffee, tea, bananas, rubber, coconut, palms, cocoa, mangoes); post-weed emergence but pre-crop-emergence in a wide range of crops (including vegetables, beet, lucerne, okra, soya beans, figs, kiwi fruit, olives, cucurbits, cereals, cotton, etc.); on non-crop arrears; immediately pre-harvest in ripened cereals; in cereal stubble; and in pasture renovation. Also used for pre-harvest desiccation of cotton, cereals, peas, beans, etc.; for destruction of rye sown to prevent wild erosion of the soil; for control of suckers on fruit trees; and for aquatic weed control.

Degradation and metabolism:

Environmental: Strongly adsorbed to soil. Microbial degradation is the major cause of loss from soil, with liberation of carbon dioxide. Half-life in soil is normally less than 60 days. Half-life in pond water from 12 days to 10 weeks.

In plants: Glyphosate is not metabolised in plants.

Chlorpyrifos

Chemical names: *O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate (IUPAC)

O,O-diethyl *O*-(3,5,6-trichloro-2-pyridinyl phosphorothioate (CA)

Chemical family: organophosphorus; pyridine

Molecular formula: $C_9H_{11}Cl_3NO_3PS$

Molecular weight: 350.62

Corrosiveness: corrosive to copper and brass

Stability: Stable in neutral and weakly acidic media. Hydrolysed by strong alkalis.

Solubility: in water at 25^{o}C, ca 2 mg/L. In Benzene 7900, acetone 6500, chloroform 6300, carbon disulphide 5900, diethyl ether 5100, xylene 4000, dichloromethane 4000, isooctane 790, methanol 450 (all in g/kg at 25^{o}C).

Mode of action: Non-systemic insecticide with contact, stomach, and respiratory action. Cholinesterase inhibitor.

Uses: Control of soil insects and some foliar insect pests on a wide range of crops, including pome fruit, stone fruit, nut crops, strawberries, figs, bananas, vines, vegetables, potatoes, beet, tobacco, soya beans, sunflowers, sweet potatoes, ground nuts, rice, cotton, lucerne, cereals, maize, sorghum, asparagus, glasshouse and outdoor ornamentals, mushrooms, turf, and in forestry. Control of insect pests in stored products, household insect pests (including ants and cockroaches), flies and other insects in animal houses, and mosquitoes (adults and larvae). Also used as an animal ectoparasiticide.

Degradation and metabolism:

Environmental: In soil, chlorpyrifos is slowly degraded, with a half-life of ca. 80-100 days, to 3,5,6-trichloro-2-pyridinol, which is subsequently degraded to organochlorine compounds and carbon dioxide.

In animals: In rats, dogs and other mammals, following oral administration, rapid metabolism occurs, with the principal metabolites being 3,5,6-trichloro-2-pyridinol and monoethyl chlorpyrifos. Excretion is principally in the urine.

APPENDIX 2

MEDIA RECIPES

Tamiya medium for algae (Vasser 1989)

KNO_3	5 g/L
KH_2PO_4	1.25 g/L
$MgSO_4 \cdot 7H_2O$	2.5 g/L
Disodium EDTA	37 mg/L
Fe solution (x1000)	1 mL
Microelements solution (x10)	0.3 mL

Fe solution (concentrated 1000 times)

$FeC_6H_5O_7$	25 g/L	or
$FeSO_4 \cdot 7H_2O$	3 g/L	

Microelements solution (concentrated 10 times)

H_3BO_3	28.6 g/L	
$MnCl_2 \cdot 4H_2O$	18.1 g/L	
$ZnSO_4 \cdot 7H_2O$	2.22 g/L	
NH_4VO_3	0.23 g/L	
MoO_3	0.18 g/L	or
$Na_2MoO_4 \cdot 2H_2O$	0.30 g/L	

Keating medium for algae (Keating 1985)

"M" solution

Disodium EDTA	5 mg/L
H_3BO_3	5.7 mg/L
$FeCl_3 \cdot 6H_2O$	1.2 mg/L
$MnCl_2 \cdot 4H_2O$	0.7 mg/L
LiCl	0.6 mg/L
RbCl	0.1 mg/L
$SrCl_2 \cdot 6H_2O$	0.3 mg/L
NaBr	0.06 mg/L
$Na_2MoO_4 \cdot 2H_2O$	0.1 mg/L
$CuCl_2 \cdot 2H_2O$	0.067 mg/L
$ZnCl_2$	0.052 mg/L
$CoCl_2 \cdot 6H_2O$	0.020 mg/L
KI	0.006 mg/L
SeO_2	0.0014 mg/L
NH_4VO_3	0.0011 mg/L

"S" solution

Glycylglycine (buffer)	250 mg/L
$NaNO_3$	150 mg/L
$CaCl_2 \cdot 2H_2O$	38 mg/L
$MgSO_4 \cdot 7H_2O$	20 mg/L
$Na_2SiO_3 \cdot 9H_2O$	145 mg/L
KCl	10 mg/L
K_2HPO_4	10 mg/L
KH_2PO_4	25 mg/L

Vitamins

B_{12}	1 μg/L
Biotin	0.75 μg/L
Thiamine (HCl)	75 μg/L

Concentrated (x6) solutions "M" and "S" can be made separately and later combined to make up Keating (A-MS) algal medium (pH=8.5 before autoclaving, 7.75 – after).

M4 medium for daphnia

"A" solution (concentrated 2000 times)

Disodium EDTA	5000 mg/L
$FeSO_4 \cdot 7H_2O$	1991 mg/L
H_3BO_3	5719 mg/L
$MnCl_2 \cdot 4H_2O$	721 mg/L
LiCl	612 mg/L
RbCl	142 mg/L
$SrCl_2 \cdot 6H_2O$	304 mg/L
NaBr	32 mg/L
$Na_2MoO_4 \cdot 2H_2O$	126 mg/L
$CuCl_2 \cdot 2H_2O$	33 mg/L
$ZnCl_2$	26 mg/L
$CoCl_2 \cdot 6H_2O$	20 mg/L
KI	6.5 mg/L
Na_2SeO_3	4.38 mg/L
NH_4VO_3	1.15 mg/L

"B" solution (concentrated 100 times)

$CaCl_2 \cdot 2H_2O$	29.38 g/L
$MgSO_4 \cdot 7H_2O$	12.33 g/L
KCl	580 mg/L
$MaHCO_3$	6.48 g/L

"C" solution (concentrated 1000 times)

$Na_2SiO_3 \cdot 9H_2O$	1 g/L
$NaNO_3$	27.4 mg/L
K_2HPO_4	18.4 mg/L
KH_2PO_4	14.3 mg/L

Vitamins (concentrated 1000 times)

Thiamine (HCl)	75.0 mg/L
B_{12}	1.0 mg/L
Biotin	0.75 mg/L

Solutions A-C prepared individually with Milli-Q water. Vitamins solution can be stored frozen.

Total hardness of the medium is 2.5 mmol/L, alkalinity 0.9 mmol/L, conductivity 610 µS/cm, pH=8.2±0.2.