ECOTOXICOLOGY OF NEOTROPICAL FRESHWATER ZOOPLANKTON SPECIES EXPOSED TO TOXIC MIXTURES

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By

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Declaration

This thesis has been composed in its entirety by the candidate and no part of this work has been submitted for any other degree.

Abstract

Chemical pollutants in the environment rarely occur as single compounds, but rather as more or less complex mixtures. Aquatic ecosystems near agricultural areas are particularly prone to impact from simultaneous or sequential exposures to combinations of different pesticides, yet environmental regulators normally limit risk assessment to the effects of individual chemicals. Reactive substances such as pesticides or metals have been found to produce interactive effects that cannot be predicted from effects of individual compounds. The precise effects of a mixture will however depend on its composition (in terms of substances present and their relative amounts) as well as the organisms and toxicity parameters investigated.

For the assessment of pesticide impacts on a sensitive wetland system in south-east Mexico a standardised bioassay with a standard cladoceran test species, *Ceriodaphnia dubia*, and two locally isolated tropical species, *Ceriodaphnia cornuta* and *Simocephalus vetulus*, was developed. The bioassay determined inhibition of cladoceran feeding rates as a sensitive sublethal endpoint for assessing effects of low, environmentally realistic concentrations of three pesticides and one metal. Zooplankton feeding is an important ecological parameter in aquatic systems, influencing rates of nutrient cycling and affecting all trophic levels.

The feeding inhibition bioassay demonstrated feeding rates of the cladocerans to be significantly reduced at sublethal exposure concentrations to aqueous solutions of the pyrethroid pesticide deltamethrin, the herbicide atrazine, and the non-essential metal cadmium. The organophosphate pesticide chlorpyrifos did not inhibit feeding at sublethal concentrations. Median 24 h feeding inhibition values ($24hIC_{50}s$) compared to lethality values ($24hLC_{50}s$) for *C. dubia* were 0.19 $\mu g\ L^{-1}$ deltamethrin (IC_{50})

compared to 0.49 μg L^{-1} (LC_{50}), 5.31 μg L^{-1} compared to 68.8 μg L^{-1} cadmium, and 13.23 mg L^{-1} compared to 30 mg L^{-1} atrazine, when food was present at equal concentrations. LC_{50}s were established in the presence and absence of food, for 24 and 48 hours, and differences in LC_{50}s and IC_{50}s between species were found.

The effects of binary and tertiary mixtures of deltamethrin, atrazine and cadmium on feeding rates were assessed by comparing observed to predicted toxicity (% feeding inhibition). All binary and tertiary mixtures were found to produce a greater-than-additive effect, with the binary and tertiary mixtures of atrazine and deltamethrin producing a pronounced more-than-additive effect, with very high mixture effects at lower individual concentrations, levelling off exponentially towards the total inhibition of feeding. Individual compounds below their NOEC were found to contribute to the overall toxicity of the mixture

Findings show that interactive effects of mixtures of reactive chemicals can be assessed from studying sensitive sublethal endpoints, such as feeding inhibition. Effects of binary and tertiary mixtures can differ from those expected from either individual effects or models such as the concentration addition (CA) or independent action (IA) models. The establishment of a database on combination effects of different groups of compounds could allow the use of this bioassay as a predictive tool for assessing effects of mixtures of pesticides and metals on individuals and population dynamics of zooplankton.

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CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Aquatic toxicology is an area of growing concern for scientists worldwide. Continuing human population growth and its demands for industrialisation and development lead to natural systems being increasingly affected by the negative impacts of modern civilisation. One area of major concern is the contamination of the planet's aquatic systems with a multitude of anthropogenic chemicals. Pesticides introduced into aquatic systems either purposefully for the control of excessive plant growth, insect vectors, or diseases and parasites in aquaculture, or through runoff, spray-drift or groundwater leaching, are an important group of aquatic contaminants due to their toxicity and omnipresence (Rand, 1995). Metals are also ubiquitous as pollutants, being released into the environment through mining and smelting processes as well as multiple industrial sources, and entering aquatic systems through wet and dry deposition, urban runoff, and various effluents, accumulating in organisms and sediments due to their persistence and their specific chemical properties which affect accumulation and sediment sorption (Nriagu, 1990). It has long been recognised that chemical pollutants in aquatic systems are present as mixtures of varying complexity rather than as single substances. However, this fact is still only beginning to be considered by regulators, since the assessment of the toxicity of such mixtures is very difficult due to the sheer number and varying amounts of the different chemicals present (EIFAC, 1987; SCOPE, 1987; Steevens & Benson, 2001).

The effects of chemicals on individuals, populations, communities, and ecosystems depend on a multitude of different factors, and differences in climate, exposure regimes, as well as species-specific senstivities, require to be taken into account when investigating and predicting consequences of aquatic pollution. So far, most ecotoxicological research has been carried out in temperate countries, involving species and exposure regimes typical of those areas. However, developing countries in tropical areas are increasingly feeling the same pressures on their natural systems, as industrialisation and intensive agricultural practices are progressing (Farnworth & Golley, 1974; Henriques *et al.*, 1997; Peters *et al.*, 1997). Therefore, there is a growing need for the investigation of pollutant effects on tropical aquatic systems, involving locally representative species, compounds and investigative tools (Castillo *et al.*, 1997; Castillo, 2000).

This study is part of a project funded by the European Union, studying the effects of agrochemicals on tropical wetland systems. The area of study that this work is involved in is a large river system in the Yucatan Peninsula of Mexico: the Rio Palizada river delta, which includes a large tidal lagoon called Laguna de Terminos and extensive riverine wetlands. Wetlands in this area are affected by pesticide runoff from many farmed areas, including large citrus fruit and tobacco plantations, as well as numerous smaller farmed areas planting vegetables. The aim of this study was to identify local species of zooplankton and to develop a bioassay that could be used to assess the effects of low, environmentally relevant concentrations of combinations of pesticides, using those local species and a more established standard test organism, the cladoceran *Ceriodaphnia dubia*. This bioassay was developed initially for the use in the laboratory, with the aim of adapting it for subsequent *in situ* studies.

The relative effects of a selection of compounds on the different species will be evaluated, and the potential assessment of mixture effects of those compounds on a sublethal, ecologically relevant parameter will be determined.

1.2 THE TROPICS AND TROPICAL AQUATIC SYSTEMS

The tropics extend from the tropic of cancer at 23°27′ N to the tropic of Capricorn, 23°27′ S, comprising three main geographical regions: Central and South America, Africa, and Australasia. The tropics share with other latitudinal belts the prevalence of three environmental cycles of fixed period- the diel, lunar and annual. Discussion of tropical distinctiveness has centred on the annual (seasonal) cycle, with occasional emphasis on the diel one (Talling & Lemoalle, 1998).

The absence of much environmental seasonality typical of high latitudes, most notably the thermal effects of a marked radiation minimum, has profound implications for population ecology. Tropical cycles are more often ruled by rainfall patterns, leading to a very different seasonality than in temperate regions.

Within the latitudinal region of the tropics, other gradients can have important consequences on the climate, such as East to West climate gradients, often related to transverse mountain ranges, (e.g. Andes, Ghats of India) and a transition from oceanic to continental conditions. Altitudinal gradients, which affect temperature and rainfall, are also important.

Tropical freshwater systems are very distinct from those in temperate regions, in terms of their climatic, physical and chemical conditions as well as their ecology and biology.

The scientific exploration of tropical inland waters (tropical limnology) has mostly occurred in the twentieth century, especially the second half, seriously lagging behind

that of temperate limnology. Most early work in the tropics, before 1925, was based on short-term expeditions with taxonomic, faunistic and floristic aims, which did not greatly advance the knowledge of ecological dynamics. Since 1980 the comparative and generalised aspects of tropical limnology have been developed considerably, with studies requiring a sufficient background of site-specific or region-specific in-depth studies (Lewis *et al.*, 1995; Talling & Lemoalle, 1998). More work has been carried out on the ecology of large rivers, the chemical fluxes of which are of considerable global interest, than on that of small streams and their invertebrate fauna. The biology and ecology of fish populations has also received a considerable amount of research effort. Tropical wetlands have attracted attention, and, in recent years, much work has concentrated on floodplain systems.

1.2.1 Environmental conditions influenced by tropical latitude

The environmental conditions in tropical freshwaters are influenced via three main pathways:

- **Solar radiation input:** this is the primary influence, with important consequences for the temperature regime. The natural daylength photoperiod is precise and of limited seasonal range, with minimum and maximum values, at the northern and southern boundaries of the tropics, of 10.6 and 13.6 hours.
- **Geostrophic influence:** the effect of the earth's rotation (Coriolis force) is minimal, affecting the motion of large moving masses of fluids whether in the atmosphere, oceans or lakes. The reduction of this force allows for lakes a greater effectiveness of wind-induced vertical mixing, enhancing the depth of an upper mixed layer (Lewis *et al.*, 1995).
- **Air-mass circulation:** influenced by latitudinal belts of pressure differentials. These include higher-pressure regions in the subtropics and a seasonally migrating equatorial trough and intertropical convergence zone (ITCZ) of lower pressure and upwelling. The results are a seasonality in tropical wind patterns as well as a broad tropical belt of generally elevated and often very seasonal rainfall (Talling & Lemoalle, 1998).

In addition, there are other features connected with the chemical denudation and water-leaching of tropical land masses, and some influence of the modern tropical environment on chemical pathways (e.g. silicate weathering, soil laterisation, denitrification) and their chemical species (e.g. silicic acid, nitrate), which in varying concentrations enter freshwaters.

1.2.2 Chemical composition of tropical soils and inland waters

Tropical rivers tend to have a lower total ionic concentration compared to temperate ones, with much lower and less dominant levels of calcium and bicarbonate ions. Instead, tropical freshwaters often contain higher levels of sodium and chloride ions, as well as silicate and iron. These differences are the product of geological and climatic variation, which in turn indicate some fundamental differences between tropical and temperate environments.

The interaction of rainfall, rocks, soil, and vegetation, is of great importance for the chemical composition of freshwaters and the concentrations of minor chemicals, some of which are important as plant nutrients. The high silicate levels are due to the interaction of a generally high soil pH, which increases the solubility of silicate, as well as the high temperatures. The pH of tropical soils are generally between 5.8 and 7.5, compared with temperate soil pH of between 4.0 and 5.8 (Payne, 1986). The pH in tropical rivers also tends to be variable, between 4.3 and 7.5, due to the generally low buffering capacity.

The high nutrient leaching of tropical soils is a result of a combination of high temperatures, prolonged and intense sunlight, and high rainfall, leading to extremely weathered soils, which are low in available ions. This means that the amounts of essential chemicals such as nitrogen and phosphorus that are accumulated in the vegetation are extremely important. Tropical wetlands are areas with abundant plant growth, and often associated with large quantities of decaying organic material under conditions of low flow. Nutrients like nitrates and phosphorus can often be very reduced due to their utilisation by plants. The extreme decomposition of this organic material produces conditions of very low oxygen levels and acidic pH, which can lead to the release of iron from organic complexes in the sediment. Floodplains on rivers

can have similar effects on ionic composition of water due to large amounts of decomposition, which occurs when surrounding terrestrial areas are flooded. Conditions in these floodplains are very variable, with phytoplankton blooms occurring as a result of flooding with nutrient-rich river water, which in turn quickly deplete nutrients. The rapid recycling of nutrients by animals seems to generally increase fertility and productivity in these waters (Lewis *et al.*, 1995; Talling & Lemoalle, 1998).

Concentrations of organic suspended solids in tropical waters (rivers) are often very high, with the typical brown humic and fulvic acids derived from the breakdown of leaves and other plant material sometimes leading to conditions of greatly reduced transparency. However, the concentrations of inorganic ions are generally low, leading to low conductivities ($<100\mu S\ cm^{-1}$).

Nutrient concentrations in rivers are also highly seasonal, and during the rainy season, nutrient concentrations can become greatly reduced due to dilution, whereas the suspended solids can increase with the runoff from soils. In lakes and river deltas, relatively high nutrient and ion concentrations can sometimes be achieved from the inflow from estuaries and rivers as well as leaching from soils and with runoff from the rain, and they concentrate in lakes due to evaporation, biological turnover and interactions with the sediments (Payne, 1986; Talling & Lemoalle, 1998).

The often pronounced thermocline in lakes can have the effect that, while algae and detritus sink to the bottom, gases and oxygen cannot diffuse down, leading to anoxic conditions in deeper layers of the water body and in the sediment. Large amounts of organic material, which typically accumulate on the bottom of lakes and ponds, can bind and sequester chemicals, which can become resuspended in conditions of drying out, flooding, heavy rainfalls or storm events.

Decomposition and mineralisation processes can be faster in the tropics due to higher temperatures and high humidity, and the capacity of tropical systems to absorb and break down organic waste from pollution can be greater. However, these capacities are limited due to the generally already higher amounts of organic matter present (Payne, 1986; Lewis *et al.*, 1995; Talling & Lemoalle, 1998).

1.2.3 Tropical zooplankton

Temperature and levels of humidity are potentially factors that affect growth rates and stage-duration for many organisms. These climatic factors being generally high and relatively constant in the humid tropics lead to the possibility of higher frequencies of cyclic responses than in temperate regions. The absence of a pronounced seasonality allows organisms a continuous, unphased reproduction and survival, yielding a population structure without marked discontinuities of age frequency (Talling & Lemoalle, 1998). These short generation cycles enable zooplankton populations to respond faster to changes. Where changes are minimal, zooplankton populations are stable, and reproduction and survival are continuous (Talling & Lemoalle, 1998). The appearance of males and resting eggs within cladoceran populations is often related to events of water bodies drying out.

Cladoceran populations in the tropics are, as in temperate systems, dependent on the phytoplankton population dynamics. The phytoplankton is inherently susceptible to changes in both the radiation intensity / temperature as well as water balance. The reactions upon physical and especially chemical environmental factors can be profound, and are often cyclic in time. Biotic interactions including grazing can introduce further temporal change. The main environmental factors affecting phytoplankton populations are restratification events, and in shallow lakes and rivers, the main effects are through major, usual seasonal, water inputs, which lead to a

deepening of the water column, reduced light penetration due to introduced silt, washout effects, and more positively, injection of nutrients (Lewis *et al.*, 1995).

Zooplankton population dynamics are also influenced by predation and interspecific competition, which can lead to species-specific diel migration patterns in cladocerans (Zaret & Suffern, 1976; Matsumura-Tundisi *et al.*, 1984; Talling & Lemoalle, 1998).

Cladocerans, especially the genus *Daphnia*, are uniquely effective grazers of phytoplankton in freshwater systems of the world. Their activity determines relationships among chlorophyll a, nutrients, phytoplankton diversity and the magnitude of trophic cascades (Tessier & Bizina, 2001). Their foraging mode explains much of their ecological dominance, as they consume a broader range of particles than rotifers, copepods and also other cladocerans, such as the *Bosminiidae*. The filtration rate as well as size of particles consumed by cladocerans (e.g. *Daphnia*) increases with body size. Feeding efficacy not only depends on body size but also on the ability of larger species to be more efficient grazers since they can digest algal cells with resistant protective structures such as thick cell walls or gelatinous sheaths (Tessier & Bizina, 2001). However, filtration rate and feeding efficacy seem to be species-specific (Tessier & Bizina, 2001).

The cladoceran community in the tropics generally differs from that in temperate regions. Zooplankton and specifically cladoceran communities in tropical freshwaters are reportedly composed of smaller species, with a lower mean body size, than communities in temperate areas. This smaller average size is partly due to the absence of the larger *Daphnia*, which has been reported for Africa (Dumont, 1980), Asia (Fernando, 1980a), as well as for South America (de Infante, 1985) and North Australia (Tait *et al.*, 1984).

Some differences in opinion exist concerning the species diversity of tropical cladocerans. Some reports describe the tropical cladoceran communities as less diverse than temperate ones, as most of the large *Daphnia* as well as all known predacious cladocerans are absent (Fernando, 1980b; Fernando *et al.*, 1987). Others point out that tropical cladoceran communities consist not of less species, but different ones, with the numerous *Daphnia* species that are so dominant in temperate areas replaced by more sidids, moinids and bosminids (Dumont, 1994). Both the sizes of individuals as well as total species richness decline towards the equator for the three major genera: *Daphnia, Ceriodaphnia* and *Diaphanosoma*. On the other hand, *Ceriodaphnia* are much more common in the tropics than in temperate regions, and many authors point out that new species are still being described. The species *Ceriodaphnia cornuta*, for example, which is ubiquitous throughout tropical freshwaters, is most probably a conglomerate of different taxa, possibly composed of up to seven species worldwide (Dumont, 1994).

Reports comparing data on species diversity found throughout the subtropics and tropics show that at least half of the presently known cladoceran species occur exclusively in the tropics and subtropics, often with specific groups limited to particular geographical subzones (Dumont, 1994). Comparing the numbers of species found in average temperate and tropical lakes, Dumont could not establish a consistent pattern of difference in species richness.

Some studies have reported that cladoceran communiies in the limnetic (pelagic) zone of tropical lakes and water bodies do not have fewer species, but exist at lower population densities, which is considered a consequence of the higher prevalent predation pressure in tropical freshwaters. Different explanations are offered for the absence of the larger *Daphnia* and the generally smaller size of cladocerans in the

tropics. Generally, most of the larger *Daphnia* inhabit temporary ponds, while large permanent lakes are populated by small and medium sized species of cladocerans. Larger *Daphnia* are more effective grazers that can outcompete smaller species, however they are also much more vulnerable to predation from planktivorous fishes. In the tropics, the effects of predation usually override those of competition (Dumont, 1994). In tropical inland waters, fish are much more numerous, and a large proportion of them remains planktivorous throughout their life. There is also no interruption in predation during the winter. Many researchers therefore regard the intense (and uninterrupted) temperature-dependant predation in tropical freshwaters as the main reason for the absence of larger *Daphnia* species (Fernando, 1980a; Fernando, 1994; Dumont, 1994).

The high temperatures however have other direct and indirect effects on the cladoceran species composition. A high water temperature certainly limits the larger *Daphnia.* Many studies have found that high temperatures seem to negatively affect the larger species, reducing their fecundity and feeding efficiency (Fernando, 1980a; Moore & Folt, 1993; Moore *et al.*, 1996; Gillooly & Dodson, 2000).

Studies measuring cladoceran performance at different temperatures show that a net energy deficit can occur at higher temperatures, resulting from respiration rates that are more accelerated relative to rates of ingestion and excretion (Gophen, 1976).

Several recent studies show a strong interaction between body size, food concentrations and elevated temperatures. The threshold food concentration of an animal describes the food concentration where respiration exactly balances assimilation, so that no net energy is available for individual growth or reproduction. Work by Lampert (1977) on *D. pulex,* and by Vidal (1980) on the marine copepod *Calanus*, show that threshold food concentrations increase at a faster rate for larger

individuals as water temperature increases, leading to energy deficits for large animals at higher temperatures. For cladocerans at least, this could be due to the action of thoracic limbs which are used both for feeding and ventilation. At elevated temperatures, the increased oxygen demand, exacerbated by lower dissolved oxygen levels in the water, instigates the thoracic limbs to beat more rapidly, causing the gut to fill faster. However, rates of gut passage may not be able to increase simultaneously, limiting the rate of food processing and ultimately uptake (Gophen, 1976).

This would suggest that larger individuals, and possibly larger species, are less successful at elevated temperatures unless food is abundant. Abundance of phytoplankton in tropical lakes and freshwater bodies is variable, and large blue-green algae, which cannot be consumed by the *Daphnia*, are often dominant.

Moulting could also constrain the body size of cladocerans, as it has been shown that the cost of moulting in crustaceans increases exponentially as the animal becomes larger (Moore & Folt, 1993). At elevated temperatures, development is accelerated, leading to more frequent moults. Consequently, the combined costs of development (moulting) and respiration at higher temperatures could reduce the energy available for somatic growth and reproduction. The smaller body size of cladocerans and copepods at higher temperatures is often associated with a reduction in fecundity, which would support this explanation (Moore & Folt, 1993).

Whatever the main reasons are, the mean body sizes of cladocerans seem to be strongly related to latitudinal patterns. Gillooly and Dodson (2000) carried out a thorough analysis of available data on the size of cladocerans in over 1100 water bodies throughout the Western Hemisphere and found that the mean body length of cladoceran species is greatest in North temperate regions, declining toward the poles

and equator. Mean cladoceran size is greatest in regions where the mean annual surface temperatures of lakes ranges from 6 to 8°C, both in the Northern and Southern Hemisphere. However, in lakes at higher altitudes within the tropical zone, where temperatures are comparable to those in temperate regions and seasonality does occur, larger *Daphnia* species can be found (Dumont, 1994; Gillooly & Dodson, 2000).

1.2.4 Implications for tropical ecotoxicology

The fact that tropical freshwater systems are so different from temperate ones in their physical, chemical and biological parameters, suggests that it is important to consider the ecotoxicological effects of chemicals in tropical aquatic systems separately from those known in temperate aquatic systems. Traditional ecotoxicological research has been mainly developed in temperate countries, and with organisms and conditions that are relevant to temperate systems. However, organisms and conditions in tropical systems are very different, and the climatic factors that lead to those differences may also have serious implications for the ecotoxicity of anthropologically introduced toxic substances. Therefore, it is necessary to find different approaches when investigating ecotoxicological effects in tropical freshwater systems.

Very little research has been carried out on ecotoxicology in tropical systems, and not much is known so far on how the complex ecological relationships, as well as individual species, within tropical freshwater systems are being affected by the increasing amounts of anthropologically released chemicals.

When looking at the influences of climatic and environmental factors, it is important to differentiate between their influences on the fate of released chemicals, and the influences (impacts) on actual effects within ecosystems.

1.2.4.1 Fate and degradation of pesticides under tropical conditions

Studies on the fate of agrochemicals under tropical conditions come from laboratory, mesocosm, and field studies. The main climatic and environmental parameters affecting the fate, as well as ultimately the ecological effects, of chemicals and pesticides in tropical aquatic systems, which are different to temperate ones, are light, temperatures, rainfall regimes and the amounts of organic matter present. The elevated temperatures and high solar radiation affect direct and indirect photolytic breakdown processes. The often seasonal and very high as well as sudden amounts of rainfall can lead to increased pesticide runoff and leaching to groundwater, as well as affecting the concentrations of dissolved and particulate organic matter, the water hardness, pH and dissolved oxygen levels.

Many studies have found that pesticide degradation rates can be higher and faster under tropical conditions. Generally, the solubility of all chemicals is temperature dependent, increasing with higher temperatures. The mobility of chemicals, their uptake and removal rates are all increased at higher temperatures and humidity (Klein, 1989). Increased light intensity enhances rates of degradation either directly or via the formation of active species (photolysis). The rates of hydrolytic breakdown of chemicals are also temperature, as well as pH, dependent. Hydrolysis rates are higher at a lower pH, and are greatly increased at high temperatures, making it a significant parameter of influencing the fate of chemicals in hot climates, whereas it is less significant compared to other loss processes in temperate climates (Klein, 1989). Although hydrolysis can function as a potent detoxification mechanism for a number of pesticides, in this way reducing their bioaccumulation potential, it is generally a transformation process only, and hydrolytic transformation of chemicals can lead to derivatives with unpredicted environmentally relevant physico-chemical and

toxicological properties. Therefore hydrolysis can both increase and decrease the toxicity of a pesticide (Klein, 1989).

In studies on tropical rice paddy ecosystems, hydrolysis has been found to be a major route of chemical degradation for several pesticides, especially in combination with changes in pH and aerobic / anaerobic conditions, which are common due to the practice of intermittent flooding and drying cycles (Abdullah *et al.*, 1997). However, the products of hydrolytic degradation will also accumulate, and further degradation rates can be affected by the anaerobic conditions in flooded soils, which can lead to either a faster or a slower degradation rate depending on bacteria present and on the individual chemical compounds.

Volatilisation of pesticides may also be increased in tropical agraoecosystems, and typically volatile pesticides such as DDT and HCH may be lost more rapidly (Abdullah *et al.*, 1997).

Generally it has been found that the 'loss' of pesticides in a tropical agroecosystem such as the rice paddy field via these different environmental pathways varies greatly depending on the compound, but can be faster under tropical conditions (Armbrust, 1999). Sethunathan also found the persistence of pesticides such as organochlorines, organophosphates and carbamates to be shorter under tropical, hot and humid conditions than under colder or drier ones (Sethunathan, 1989).

However, many studies on the degradation and fate of pesticides in the environment conducted in the tropics only include the parent compounds, and not degradation products, limiting the interpretation of available data (Castillo *et al.*, 1997).

Microbial degradation rates are also generally faster in tropical regions, as higher temperatures, high moisture and the longer growing seasons favour microbial activities. On the other hand, higher temperature does not automatically guarantee

higher microorganism populations or activities. In a study on pesticide residues, gathered for registration purposes in the USA, where a given pesticide was applied to identical crops in different climatic regions within the USA, no consistent trend, indicating that the disappearance of the pesticides was faster in the hot and humid subtropical regions than in the temperate ones, could be found (Matsumura, 1989; Viswanathan & Krishna Murti, 1989).

Another topic of discussion is the role played by the organic matter. Tropical aquatic systems usually contain high amounts of organic compounds, both as particulate organic carbon (POC) as well as dissolved organic carbon (DOC) (Talling & Lemoalle, 1998). The concentration of total organic carbon in freshwater systems is highly variable, ranging from $<1 mg\ C\ L^{-1}$ in alpine streams to $>20 mg\ C\ L^{-1}$ in tropical or polluted rivers. In tropical inland waters, a large proportion of this organic matter is usually in the form of fulvic and humic acids.

The behaviour of anthropogenic chemicals is strongly affected by the organic compounds. The solid forms can take up pollutants, such as heavy metals and organic chemicals, and enhance their descent through water columns to the underlying sediments. They can also make pollutants more available to filter-feeding organisms. Similarly, some of the dissolved forms can form complexes with inorganic cations and some organic substances, as well as affect sorption processes with solids, or bioaccumulation by aquatic organisms (Landrum *et al.*, 1987). Some of those lipid-soluble organic complexes can become more toxic in this way than the inorganic metals. Additionally, the reducing abilities of some of the organics can involve them in redox reactions with pollutants (Klein, 1989).

Therefore, organic compounds can either increase or decrease the bioavailability of chemical contaminants to aquatic organisms, acting either as sink or as source.

1.2.4.2 Effects of tropical conditions on ecotoxicology of chemicals, especially pesticides

Multiple studies have investigated the effects of temperature and light intensity on the toxicity of chemicals to various aquatic organisms. Many of them report an increase of toxicity at increased temperatures (Cairns *et al.*, 1975; Sprague, 1985; Brecken-Folse *et al.*, 1994; Howe *et al.*, 1994; Monserrat & Montserrat, 1995; Song *et al.*, 1997).

Factors that can increase toxicity of pesticides to aquatic organisms at higher temperatures are the higher solubility of chemicals in water, a faster rate of uptake and blood flow, and increased bioconcentration. Additionally, levels of dissolved oxygen decrease at elevated temperatures, with limiting oxygen availability enhancing the negative effects of the toxicant (Sprague, 1985; Viswanathan & Krishna Murti, 1989). However, a high solubility of a chemical does not necessarily lead to a high toxitity, as studies by Könemann (1981) with QSARs have shown.

In conclusion, there are several important environmental parameters that differ between temperate and tropical aquatic ecosystems, influencing the effects of pollution on their function and stability. Rates of biological uptake and release can be higher in tropical aquatic systems compared to temperate ones, as can be rates of physiochemical and biological degradation. The rate of oxygen depletion is also higher, as are the biological impact of nutrients and suspended solids. Solubility of liquids and solids is higher, and solubility of gases is lower in tropical systems than in temperate ones, and toxicity thresholds can also be lower in tropical systems (Wolanski, 1992).

Evaluating all those factors mentioned, Saenger & Holmes (1992) argued that tropical organisms might be closer to their limits of adaptation and thus respond differently to externally imposed stress. Due to the relatively uniform and stable conditions in the

tropics, "stress" is generally lower than in temperate systems. Tropical forms may, in some circumstances, be more vulnerable to physical and chemical stress, but more resilient in their response to biotic and abiotic disturbance (Saenger & Holmes, 1992). Generally, many scientists agree that more research is needed for a better understanding of the differences in distribution, degradation, bioavailability, and toxicity of pesticides between tropical and temperate environments, including studies on impacts of repeated or continual low-level exposure to mixtures of pesticides, and involving local species for toxicity testing (Castillo *et al.*, 1997; Henriques *et al.*, 1997; Lahr, 2000).

1.3 TOXICITY OF MIXTURES

The toxicity of mixtures depends on their concentration and composition and on the chemical and physical properties of the individual components, their degradation time and degradation products.

Different models and concepts have been developed to deal with the difficulties involved in studying mixture toxicity, since it is virtually impossible to establish toxicological characteristics for each individual chemical and impracticable to actually test the combination toxicity of all chemicals applied jointly considering the enormous number of possible combinations. Models to predict toxicity of mixtures of chemicals based on their structure-activity relationships have been mainly developed for the large group of non-ionised, relatively inert chemicals that induce acute toxic effects through an unspecific mode of action usually referred to as general anaesthesia or narcosis, such as chlorobenzenes, alcohols, and ketons (Könemann, 1981). However these models have proved less successful for predicting mixture toxicity of

chemicals which cause toxicity through a specific mode of action and at very low concentrations, such as most pesticides.

Mixtures of chemicals are generally expected to induce greater biological effects than those caused by the same concentration of the single compounds (EIFAC, 1987; SCOPE, 1987). However, whether this is the case depends on the characteristics of the individual compounds present in the mixture.

Two different reference models for predicting the toxicity of mixtures of chemicals that are commonly used are the 'concentration addition model' and the 'independent action model'. The applicability of each model depends on the fact whether the chemicals in combination are deemed 'similar' or 'dissimilar'. Calamari and Vighi (1991) state in a report to the Scientific Advisory Committee on Toxicology and Ecotoxicology of the European Community (EU) that, on the basis of available information, the reference model of 'concentration addition' might provide a reasonable expectation for combination effects of 'similar acting chemicals'. A proposal was derived for the establishment of water quality objectives from this reference model. However, controversies exist concerning the different understandings of the term 'similar acting chemicals', and concerning the grouping criteria for chemicals with unknown mechanisms or even unknown modes of action. Also, there are still disagreements about the reference models to be used for the assessment and prediction of combination effects from dissimilar chemicals (Greco *et al.*, 1992; Grimme *et al.*, 1996; Altenburger *et al.*, 2000).

Another area of controversy is whether low, nonsignificant, or nonobservable effect concentrations of dissimilar acting chemical can contribute to the overall toxicity of mixtures.

For combinations of reactive chemicals such as pesticides as well as metals, very different effects have been observed, from less than additive (antagonistic) effects over simple additivity to more than additive (synergistic) effects.

1.3.1 Fundamental concepts and approaches

The way in which toxic substances affect aquatic organisms depends on many different factors. External, chemical and physical factors such as water chemistry, pH, temperature, light, the presence of humic acids influence the reactivity and therefore toxicity of chemicals in the aqueous phase. Secondly, internal physiological factors including adsorption (uptake), transport and distribution internally, metabolic transformation, accumulation, and excretion, may all influence the amount of toxicity of the chemical and its metabolites present within the organism. These processes also may be affected by other water quality characteristics of the ambient environment of the organism that are not necessarily harmful in themselves. When an organism is exposed to two or more potentially toxic substances, there is the possibility of interaction between different physiological processes within the organism, including those affecting the chemicals' adsorption, distribution, transport and release from different compartments, action on receptor sites, metabolism and elimination. All of these may contribute to the response of the whole organism, such as death, growth, avoidance behaviour, and bioaccumulation.

The problem of mixtures of poisons was recognised by a few early workers who mainly carried out work with fish and found that the toxic effects of mixtures could be often calculated by a summation of their individual toxic fractions (Bucksteeg *et al.*, 1955).

Since then, more work has been carried out on effects of mixtures of toxicants on fish and aquatic invertebrates, but much more detailed and comprehensive data is required

for monitoring and modelling the effects of mixtures of chemicals on ecosystems. Data on priority chemicals and mixtures should be obtained from a combination of laboratory and field studies, and consideration should be given to site-specific factors appropriate to the particular ecosystem.

1.3.2 Different chemicals in mixtures

Toxic substances can be grouped in different ways. They can be classified by their mechanisms of action, their target sites, their effects, use, physical state, chemistry, toxicity potential, or impact on aquatic resources (Rand, 1995).

1.3.2.1 Metals and Metalloids

Heavy metals are a group of metallic elements with atomic weights greater than 40 and are characterised by similar electronic distribution in their external shell. These exclude alkali earth metals (e.g. calcium, magnesium), alkali metals (e.g. sodium, potassium), lanthanides and actinides. The broader definition of *trace metals* includes both heavy metals and the latter metals. *Metalloids* are nonmetallic elements, such as silicone and arsenic, which possess many properties similar to those of metals. In aquatic systems, the heavy metals of greatest concern are copper, cadmium, zinc, mercury, and lead. These elements are toxic to organisms above specific threshold levels, but many of them are essential for metabolism at lower concentrations. Other elements of concern are aluminium, chromium, selenium, silver, arsenic, and antimony, which have contributed to serious problems in freshwater, estuarine, and coastal systems. Metal contamination in the aquatic environment stems mainly from industrial processes such as mining and smelting, paint and dye manufacture, and from tanks and pipes in domestic systems. The toxicity of metals varies with aquatic

species and environmental conditions; water quality (e.g. hardness, pH) and the presence of humic acids greatly affect the chemical speciation of metals.

1.3.2.2 Inorganic Chemicals

Inorganic, nonmetallic toxic agents include a variety of elements, such as chloride, chlorine, nitrogen, phosphorus, and boron, as well as ammonia, arsenic, nitrites, nitrates, and sulfides. The chemical and toxicological properties of the inorganics differ and, as with the metals, may vary with aquatic conditions (e.g. pH, temperature) as well as relative to specific organisms.

1.3.2.3 Organic Chemicals

The organic chemicals comprise a large variety of different chemicals concerning their toxicity characteristics, bioavailability and fate (in the environment). Certain groups can be classified.

Polychlorinated Biphenyls (PCBs) constitute of a basic biphenyl molecule with various substitutions of chlorine atoms. They are manufactured and widely used because of the extreme stability of many of the isomers. Because of this stability and their general hydrophobic nature, PCBs released into the environment have dispersed widely, some accumulate in living organisms, and cause many adverse effects. Many countries have therefore banned the production of PCBs and eliminated or severely reduced their use.

Chlorinated dioxins and *chlorinated furans*, which are formed naturally through natural combustion processes, are also by-products of anthropogenic actions such as combustions and certain manufacturing processes, like the production of PCBs and chlorophenols. A variety of adverse effects have been reported to result from elevated levels of dioxins and furans in organisms.

Organic solvents include a wide variety of aliphatic and aromatic hydrocarbons both halogenated and nonhalogenated (e.g. benzene and related aromatic chemicals).

Polycyclic aromatic hydrocarbons (PAHs) comprehend a wide range of naturally occurring and non-naturally occurring chemicals. The largest fraction is a result of human activities, sources are municipal and industrial effluents, petroleum spills, combustion of fossil fuels, atmospheric deposition, and wastewater released from oil refineries and tankers. The general toxicity of PAHs varies, some are highly toxic mutagens, carcinogens or teratogens. Many aquatic organisms can metabolise and detoxify certain PAHs, but some of these compounds become more toxic when activated through metabolism. Because of their generally hydrophobic nature, most PAHs in water do not occur in dissolved form but are associated with particulate matter and concentrate in sediments.

Synthetic detergents such as alkylbenzene sulfonates are effective surfactants, but are toxic to biota as not all are readily degradable. Linear alkylbenzene sulfonate (LAS) is biodegradable, and has replaced most of the older, non-biodegradable sulfonates. Other surfactants used as components of oil-spill dispersants are also often very toxic to aquatic organisms.

Organic chemicals can also be classified by their modes of action:

Narcotics (various chemicals with an unspecific toxic action)

Polar Narcotics (various chemicals)

Respiratory Uncouplers (e.g. some phenols)

Acetylcholinesterase (AChE) Inhibitors (e.g. organophosphate pesticides)

Membrane Irritants (reactive chemicals such as benzaldehyde)

CNS Convulsants (e.g. pyrethroids)

Respiratory Blockers (e.g. rotenone)

Dioxins (e.g. TCDD) (Rand, 1995)

McCarty & Mackay (1993) give a more detailed review on the grouping of chemicals depending on their toxicological modes of action.

The modes of action can be divided into two general mechanisms: narcosis and specific action. Narcosis describes a generalised depression in biological activity due to the presence of toxicant molecules. The exact mechanisms and sites of action are still unclear, but the cell membranes seem to be involved (Franks & Lieb, 1997). Narcosis is usually reversible by stopping the exposure before death and allowing the organism to recover by eliminating the chemical.

Body residues and modes of toxic action can help to better interpret and explain mixture toxicity (McCarty *et al.*, 1992). Although mixture interactions may be present, many organic chemicals show approximate additivity in mixtures. Hermens *et al.* (1984; 1985) and Deneer *et al.* (1988a) reported from a variety of mixture studies with equitoxic contributions from three to 50 organic chemicals that mixture toxicity was generally additive. They found that with larger numbers of chemicals in mixture, generally closer approximations of concentration addition were achieved, both with acute and chronic endpoints, and unaffected by the presence of chemicals with different specific modes of toxic action in the mixture.

An explanation for this phenomenon was suggested by McCarty and Mackay (1993). When non-narcotic organic chemicals are present below their threshold for specific toxic action (i.e. below about 0.3 to 0.02 of their threshold LC_{50}) they do not express a specific toxic action, but rather contribute to narcotic activity. In this way, simple addition of the components of the mixture, rather than any interaction between specific modes of toxic action, produces the biological response.

1.3.2.4 Pesticides

Pesticides are a diverse group of chemicals, their structures ranging from simple inorganic substances to complex organic molecules. Some are natural derivatives of plants (e.g., pyrethrins) and others are synthetic derivatives of natural products or completely synthetic substances chemically manufactured (Hayes & Laws, 1991). Since pesticides, unlike most toxic agents, are being synthesised and applied to kill or control organisms, they are all toxic to some forms of life (Deneer, 2000). They enter aquatic systems by various means: incidentally during manufacture, during their application (i.e., through aerial spray drift), and through surface water runoff from agricultural land after application. Some are also deliberately introduced to kill weeds or algae, vectors of human disease, or parasites and diseases in aquaculture (Rand, 1995).

Effective pesticides are designed to be selective in their effects, producing extremely toxic effects only to the target organisms, yet few are absolutely specific, and other closely and less closely related species may be affected. The mode of application of pesticides varies according to the circumstances, and formulations often contain other components that may be toxic as well. Biodegradability of pesticides varies greatly, and the degradation products of some can be more toxic than the original compound (Hayes & Laws, 1991).

1.3.3 Mixture interactions

Exposures to mixtures may result in toxicological interactions (Alabaster & Lloyd, 1980; Calabrese, 1991). A toxicological interaction is one in which exposure to two or more chemicals results in a biological response quantitatively or qualitatively different from that expected from the action of each of the chemicals alone. Interaction between chemicals can occur by mechanisms such as alteration in

absorption, protein binding, and biotransformation or excretion of one or more of the interacting chemicals. The multiple chemical exposures may be sequential or simultaneous in time and the altered response may be greater or smaller in magnitude. Simultaneous exposure to two chemicals may produce a response that is simply additive of the individual responses or one that is greater or less than expected from addition of their individual responses. Several terms have been used to describe toxicological interaction:

Additive effect: occurs when the combined effect of two chemicals is equal to the sum of the effects of the individual chemicals applied alone. The additive effect is most commonly observed when two chemicals are applied together.

More-than-additive (synergistic) effect: occurs when the combined effect of two chemicals is much greater than the sum of effects of the individual chemicals applied alone.

Potentiation occurs when one chemical has a toxic effect only when applied with another chemical.

Less-than-additive (antagonistic) effect: occurs when two chemicals, applied together, interfere with each other's action or one interferes with the other chemical.

Interactions may be different or even opposite depending on the magnitude of the exposure, e.g. additive at low doses and antagonistic at high doses, so discussion and especially prediction of interactions require clear information about the magnitude of the exposures in question (Hermens 1982).

There are four types of antagonism: functional, chemical, dispositional, and receptor antagonism. Functional antagonism occurs when two chemicals counterbalance one another by opposite effects on the same physiological function. Chemical antagonism is a chemical reaction between two chemicals to produce a less toxic product.

Dispositional antagonism occurs when the absorption, biotransformation, distribution or excretion of a chemical is changed so that the concentration and/or duration of the chemical at the target site are decreased. Receptor antagonism occurs when two chemicals that bind to the same receptor site produce less of an effect when applied together than the sum of their individual effects, or when one chemical antagonises the effect of the second chemical. (Rand, 1995).

1.3.4 Models and terminology

Many different authors have described methods of modelling and data analysis for types of combined effects that occur. As a result, there has been some confusion about the terminology. The fundamental description of the terminology and classification of mixtures was made by Bliss (1939), which was then elaborated in a number of publications by Plackett and Hewlett (1952, 1963, 1967) and Hewlett and Plackett (1959), who defined four types of joint action with respect to quantal responses (see Table 1.1).

Table 1.1 Types of joint action of chemicals in mixtures

	Similar Joint Action	Dissimilar Joint Action
Interaction absent	Simple Similar Action	Independent Action
Interaction present	Complex Similar Action	Dependent Action

A joint action is defined as similar or dissimilar depending on whether the sites of primary action of two chemicals are the same or different, and as interactive or non-interactive depending on whether one chemical does or does not influence the biological activity of the other. With mixtures of more than two chemicals, interaction

can be problematic to classify because there can be different types of interaction between the different chemicals. Therefore, a mathematical description of the joint toxicity of a mixture of more than two compounds is only possible in a few cases, for which the absence of interaction is a requirement.

Simple Similar Action or Concentration Addition Model

This model anticipates that the modes of action of each toxicant in the mixture are qualitatively identical, even though the common effect may be produced by a different concentration of each. The chemicals in the mixture are expected to have a common site of action (Bliss, 1939; Plackett and Hewlett, 1963), theoretically one chemical acts like a dilution of another, meaning that the effect can be obtained by replacing one chemical totally or in part with the equieffective amount of another chemical. However, there exists a certain amount of controversy as to the similarity criteria of the chemicals in the mixture.

Concentration addition is expressed mathematically as

$$C_{eff} = \sum_{i=1}^{n} (c_i/EC_{x_i}) = 1 \qquad (1.1)$$

Where: C_{eff} = overall effective concentration, n = number of mixture components, c_i = actual respective concentration of compound i in the mixture, EC_{x_i} = effective concentration of compound i, i.e. the concentration at which the studied effect, e.g. 50% mortality among the test organisms, occurs if the organisms are only exposed to compound i (singly).

Each fraction (c_i/EC_{x_i}) represents the concentration of a mixture component scaled for its relative toxicity and is described as the *toxic unit* of that component (Altenburger *et al.*, 2000). Thus, each component in the mixture can be replaced by another without

changing the overall toxicity as long as the sum of toxic units remains the same. Concentration addition holds true if, at a total concentration of the mixture provoking *x*% effect, the sum of the toxic units equals one. Otherwise, more or less than additive effects are expected.

Toxic Unit: The actual concentration of a chemical in the mixture divided by its threshold effect concentration (usually the EC_{50} of the compound). In an *equitoxic* mixture, all components are present in the same ratio of their toxic units.

To get a complete response curve for a mixture, the mixture ratio must be kept constant and the total concentration of the mixture is varied.

Concentration addition has been found well suited for the prediction of the toxicity of multiple mixtures of similarly acting chemicals, i.e. unspecifically acting substances, mainly organic, nonreactive chemicals with narcotic properties (Könemann, 1981; Hermens *et al.*, 1984).

Quantitative structure-analysis relationships (QSARs) were first used in aquatic toxicology by Veith *et al.* (1979), and support evidence of simple similar action of similarly acting chemicals. QSARs relate toxicity data to physico-chemical properties of the chemical compounds, such as hydrophobicity, as expressed by the octanol: water partition coefficient, the K_{OW}, the bioconcentration factor BCF, and the tendency of chemicals to be reduced, as expressed by the electrochemical reduction potential or the Hammet constants σ (Hermens *et al.*, 1984).

Joint toxic effects of mixtures of narcotic chemicals have been found to be adequately described by concentration addition when employing relatively insensitive criteria such as acute lethality. For more sensitive criteria, e.g. growth, the joint toxic action of this type of mixture has been reported to be only partially additive (Hermens *et al.*, 1984; Deneer *et al.*, 1988a).

However, QSARs classify pollutants in rough, large groups, and scientist such as Calamari and Vighi (1991) have pointed out the need to make more precise distinctions between classes of chemicals, proposing that even for the chlorinated aliphatic hydrocarbons, a division into six subgroups should be performed before mixture toxicities within each of these groups can be considered (Grimme *et al.*, 1996).

Opposing views exist concerning the question whether combination effects occur experimentally when the components of a mixture are present in concentrations at or below their individual no-observed effect concentrations (NOECs). The European Inland Fisheries Advisory Council (EIFAC), in a review on the existing evidence of aquatic toxicities of chemical mixtures (1980), proposed that concentrations below 0.1 of the EC_{50} of a given pollutant do not contribute to an overall mixture toxicity. Research by Könemann (1980), Hermens *et al.* (1984) and Deneer *et al.* (1988) challenged this view, supported by evidence from toxicity test systems with mixtures of 50 different chemicals with an anticipated similar, "anaesthetic" toxic mode of action, and testing their mixtures in equitoxic ratios of the compounds. From these studies it has been concluded that concentration addition applies to mixtures of organic chemicals with a narcotic mode of toxic action at concentrations as low as 0.0025 of their LC_{50}s, or below their NOECs.

However, this phenomenon has only been described for nonreactive, nonionised chemicals.

Response Addition or Independent Action Model

This model is based on the assumption that the constituents of a given mixture act independently in a statistical sense, each acting on a different physiological or

biochemical system, having different molecular acceptor sites, but contribute to a common response.

It can be mathematically expressed as:

$$E(c_{mix}) = E(c_1 + \ldots + c_n) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \qquad (1.2)$$

Where: $E(c_{mix})$ = predicted effect (scaled from 0-1) of an n-compound mixture, c_i = concentration of the ith compound, and $E(c_i)$ = effect of that concentration if the compound is applied singly.

The concept of independent action can be specified by including a so-called coefficient of correlation (generally shown as r) to account for possible correlations between the susceptibilities of the individuals within a population (tolerance correlation). It was originally only formulated for binary mixtures (Bliss, 1939; Hewlett & Plackett, 1959), with the coefficient ranging from –1 (completely negative correlation) to +1 (completely positive correlation). Extending this approach to multiple mixtures is difficult because many different possible patterns of correlation may exist (Backhaus *et al.*, 2000).

If r equals zero, and tolerances are not correlated, the result is as described in Equation 1.2. The probability of survival at exposure to a mixture can be obtained by multiplying the probabilities of survival of the organism to each individual compound. If the susceptibilities are completely positively correlated (r = +1), the effect provoked by the given mixture equals the effect of the most toxic compound (the so-called no addition case). If r = -1, the mixture effect equals the sum of the individual mixture components (effect summation). In the basic case (r = 0), the predicted mixture toxicity is higher than in the case of no addition but lower than calculated if

using the sum of the effects of the individual components. It is unclear at the moment whether the situations described by no addition and effect summation are more than theoretical concepts, but the basic situation with no correlation of susceptibilities has been attributed to a more general meaning (Greco *et al.*, 1992).

Both concepts, concentration addition and independent action, predict the toxicity of a mixture on the basis of the toxicities of the single components. For both predictions it is necessary to exactly know the quantitative and qualitative composition of the mixture; but the way they operate is different. Concentration addition permits the prediction of an effect concentration of a mixture using known effect concentrations of the single compounds. On the contrary, independent action depends on known effects of the individual substances for predicting the overall effect of a mixture. Since the predicted mixture effect $E(c_{Mix})$ is always greater than the effects of the mixture components $E(c_i)$ alone, it is necessary, in the case of multiple mixtures, to describe rather low effects of the single compounds. Thus the use of this concept for multiple mixtures requires large amounts of reliable toxicity data with little variance for every component of a mixture.

Approaches for dealing with mixtures composed of dissimilarly acting chemicals are controversial. Since organisms are structured entities, it has been assumed that independent action is a rather unlikely type of joint action on complex effect levels, such as death or inhibition of reproduction (Broderius *et al.*, 1995). Other studies suggest that independent action is a more suitable model for predicting the toxicity of mixtures of dissimilarly acting chemicals (Backhaus *et al.*, 2000). In experimental studies with mixtures of chemicals with different modes of toxic action, concentration addition often resulted in a slight overestimation of the observed mixture toxicity, though these deviations were mostly judged not relevant (Broderius *et al.*, 1995;

Altenburger *et al.*, 2000). Both concepts anticipate that the overall effect of a mixture is increased by the addition of more compounds.

1.3.5 Classification of mixture potency

In an approach to characterise joint effects, the sum of TU_i, (see Eq. 1.1), described as *M*, has been used (EIFAC 1987). When M = 1 the joint effect is considered to be simply additive; when M < 1, more than additive (synergistic); and when M > 1, less than additive (antagonistic).

Ideally, a scale for the toxicity of a mixture should provide constant values for the two reference points of 'addition' and 'no addition', independently of the number of compounds in the mixture and the ratio between the concentrations in terms of their toxic units, and it should have a logarithmic form because of the log-normal distribution of toxic concentrations with reference to the toxic response. With this in mind Könemann (1981) proposed the Mixture Toxicity Index (MTI) to overcome some of the problems of devising a widely applicable scale (see Equation 1.3).

$$\mathbf{MTI = \log M_0 - \log M / \log M_0 = 1 - \log M / \log M_0} \qquad \mathbf{(1.3)}$$

The following scale applies to the Mixture Toxicity Index:

Antagonism: negative value

No addition: 0

Partial addition: >0 to < 1

Addition: 1

Supra addition: >1

This index indirectly takes into account the number of substances in the mixture, especially when these are present at low, equitoxic concentrations. However, for very low concentrations of chemicals, it is sometimes difficult to be able to determine concentrations exactly.

1.4 CHEMICALS USED IN THIS STUDY

Three pesticides with different chemical modes of action were chosen. All these compounds were reported to be used in the study sites, and are of varying use and importance in agriculture world-wide. Additionally, one metal was included to compare combination effects between organic chemicals and metals. Cadmium was chosen because of its high toxicity and the fact that it is an important pollutant in many aquatic systems.

Data on important chemical properties of the compounds is given in Table 1.2.

1.4.1 Chlorpyrifos

Chlorpyrifos (*O,O*-diethyl *O*-3,5,6-trichloro-2-pyridil phosphorothioate) is an organophosphate insecticide, a group of chemicals originally developed in the 1950s and today much reduced in their use due to their high acute mammalian toxicity. The organophosphate pesticides can be divided into four classes:

1 Phosphates (e.g. mevinphos)

2 Phosphonates (e.g. trichlorfon)

3 Phosphorothioates (e.g. chlorpyrifos, methyl-parathion, diazinon)

4 Phosphorodithioates (e.g. malathion)

Although OPs are a broad class of chemicals structurally, they are all acutely toxic to animals through interference with cholinergic nerve transmission. Some OPs can directly cause acetylcholinesterase (AChE) inhibiting effects (phosphate class),

whereas others are poor AChE-inhibitors and become very toxic only after metabolic activation by cytochrome P-450-dependent monooxygenases (phosphorothioate and phosphorodithioate classes) (Hayes & Laws, 1991; Belden & Lydy, 2000). The result of this bioactivation is the replacement of sulfur with oxygen within the chemical structure of the OP (desulfoxidation), and the ensuing metabolite (*O-analog*) is a much stronger inhibitor of AChE. It is structurally similar to compounds in the phosphate class of OPs

Chlorpyrifos is a broad spectrum insecticide. It is widely utilised in agricultural and urban applications. In agriculture chlorpyrifos is applied for the control of most insect pests, on grain, cotton, field, fruit and vegetable crops. As an example, 1.4 million and 35,000 kg of chlorpyrifos respectively were used in the major agricultural areas of the Central and Imperial Valley of California in 1990 and 1991 (Bailey *et al.*, 1997). It is also used for many urban purposes, e.g. in dormant sprays, foundation and landscape applications, restaurant and building pest control and in pet products. Potential environmental concentrations of chlorpyrifos, based on measured spray drift emissions from horticulture, in drainage canals in the Netherlands of 4 to 50 $\mu g\ L^{-1}$ could be expected under worst-case conditions (Kersting & Van Wijngaarden, 1992).

1.4.2 Deltamethrin

Deltamethrin ((S)-alpha-cyano-3-phenoxybenzyl-(1R)-cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-carboxylate is a pyrethroid insecticide. Synthetic pyrethroids were first described as a new class of pesticides in 1973 (Mokry & Hoagland, 1990). They are chemical derivatives of natural pyrethrins. Synthetic pyrethroids combine high insect and low mammalian toxicity with greatly increased photostability as well as rapid biodegradability, leading to their wide and increasing use in agriculture.

Deltamethrin belongs to the Type II class of pyrethroids, the 'new generation' of pyrethroids that have been developed during the 1980s, it is one of the most effective pyrethroid insecticides. Like pyrethrum and all the pyrethroids, deltamethrin is a sodium channel toxin; it acts by blocking open sodium channels, which results in rapid paralysing of the nervous systems of arthropods (Hayes & Laws, 1991). Its low mammalian toxicity is due to their ability to rapidly hydrolyse the active compound to its inactive acid and alcohol components.

Synthetic pyrethroids represent 20 to 25% of the world foliar insecticide market (Hayes & Laws, 1991). Deltamethrin kills insects on contact and through digestion, it is used to control a wide variety of insect pests, and is applied to fruit, greenhouse and field crops. The recommended dose for agricultural use is five to 17.5grams of active ingredient per hectare, which is 200 times lower than the one for DDT (Xiu *et al.*, 1989).

1.4.3 Atrazine

Atrazine (2-chloro-4-ehylamine-6-isopropylamino-S-triazine) is a chlorotriazine. It acts by inhibition of the Hill reaction involved in the photosynthesis process (Hayes & Laws, 1991). Atrazine is a selective pre-and postemergence herbicide used to control broadleaf and grassy weeds on crops such as maize, sorghum, sugarcane, pineapple, christmas trees, and other crops, and in conifer reforestation plantings (U.S. Environmental Protection Agency, 1994). It is also used as a nonselective herbicide on non-cropped industrial lands and on fallow lands, as well as for selective control of pondweeds, especially submerged plants. Atrazine is now the second largest selling herbicide in the U. S. with an annual application of around 40 million kg (Hayes & Laws, 1991). It is currently a cause for concern because it is very persistent and accumulates in groundwater and has been banned in many European countries.

1.4.4 Cadmium

Cadmium is a non-essential metal, and one of the most toxic metals to many organisms. It interferes with the calcium and zinc metabolisms by binding strongly to receptors, decreasing the uptake of essential nutrients (IPCS INCHEM, 1992). Cadmium baseline levels are low in nature, the main anthropogenic sources are metal ore smelting plants, coal combustion processes, as well as chemical manufacturing of textiles, plastics and paints (Nriagu, 1990).

Table 1.2 Some chemical properties and CAS numbers of the organic chemicals used in this study. Kow – octanol-water partition coefficient, Koc- organic carbon water partition coefficient

Compound	Molecular formula	CAS number	Chemical Family	MW[1)]	Water solubility at 25°C (mg/L)[2)]	Log Kow[2)]	Log Koc [2)]
Atrazine	$C_8H_{14}ClN_5$	1912-24-9	Triazine	216	214	2.8	2.4
Chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$	2921-88-2	Organo-phosphorus	349	2.9	4.7	3.9
Deltamethrin	$C_{22}H_{19}Br_2NO_3$	52918-63-5	Pyrethroid	500	0.008	6.2	4.7

[1)]Molecular weight; [2)]Values estimated using SRC EPIWIN online software; http://esc.syrres.com.

1.5 AIMS OF RESEARCH

To be able to assess the risk of contamination to aquatic systems it is important to determine the combination effects of chemicals which are likely to appear in the environment as mixtures rather than single compounds. Studies so far have mainly concentrated on lethal effects. In this study, it was investigated whether mixtures of different reactive chemicals would show interactive effects at sublethal concentrations.

Cladocerans are important constituents of freshwater zooplankton, and feeding inhibition in *Daphnia* is a sensitive and ecologically relevant endpoint for assessing toxic effects. In this study, a standard test organism and two locally isolated species from the study site in Mexico were compared in terms of their sensitivity and general test performance. The test organisms and type of bioassay employed for the study are described in Chapters 2 and 3.

Small tropical streams and lakes are suitable investigation areas for studying effect of mixtures of toxic substances. Tropical regions, Central America among them, usually have an agricultural-based economy and depend on the intensive use of pesticides to improve their productivity. However, regulatory infrastructure is often underdeveloped or not adequately enforced (Murray, 1994). Lack of adequate training and safety equipment lead to safety measures aimed at minimising the negative impacts of compounds frequently not applied. As a result, high amounts of agrochemicals reach streams and lake systems adjacent to agricultural areas.

The overall objectives of this study were the development of a sublethal bioassay with a standard toxicity test species and a tropical, locally relevant organism investigating the effects of three pesticides which are of great relevance in the study site as single

exposures and as binary and tertiary mixtures. Feeding inhibition as a sensitive sublethal endpoint has never been assessed using a tropical species, as indeed toxicity bioassays with tropical species are very rare. Mixtures of agrochemicals are of great importance in tropical freshwater ecosystems because intense agriculture and often a lack of regulation lead to such systems being exposed to multiple toxic compounds through runoff and spraydrift, simultaneously or in sequence. The three pesticides investigated in this study, chlorpyrifos, deltamethrin, and atrazine, have all been reported of being commonly used by farmers in the vicinity of the study site.

The main aims of this study were:

1. To identify and set up a healthy laboratory culture of one or two local freshwater zooplankton species from the study site in Mexico (Chapter 2)
2. To develop a functioning sublethal feeding bioassay for the standard toxicity test species *Ceriodaphnia dubia* and the tropical species (Chapter 2)
3. To determine the relative toxicity of the 4 test compounds to the 3 species through aqueous exposure, establishing LC_{50} values (Chapter 3)
4. To establish the relative toxicity of the 4 test compounds at sublethal concentrations, using the sublethal endpoint inhibition of feeding, establishing IC_{50} values (Chapter 4)
5. To determine potential interactive effects of sublethal concentrations of the different compounds through exposure of the test organisms to different binary and tertiary combinations of the test compounds (Chapter 5)

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 INTRODUCTION

This section contains general methods of experimental materials, glassware preparations, and analysis protocols for experimental media and exposure solutions. It also includes general protocols for the collection, culturing and maintenance as well as data on life-cycle parameters of the test organisms, the cladocerans *Ceriodaphnia dubia, Ceriodaphnia cornuta* and *Simocephalus vetulus.* Some descriptions of experimental test systems are also given, with more specific protocols provided in the relevant chapters.

2.1.1 Maintenance and standardisation of cladoceran cultures in the laboratory

A key element for carrying out any laboratory toxicity tests is the reproducibility of experiments and results (Baird *et al.*, 1989a; Bradley *et al.*, 1993). Many guidelines have been proposed to standardise acute and chronic toxicity tests utilising cladocerans within and between laboratories (American Society for Testing Materials (ASTM), 1980; O.E.C.D., 1981; Horning & Weber, 1985; APHA-AWWA-WEF, 1995). However, variation within and between laboratories still exists, the two main sources of this variation being genotypic differences between test organisms, and environmental differences in the culture conditions.

Many studies on *Daphnia magna* have shown genetic variation to be present among different clones cultured in separate laboratories, and this variation can affect the animals' tolerance to toxic stress as well as their general performance (Baird *et al.*,

1989b; Baird *et al.*, 1990; Baird *et al.*, 1991; Barata & Baird, 1998; Barata *et al.*, 1999; Barata *et al.*, 2000). Clones are therefore cultured to eliminate variability from different genotypes within one laboratory population, although different opinions still exist concerning the environmental relevance of culturing of a single clone for use in laboratory bioassays (Baird, 1992; Baird, 1993; Calow, 1992; Forbes & Depledge, 1993; Forbes & Forbes, 1993).

Despite an abundance of culture protocols for maintaining *Daphnia* and *Ceriodaphnia* species, no standardisation between laboratories exists so far (Baird *et al.*, 1989b). Therefore it can be difficult to produce neonates of consistent quality and quantity for testing.

The most important aspects of culture conditions which can lead to variability in performance of test organisms, are the medium, its additives, and the food. Culture media can be either natural (e.g. filtered pond water, well water) or synthetic (Baird *et al.*, 1989a). The quality of the water affects longevity and reproduction, and can also have an effect on the tolerance of test organisms to toxicants (Cowgill, 1987; Winner & Whitford, 1987; Winner, 1989; Belanger *et al.*, 1989; Patterson *et al.*, 1992). Natural waters are sometimes used for cladoceran culture, however, because of the effects of water chemistry on the toxicity and bioavailability of chemicals and the physiological sensitivity of test animals, it is an accepted practice to use chemically defined reconstituted waters for toxicity testing (Winner & Whitford, 1987; Winner, 1989).

The use of synthetic media alone has been found to lead to poor reproduction and survival in cultures of *D. magna* (Bradley *et al.*, 1993) and *C. dubia* (Winner & Whitford, 1987; Keating & Caffrey, 1989; Cooney *et al.*, 1992a; 1992b). For this reason, organic additives, such as seaweed or soil extracts, yeasts, cereal leaves or

vitamin and mineral solutions, are added to synthetic media to provide essential nutrients (Keating, 1985; Winner, 1989; Cooney *et al.*, 1992). However, as these additives vary in their composition, they can affect toxicity and bioavailability of tested chemicals in an unpredictable fashion and are generally added to culture media rather than test solutions.

The diet of cladoceran test organisms is of equal importance, as the quality and quantity of food directly affect longevity, growth and reproduction (Cowgill, 1987; Stevenson & Watts, 1984; Winner & Whitford, 1987; Cooney *et al.*, 1992) as well as sensitivity to chemicals (Belanger *et al.*, 1989; Patterson *et al.*, 1992; LaRocca *et al.*, 1994).

Early protocols for the culturing of *C. dubia* describe a simple yeast diet (Mount & Norberg, 1984), later protocols recommend an artificial diet composed of a mixture of yeast, trout chow and Cerophyl (powdered cereal leaves) (Horning & Weber, 1985). However, investigations into standardisation of *C. dubia* culturing methods carried out by different researchers in several laboratories have shown that a diet including a species of green microalga, such as *Selenastrum capricornutum*, *Chlamydomonas* spp. or *Chlorella* spp., has generally led to healthier cultures, producing more broods and healthier neonates (Winner, 1989; Belanger *et al.*, 1989; Cooney *et al.*, 1992; Patterson *et al.*, 1992; LaRocca *et al.*, 1994), green algae forming a large part of the natural diet of cladocerans.

Work carried out on the culture of *D. magna* and *C. dubia* at Stirling has indicated that a diet of the green alga *C. vulgaris* with the addition of a seaweed extract solution produces optimal conditions for animals cultured in reconstituted water.

The quantity of food is also very important, as cladocerans are highly sensitive to alterations in ration level. Food concentration affects growth, time until first brood, as

well as brood size and viability, and can also have effects on energy allocation patterns (Taylor, 1985; Stevenson & Watts, 1984; Baird *et al.*, 1989a; Enserink, 1995), as well as influencing sensitivity to toxicants (Cowgill, 1987; Baird *et al.*, 1989a). The determination of the optimum food concentration for the culture of a cladoceran species is therefore essential, and this will depend on the individual species and the culture conditions, especially temperature.

2.2 LABORATORY CULTURES

2.2.1 Glassware preparation

All glassware and containers used in culturing, solution and media preparation, and experiments, were soaked in a 5% nitric acid solution for 8 hours prior to use, and then washed seven times in ultra pure Milli-Q® water (Millipore, Milli-Q® ultra pure, 18MΩ cm^{-1} resistivity, passed through 4 cartridges and 3 filters to remove all bacteria, organic and inorganic impurities.) Alternatively glassware was cleaned in a dishwasher with an acid rinse cycle. All glassware used in cadmium experiments was either soaked in the above mentioned acid bath or cleaned in the dishwasher.

Glassware contaminated with organic chemicals was soaked in or twice rinsed with a 1:1 mixture of methanol and acetone, followed by 3-5 rinses with Milli-Q® water.

Ordinary glassware used in animal culture without the use of toxic substances was soaked for at least 8 hours in a 2-5 % solution of Decon and rinsed 3 times with Milli-Q® water. Washed items were then either oven dried or allowed to air dry on a clean drying rack in a dust free environment. The mouths of dry flasks and jars were covered in either parafilm or aluminium foil for storage

2.2.2 *Chlorella vulgaris* culture

C. vulgaris is a non-motile, unicellular species, frequently used for culturing daphnids (Lampert, 1987).

Animals were fed on a diet of the green alga *Chlorella vulgaris* Beijerinck, strain CCAP C211/12, obtained from the Institute of Freshwater Ecology (Windermere Laboratory, Ambleside, Cumbria.)

The media used to culture *C. vulgaris* consisted of a 1:1 mixture of *Euglena gracilis* Medium (EGM) and Jaworski's Medium (JM). EGM is an organically enriched medium, which allowed rapid growth of *C. vulgaris*, but also promoted bacterial growth, so great care was required to avoid contamination. JM is an inorganic medium containing the necessary trace elements and vitamins for good algal growth. Recipes and preparations are given in Table 2.1. All media were made up using Milli-Q® water. Stock solutions and chemicals for 3 litres of each medium (EGM/ JM) were added to a 6L round glass flask and were made up to a volume of 6L by adding Milli-Q® water.

C. vulgaris was cultured using an axenic bulk culture method. A 10 mL algal inoculate (obtained from the Institute of Freshwater Ecology) was subcultured by inoculating 50 mL of sterile medium, in a 100 mL conical flask, under axenic conditions. All media were autoclaved prior to inoculation at 15psi at 120°C for 20 minutes, and allowed to cool overnight. All flasks were stoppered with cotton wool and aluminium foil. The inoculated flask was then placed in an orbital incubator (Cryotechnics), and shaken at 140 rpm at 17°C under constant light intensity (2000 lux), to maintain the algae in their exponential growth phase. After 7-10 days the culture was suitably dense to allow subculturing, which is apparent by a dark green colour.

One litre of medium was prepared and poured into three conical flasks for subculturing, one 500 mL flask received 400 mL medium and two 250 mL conical flasks were filled with 200 mL each. Flasks were autoclaved and inoculated under axenic conditions, using the stock culture in the 100 mL flask. Of this stock, approximately 50 mL was used to inoculate the 500 mL flask and 25 mL for each of the 250 mL flasks. The second set of inoculated flasks were then placed in the orbital incubator and the cultures were allowed to grow for 7 – 10 days to reach a high enough cell density for inoculating the main bulk culture.

The 6 L bulk culture flask was set up as in Figure 2.1. A rubber bung was fitted to the 6 L round flask with two glass tubes as air inlet and outlet attached to it. Air filters (0.2 μm PTFE membrane filters, Acro 37 TF, Gelman Sciences) were connected to the glass tubes with silicone rubber tubing, and the filter attached to the inlet pipe was connected to an air pump with a longer piece of rubber tubing. This allowed contamination free aeration of the algal culture, preventing cell clumping and promoting better algal growth.

6 litres of the medium were prepared in the glass flask of which one litre was decanted off for sub-culturing of the maintenance cultures. One 500 mL and two 250 mL conical flasks were filled with the decanted medium as before and autoclaved together with the 6 L flask containing 5 L medium. For autoclaving of the 6 L flask, the open ends of rubber tubing were closed up with cotton wool and aluminium foil, foil placed around the bung, and the bung loosened slightly before being put into the autoclave to avoid excessive pressure build up during autoclaving. On autoclave completion, the flask was resealed with the bung and clamps on the air tubes were clamped shut to prevent medium from reaching the filters. After cooling down over night, pressure inside the flask had equalised and the clamps could be opened again.

The 6 L round flask was inoculated with the entire contents of the 500 mL conical flask of the dense algae culture, and the contents of one of the 250 mL conical flasks were used to inoculate the three new small conical flasks with freshly autoclaved medium. The second 250 mL conical flask was kept as a backup culture and later discarded if not required. The three small flasks were transferred to the orbital incubator to grow for inoculating the next bulk culture and sub cultures. The 6 L flask was placed on a bench and gently aerated at 2-4 L air min^{-1} through the inlet filter at a temperature of 20°C under a constant light intensity, provided by a 20 W white fluorescent tube. This algae culture was left to grow under these conditions for 5-8 days until dark green in colour. If the culture showed signs of contamination with fungi or bacteria, such as cloudiness, foaming or a foul smell, it was discarded.

C. vulgaris from this main culture flask was concentrated for food use by spinning it down on a bench top centrifuge in 250 mL centrifuge tubes, at 3300 rpm for 20 minutes (Sigma Laboratory Centrifuges, 4-15). The supernatant was discarded and the algal pellet was resuspended in 10- 20 mL ASTM hard water (ASTM, 1980) and stored in a centrifuge tube to be spun down again. ASTM is the standard synthetic fresh water used for all culture and exposure media. It is prepared by dissolving four metal salts in Milli-Q® water (see Table 2.2).

The resuspended algae was then centrifuged another time to remove all traces of the medium, as before, but this time for 30 minutes. The supernatant was discarded and the algal pellet resuspended in ca. 50 mL ASTM. All the resuspended algae was poured into one flask and made up to a volume of about 250 mL by adding ASTM. This guaranteed that the algal solution had an equal cell count, which was then determined using an electronic particle counter (Coulter Multisizer, Coulter Electronics Ltd.), fitted with a 30 μm orifice tube. The desired algal cell concentration

was around 1 x 10^9 cells mL^{-1}. The algal solution was then stored in 50mL centrifuge tubes and either frozen at -18°C for storage, or kept in a refrigerator at 4°C for up to 5 days for use as daphnid food. Frozen *C. vulgaris* has been found to be an acceptable food alternative to fresh algae, providing suitable nutrient levels for maintaining healthy cultures (Cox 1992). The frozen (mainly dead) algae are also advantageous for use in the feeding bioassay as there is no algal cell division. The feeding bioassay works by determining feeding rates of the cladocerans by counting the algal cell concentration in the exposure solution initially and after a feeding period of 24 hours. Therefore a replication of algae cells during the bioassay is undesirable as it could lead to results being falsified.

Table 2.1 EGM / JM Media: Recipes and preparation

Chemical	Stock Solution Concentration	Amount of Stock / Media Added
EUGLENA GRACILIS MEDIUM		
Sodium acetate trihydrate	-	1.0 g L^{-1}
Lab-Lemco powder (Oxoid L29)	-	1.0 g L^{-1}
Tryptone (Oxoid L42)	-	2.0 g L^{-1}
Yeast Extract (Oxoid L21)	-	2.0 g L^{-1}
$CaCl_2$	1.0 g L^{-1}	10.0 mL L^{-1}
JAWORSKI'S MEDIUM		
1. $Ca(NO_3)_2 \times 4H_2O$	4.0 g x 200 mL^{-1}	1 mL L^{-1}
2. KH_2PO_4	2.48 g x 200 mL^{-1}	1 mL L^{-1}
3. $MgSO_4 \times 7H_2O$	10.0 g x 200 mL^{-1}	1 mL L^{-1}
4. $NaHCO_3$	3.18 g x 200 mL^{-1}	1 mL L^{-1}
5. NaFeEDTA Na_2EDTA	0.45 g x 200 mL^{-1} 0.45 g x 200 mL^{-1}	1 mL L^{-1}
6. H_3BO_3 $MnCl_2 \times 4H_2O$ $(NH_4)6Mo_7O_{24} \times 4H_2O$	0.496 g x 200 mL^{-1} 0.278 g x 200 mL^{-1} 0.20 g x 200 mL^{-1}	1 mL L^{-1}
7. Cyanocobalamin Thiamine HCL Biotin	0.008 g x 200 mL^{-1} 0.008 g x 200 mL^{-1} 0.008 g x 200 mL^{-1}	1 mL L^{-1}
8. $NaNO_3$	16.0 g x 200 mL^{-1}	1 mL L^{-1}
9. $Na_2HPO_4 \times 12H_2O$	7.2 g x 200 mL^{-1}	1 mL L^{-1}

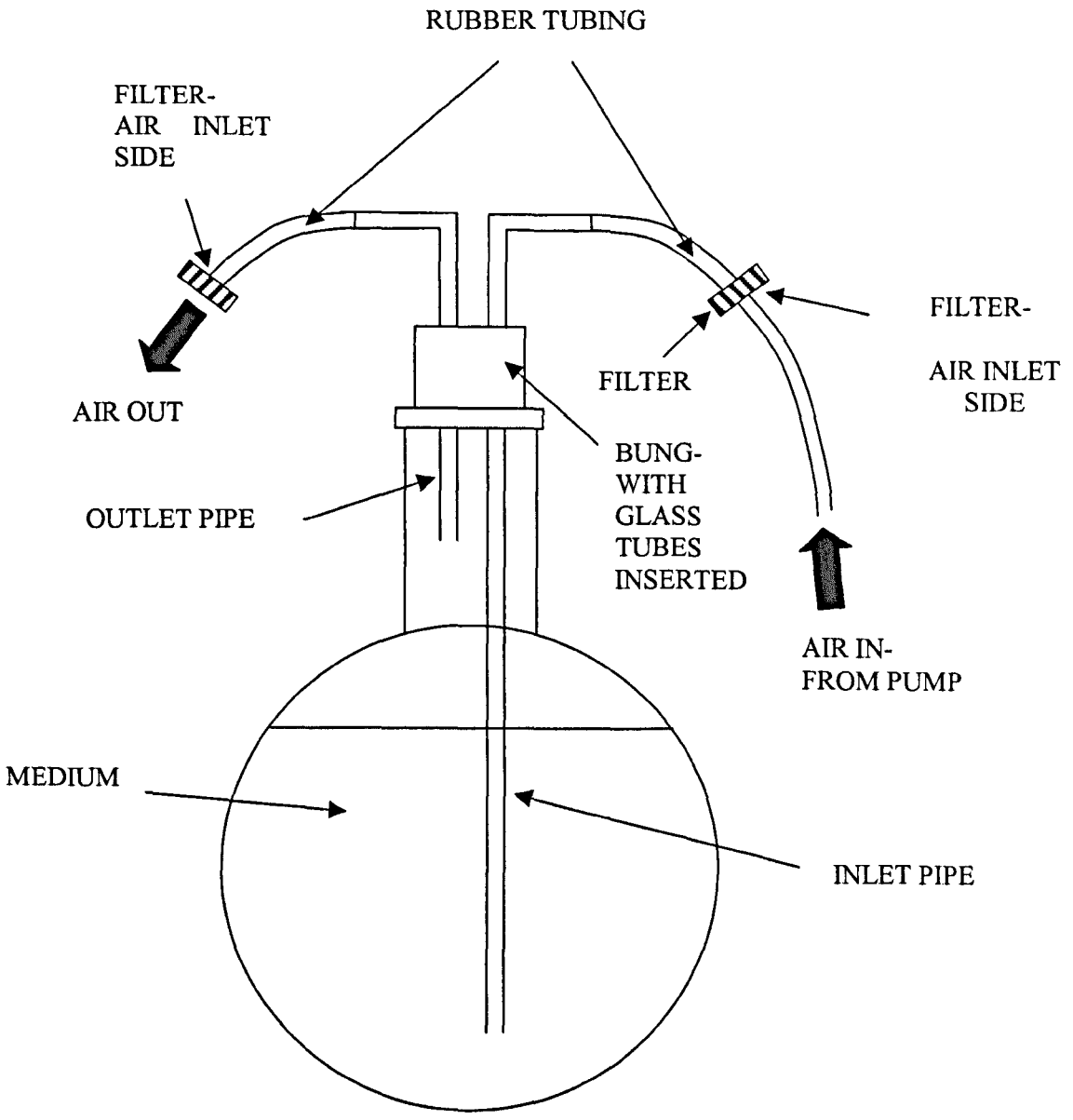


Figure 2.1: Diagram of the set-up for the six- litre algae bulk culture

Table 2.2 Quantities of AnalR grade chemicals used to prepare synthetic fresh water (ASTM hard and moderate hard water), with resulting water qualities (ASTM, 1980).

SALTS	STOCK CONCENTRATION	STOCK ADDED FOR HARD WATER	STOCK ADDED FOR MODERATELY HARD WATER
KCl	1.6 g L^{-1}	25 mL $10L^{-1}$	12.5 mL $10L^{-1}$
$MgSO_4 \times 7H_2O$	49.1 g L^{-1}	50 mL $10L^{-1}$	25 mL $10L^{-1}$
$NaHCO_3$	38.4 g L^{-1}	50 mL $10L^{-1}$	25 mL $10L^{-1}$
$CaSO_4 \times 2H_2O$	12 g $5L^{-1}$	50 mL $10L^{-1}$	25 mL $10L^{-1}$
pH	17.6- 8.0	500 mL $10L^{-1}$	250 mL $10L^{-1}$
Hardness (mg/L $CaCO_3$)	160- 180		

Table 2.3 Average composition of the organic extract Marinure added to ASTM synthetic fresh water (data supplied by Glenside Organics Ltd.)

Dry Matter	92-95 %
Organic Matter	50-55 %
Inorganic Matter	40-45 %
Chlorine	3.0 %
Sulphur	2.7 %
Potassium	2.5 %
Nitrogen	1.4 %
Calcium	1.2 %
Magnesium	0.8 %
Phosphorus	0.05 %
Iodine	1800.0 ppm
Iron	1500.0 ppm
Boron	110.0 ppm
Zinc	100.0 ppm
Manganese	13.0 ppm
Aluminium	5.0 ppm
Nickel	5.0 ppm
Copper	3.0 ppm
Cobalt	1.6 ppm
Vanadium	0.7 ppm
Cytokinines and other natural growth stimulants	130-260 ppm

2.2.3 *Ceriodaphnia dubia* culture

The *Ceriodaphnia dubia* (Richard) clone (U.S. EPA strain) was obtained from the Institute for Environmental Quality, Wright State University, U.S.A. This clone was used for all experiments and was maintained in a bulk culture system throughout the period of this research.

2.2.3.1 Culture media

C. dubia cultures were maintained in the synthetic moderately hard ASTM fresh water (American Society for Testing Materials (ASTM), 1980; Horning & Weber, 1985), see Table 2.2. The organic additive "Marinure" (recommended by Baird 1989a) was added to supply essential nutrients (see Table 2.3), which were otherwise lacking in artificial culture media (Baird *et al.*, 1991). "Marinure" is an extract of the marine seaweed *Ascophyllum nodosum* (Marinure, Glenside Organics Ltd.). The extract was supplied in a concentrated liquid form of consistent quality, and a stock solution was prepared by diluting 9 mL of the concentrate in 1000 mL of ASTM hard water (~ 1:100). The stock was filtered to remove large particles and stored in a dark bottle at 4°C to prevent photodegradation. To guarantee a consistent strangth of stock solution, the optical density (O.D.) of the solution was measured at 400 nm in a double beam UV spectrophotometer (Uvikon 810, Kontron), using a cuvette with a 1 cm pathlength. For this, the stock solution was diluted (1 in 10) with ASTM hard water, which was also used as a blank. An O.D. reading of between 0.6 and 0.7 indicated that the stock solution was at the right concentration.

The extract was added to the culture medium at a concentration of 5 mL L^{-1}. ASTM moderately hard water was prepared and left at 25°C for at least 8 hours before use.

2.2.3.2 Laboratory culture method

All daphnid cultures were maintained in a 25°C (+/- 2°C) constant temperature room, which was within the optimal temperature range for the tropical species as well as the subtropical *C. dubia.* The photoperiod was a constant 14:10h- light: dark. This constant temperature and photoperiod minimised the chances of sexual reproduction occurring (Stross & Hill, 1965).

C. dubia maintenance cultures were set up in 150 mL glass bottles containing 120 mL enriched ASTM moderate hard water and 20 neonates below 24h of age. Using neonates of the same age to set up the cultures guaranteed that animals would reach reproductive maturity simultaneously and would release broods at approximately the same time, making experimental planning easier. New cultures were set up at staggered time intervals, so that different cultures would release broods on successive days and a continuous supply of neonates for experiments was guaranteed. Depending on the experimental schedule, between six and 12 bottles for maintenance culture were kept at one time.

Each bottle was covered with a clean lid to prevent excess water loss through evaporation. Cultures were transferred to fresh medium and clean bottles three times a week (Monday, Wednesday, Friday), animals were handled at all times with clean plastic disposable pipettes to prevent contamination of the medium. Maintenance cultures were fed at an algal concentration of 1×10^6 cells mL^{-1}. The alga was added to the culture medium, (so that 1mL of an algal stock solution of 1×10^9 cells mL^{-1} was added to two litres of culture medium), and cultures received fresh food every other day.

Bottles containing adults were checked daily for neonates, which were removed. The first two broods from each adult culture were discarded, as they are known to be more

variable in size and quality (Waddell, 1993). Equal size and quality (health) of experimental animals is an essential requirement for comparability in toxicity and feeding experiments, to reduce variability in performance.

Neonates from the 3rd to the 6th brood were either taken for use in experiments, used to establish new maintenance cultures, or discarded. If they were intended for use in experiments, neonates were either used straight away (in cohorts of <24h old neonates) or separated and kept under comparable conditions as the maintenance cultures until they had released their first broods (young adult females, 4- 5 days old). After releasing their 6th brood, animals were discarded, as the quality of neonates starts to decline (Barata & Baird, 1998). New cultures were started using neonates from a 3rd brood onwards. In this way a cycle of constantly neonate-producing adult cultures was maintained.

2.2.3.3 Feeding

Animals were fed on a diet of the green alga *C. vulgaris* (as described in 2.1). Optimum algal concentrations for feeding the 3 cladoceran species when kept at 25°C were determined as described in Chapter 4. Maintenance cultures of all 3 species were fed at 1-2 x 10^6 cells mL^{-1}.

2.2.4 Mexican test species

For the adaptation of traditional toxicity tests with temperate species to a suitable organism representative of tropical systems and conditions, an appropriate test organism was required. A variety of tropical cladocerans were isolated from the test site in Campeche, Mexico, and evaluated concerning their suitability as test organisms.

2.2.4.1 Field collection

Field collection of organisms was carried out during the summer of 2000. The field site was the Rio Palizada river delta near the Laguna de Terminos, Isla del Carmen, Campeche, in the South East of Mexico (Figure 2.2). This wetlands area consists of a large river with multiple small sidearms, which form small lakes, the river eventually leading into a large tidal lagoon, the Laguna de Terminos. The main impacts on the area come from large plantations of mainly tobacco and citrus fruits, as well as many smaller farms planting fruit and vegetables. Agrochemicals are sprayed on the plantations using small airplanes, and local reports state that no great care is taken to avoid contamination of the surrounding wetlands. Pesticides also enter the rivers through runoff from fields and plantations, with rain and irrigation water.

Cladocerans were collected from small lagoons and rivers using a zooplankton net fitted with a fine end filter of 500 μm mesh size. Several sweeps of the vegetation growing in shallow water around the edges of the rivers and lagoons were made with the net to collect animals hiding amongst the vegetation. The contents of the net and filter were rinsed into a bucket of river water and the contents of the buckets were sorted in shallow white trays. Any cladocerans found were placed into screw topped jars filled to the rim with river water using a disposable plastic pipette and taken back to the lab.

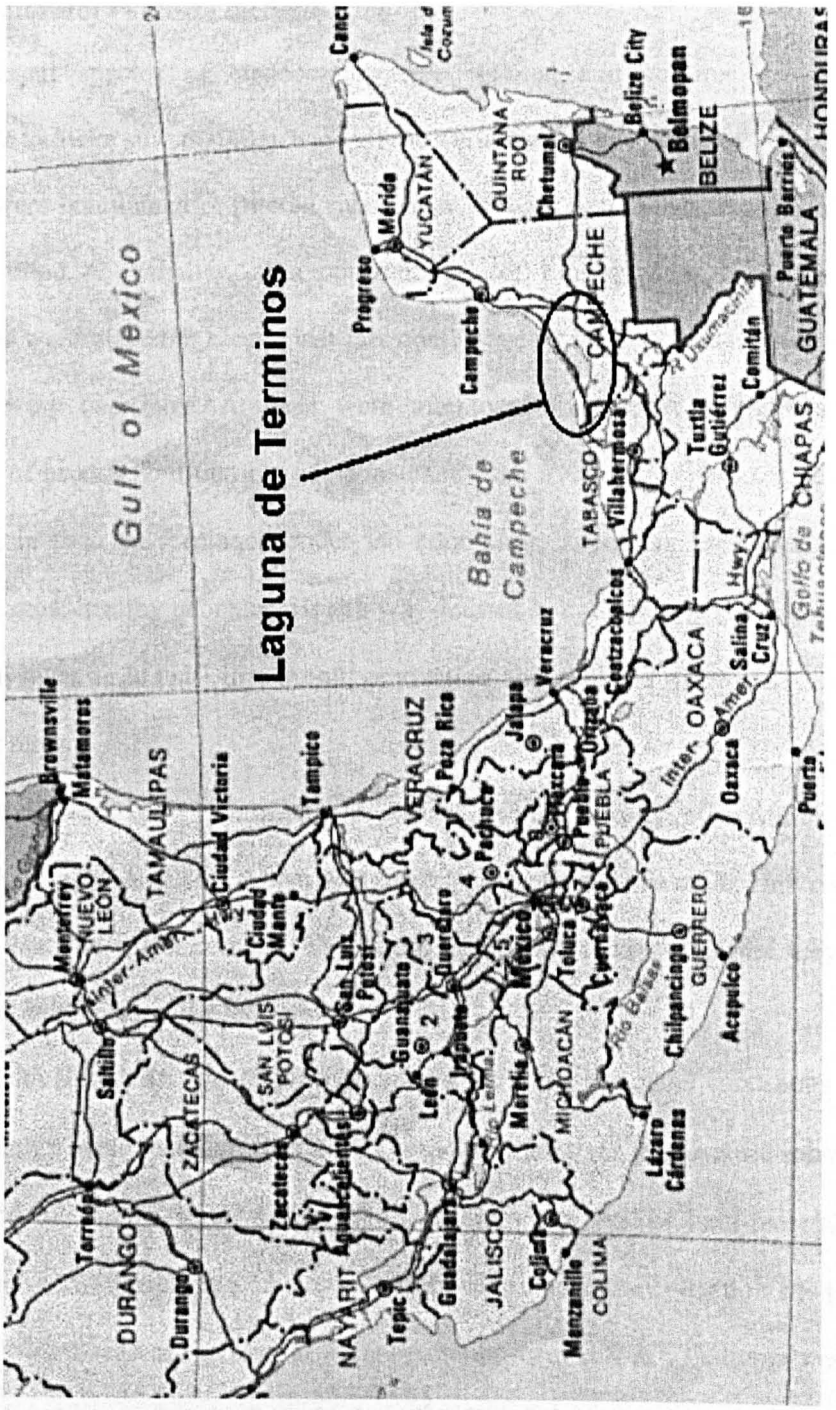


Figure 2.2 Map of field site in wetland system near Laguna de Terminos, Campeche, Mexico

2.2.4.2 Laboratory culture method

Five different species of cladocerans were isolated and cultures grown from individuals to make sure that they were all the same species and clone.

Cultures were maintained in filtered river water, and fed with either freshly defrosted or freeze-dried *C. vulgaris*, at a concentration of roughly 5 x 10^5 cells mL^{-1}. Individuals were placed in clean glass jars containing 30 mL volumes, and water was changed every two days. Animals were monitored for survival, first onset and frequency of broods, and number of neonates. Of the five species, two seemed most promising in their performance under lab conditions, appearing most healthy and producing most healthy neonates. Health was defined as continuity and comparability of survival rates, time until first brood, production and numbers of neonates, as well as feeding rates.

The two species were identified as *Simocephalus vetulus* (O.F.Müller) and *Ceriodaphnia cornuta* (Sars), which was confirmed by R. Ribeiro at the University of Coimbra, Portugal (pers. comm.). They were taken back to Stirling were they were established in cultures in the laboratory.

Cultures with *S. vetulus* and *C. cornuta* were kept under the same conditions as *C. dubia.* Animals were maintained in a 25°C (+/- 2°C) constant temperature room, at a photoperiod of 14:10h- light: dark. Culture medium was ASTM hard-reconstituted water, which most closely resembled the water conditions at the collection site (Table 2.2), with added seaweed extract at a concentration of 5 mL L^{-1}. Cultures were fed with *C. vulgaris* at a concentration of 1-2 x 10^6 cells mL^{-1}.

Maintenance cultures of *S. vetulus* were initiated in 180 mL wide rimmed glass bottles containing a volume of 150 mL and 10-15 neonates <24h old. *C. cornuta* cultures were set up in 150 mL narrow rimmed glass bottles containing a volume of 120 mL

and 20-30 neonates <24h old. Bottles were covered with clean lids to prevent excessive water loss through evaporation. As with *C. dubia,* the first two broods were discarded and only neonates from the 3rd to the 6th brood were used for experiments and setting up of new brood cultures. Cultures were transferred into fresh bottles and medium every second day (Monday-Wednesday-Friday). Six bottles were maintained for each culture at all times, and more were used depending on the numbers of animals required for experiments.

Important life-cycle data was established for *C. dubia* as well as for *C. cornuta* and *S. vetulus*. Parameters established were adult body length, rough length of neonates, age at first production of brood, intervals between broods, and numbers of neonates per brood, details are given in Table 2.4. These life-cycle variables were chosen as they are important parameters for comparing performance of different cladoceran species.

Table 2.4 Life-cycle parameters for *C. dubia*, *C. cornuta* and *S. vetulus*

Parameter	*C. dubia*	*C. cornuta*	*S. vetulus*
Body length (mm)	0.82 ± 0.06	0.43 ± 0.03	1.25 ± 0.08
Size of neonates (mm)	0.3 ± 0.05	0.2 ± 0.02	0.5 ± 0.05
Age at first brood (hours)	48 – 72	48 – 72	120 – 144
Interval between broods (hours)	24 – 48	24 – 48	48 – 72
Numbers of neonates: 1st brood	2 – 5	2 – 3	2 – 5
2nd brood	4 – 7	3 – 6	5 – 8
3rd and consecutive broods	6 – 18	5 – 15	5 – 20

2.3 EXPOSURE SOLUTIONS

2.3.1 Cadmium

2.3.1.1 Cadmium solutions

A cadmium stock solution of 10 mg L^{-1} was prepared for use in toxicity and feeding tests. Cadmium chloride ($CdCl_2$ x 2 ½ H_2O, BDH, AnalaR) was dissolved in nanopure water (Milli-Q), and well mixed. This stock solution was diluted using ASTM hard or moderate hard water (ASTM, 1980) to the required experimental exposure concentrations. The 10 mg L^{-1} stock was stored at 4°C for up to 3 days (Waddell, 1993). The stock solutions were allowed to equilibrate to room temperature before use.

2.3.1.2 Cadmium analysis

Cadmium concentrations of all prepared solutions were measured using a graphite furnace atomic adsorption spectrophotometer (ATI Unicam Model 939QZ)

Duplicate samples of 3 mL of solution were collected from each stock solution and exposure concentration before algae was added at the beginning of each experiment, and acidified with 1% of 80% HNO_3 (Aristar Grade, BDH) to preserve for later analysis. Duplicate samples were also collected after the 24h exposure to determine whether concentrations decreased during the experiments. Once acidified, samples could be stored at 4°C for several weeks. The detection limit for cadmium was 0.5 μg Cd L^{-1}.

2.3.1.3 Cadmium results

Measured cadmium concentrations differed by less than 10% from nominal concentrations, thus nominal concentrations were used for all calculations. Cadmium concentrations did not decrease notably during the 24h exposure periods.

2.3.2 Organic pesticides

Preparation of stock solutions and chemical analysis for the test substances were as follows for lethality and feeding inhibition tests. Stock solutions for all organic chemicals were prepared using the "thin-layer evaporation" technique (Barata & Baird, 2000). A concentrated stock solution was prepared for each chemical by dissolving the pure compound in acetone or acetonitrile, in a teflon-coated 30 mL plastic flask, wrapped in aluminium foil, which could then be stored at 4°C for several weeks. The required chemical concentration in acetone/acetonitrile was then aliquoted into 1 L or 2 L conical glass flasks. The solvent/test substance solution was spread over the base of the flask in a thin layer, and the solvent was then allowed to evaporate, leaving a thin layer of the test substance on the flask base. For 1 L flasks, 500 mL of relevant ASTM, and for 2 L flasks, 1 L of ASTM was added to the flask, which was then placed on an orbital shaker for 2 days to allow as much of the test substance as possible to dissolve in the ASTM. For atrazine, a large amount of test substance was required. To allow dissolving of the thin layer in the ASTM, the flask was put in a sonic bath for 30 – 60 minutes, which guaranteed a better mixing of the compound, before being put on the shaker for at least 12 hours. Nominal concentrations of each compound were prepared as explained in the individual chapters.

2.3.2.1 Atrazine

Atrazine (Riedel-de-Haën, 98% purity) has a solubility limit of 30 mg L^{-1} (Hayes & Laws, 1991). Stock solutions of 30 mg L^{-1} were prepared by dissolving the pure compound in acetonitrile in teflon flasks, and relevant serial dilutions were prepared for lethality and feeding experiments. The limit of detection was 10 μg L^{-1}.

2.3.2.1.1 Analysis

Duplicate water samples from the stock solutions and the highest 3 concentrations in each series were collected before algae were added at the beginning of experiments. Samples were also collected after 24h without algae to see whether any decrease of the compound had taken place. Test solutions were extracted from water samples and pre-concentrated in a solid-phase, Bond Elut C18 extraction column (Varian, Phenomenex). Test substances were eluted from the solid phase cartridges using HPLC grade solvents, which were subsequently evaporated in a nitrogen evaporator Model N-vap 112 (Organonation Associated Inc.), and the residual compound was resuspended in the relevant mobile phase. For atrazine, the elutent was 6 mL methanol and 6 mL water. The mobile phase was comprised of acetonitrile: water in a mixture of 70:30.

Calibration was done on a serial dilution and concentrations were determined from the slope of the calibration line.

Actual concentrations were determined using an UV detector HPLC (LCD analytical Model spectromonitor 3200 variable wavelength detector), by injecting a 100 µL sample onto a Supelcosil 22021 07 LC-ABZ 5 µm x 4.6 mm column. Flow rates were set at 1 mL min^{-1}. Wavelength determined for atrazine was 225 nm, and retention time was 8.6 mins. Recovery of test substances from water samples was determined by extracting and eluting standard solutions of a known concentration from Bond Elut

cartridges. Mean recoveries of atrazine were very high at 90-100%, and concentrations did not change notably over 24 hours.

2.3.2.2 Deltamethrin

Deltamethrin (Riedel-de-Haën, 98% purity) has a solubility limit of 2 μg L^{-1} (Hayes & Laws, 1991). Stock solutions of 2 μg L^{-1} were prepared by dissolving the pure compound in acetone in teflon flasks, and relevant serial dilutions were prepared for lethality and feeding experiments. The detection limit of deltamethrin was 1 $\mu g\ L^{-1}$.

2.3.2.2.1 Analysis

Analysis of deltamethrin was very difficult, as it is such a hydrophobic compound. Different attempts were made to pre-concentrate the compound through solid-phase Bond Elut C18 or C8 extraction columns, however, elution attempts with a variety of elutant solvents failed due to the "stickiness" of the compound. Therefore, it was only possible to analyse the highest concentration stock solutions using a liquid-liquid extraction method. For the liquid-liquid extractions, 100 mL of sample (stock solution at maximum solubility level in water) was transferred into a glass mixing bottle, and 20 mL of hexane were added. The mixture was vigorously shaken for several minutes to guarantee a good mixing, which allows the hydrophobic compound to be taken up by the hexane. The solution was then allowed to separate again and the hexane was separated from the water using a separating funnel. The hexane was then evaporated under a nitrogen evaporator and a vacuum evaporator, and the residual compound was resuspended in 1 mL of mobile phase (acetonitrile: water in a 80:20 mixture).

Actual concentrations were determined using the same equipment as for atrazine. The determimed wavelength was 205 nm, and the retention time was about 7 mins. With

this procedure, recovery of standards was 90- 95%, and that of stock solutions was 80%. Stock solutions did degrade by 15-20% over 24 hours.

2.3.2.3 Chlorpyrifos

Chlorpyrifos (BDH, 98% purity) has a maximum solubility limit of 2 mg L^{-1} (Hayes & Laws, 1991). Stock solutions of 100μg L^{-1} were prepared in teflon flasks, and serial dilutions prepared for the relevant experiments. Analysis of chlorpyrifos was not completed as this compound was not included in the feeding experiments due to its lack of effect on feeding rates at sublethal concentrations.

CHAPTER 3

LETHALITY DATA FOR THE THREE CLADOCERAN SPECIES EXPOSED TO THREE PESTICIDES AND A METAL

3.1 INTRODUCTION

3.1.1 Toxicity testing

Aquatic toxicology has been described as the study of the effects of manufactured chemicals and other anthropogenic materials and activities (toxic substances) on aquatic organisms at various levels of their organisation (Rand, 1995). It focuses mainly on those effects considered to be adverse in nature, as well as on recovery processes. Adverse effects on the organismal level include both short-term and long-term lethality (expressed as mortality or survival), as well as sublethal effects such as changes in growth, development, reproduction, and behaviour. Changes also take place at the suborganismal (molecular) level and on the supraorganismal level (communities and ecosystems). The toxicity of a chemical to a living organism depends on many factors, such as the physical and chemical properties of the compound, the concentration and duration of exposure, as well as the specific susceptibility of the organism itself (Rand, 1995).

Chemicals in water can exist in three different basic forms that affect their availability to organisms (bioavailability). They can be dissolved, sorbed to biotic or abiotic components in the water column or on the bottom, or incorporated into organisms.

Dissolved, i.e. water soluble or hydrophilic chemicals, are readily available to organisms in the water column. Poorly-soluble or hydrophobic chemicals may be sorbed on sediments, suspended organic matter or other particulates and in this way irreversibly bound and generally unavailable, and may also be in solution to a limited degree depending on their K_{OW}. However, some of the bound chemicals may be available to organisms through ingestion, especially to sediment dwelling species.

When developing new bioassays with new species, it is necessary to have a good understanding of the basic sensitivity of these organisms to a range of toxic chemicals. A precise means of expressing the toxicity of chemical substances and a quantitative method of measuring it is also required when evaluating the safety of new chemicals and compounds of interest. For this initial test measurement in toxicological evaluation, it is customary to use lethality (mortality) as an index.

Measurement of lethality is precise, ecologically important, unequivocal, and therefore useful for estimating the toxic potency of a chemical. It provides an instrument for comparing substances with different mechanisms of action, as well as comparing basic sensitivities between different organisms. Mortality and survival over a specific period of time are typical effect criteria in short-term (acute) exposure tests. Data from such lethality tests is quantal, with an all-or-none response. It is essential to have an appreciation of the lethal concentrations of a substance to a test organism before developing sublethal effect criteria which indicate toxic stress at a stage before death (Rand, 1995).

In contrast, chronic tests involve prolonged contaminant exposure periods, during which usually sublethal effects of the chemicals are monitored. These sublethal

effects are usually growth, reproduction, feeding, and behavioural changes. Chronic tests will be described in detail in chapter 4.

Lethality is measured by exposing replicate groups of test organisms to a range of concentrations of a chemical for a specified length of time. The responses as mean percent mortality for each test group are plotted against the concentration producing the mortality, usually yielding a characteristic sigmoid curve. The sigmoid curves require transformation to linear curves in order to accurately interpolate 50% response values. Probit analysis is one of the most commonly used methods to calculate these values (Finney, 1971). Each point on the curve represents an average cumulative response to the specific concentration, and each average has an associated variation due to different responses of individual organisms. The least variability between concentration responses in the curve is at the 50% level of response, therefore the concentration at which 50% of the test organisms react (the median) after a specified exposure time (e.g. 24 or 48 h) is used as a measure of the toxicity of a chemical. This LC_{50} describes the concentration estimated to produce mortality in 50% of a test population over a specific time period, usually 24 to 96 h, depending on the species. Since mortality is sometimes difficult to establish, many researchers use a definable endpoint, which indicates a serious and non-reversible toxic effect on the test animal. For cladocerans, this endpoint is generally immobilisation, which describes the animal's inability to swim after being encouraged to do so by either stirring the test vessel or gently prodding with a pipette tip for a defined period of time, 5 or 10 seconds (Day & Maguire, 1990; Rand, 1995). Immobilisation is therefore an effect endpoint, and is described as the EC_{50} (the concentration estimated to produce the relevant effect in 50% of a test population).

Aquatic toxicity tests are commonly performed with fish, invertebrates and algae. Three types of test design are generally used depending on the organism and chemical investigated. These are:

1. Flow-through tests. These tests are carried out in a test chamber where a continuous flow of water and the test substance are maintained for the duration of the test. It allows the maintaining of a constant exposure concentration as well as high oxygen levels, also removing waste products produced by the test organisms. Flow-through systems are often used for testing fish and larger aquatic invertebrates that require high dissolved oxygen levels and live in flowing water.

2. Static tests. These tests involve no renewal of the test solution, organisms are exposed to the same solution for the duration of the test. This design is much less complicated than flow-through systems and suitable for algae and invertebrates. However it is only useful for short-term experiments, as the static nature of the test can lead to a possible change in chemical concentrations due to sorption, evaporation, biodegradation or transformation, as well as reduced dissolved oxygen levels and build-up of waste products.

3. Static-renewal tests. These tests include the periodic renewal of the test solution at regular intervals during the exposure period. In this way, the contaminant concentration and dissolved oxygen levels can be maintained more constant, and waste products are removed. However, the renewal of the test solutions may cause some stress to test organisms. This type of test is useful for small invertebrate species over slightly longer test periods.

3.1.2 Aquatic toxicity testing with cladocerans

Since toxicity bioassays are developed as tools for the detection and monitoring of harmful effects of anthropogenic chemicals on individuals and populations of

organisms in natural systems, it is essential to choose organisms for testing that are sensitive to a wide range of pollutants. Daphnids, being one of the more sensitive members of the freshwater zooplankton, have been used to monitor chemicals in freshwater systems since the beginnings of ecotoxicology (Anderson, 1944; Anderson, 1980). Daphnids are freshwater microcrustaceans, commonly referred to as water fleas, which belong to the class Crustacea, order Cladocera. Cladocerans from the family Daphniidae, which include *Daphnia* spp. and *Ceriodaphnia* spp., are ubiquitous in temperate freshwaters (Berner *et al.*, 1986). During most of the year, natural populations of daphnids consist almost entirely of females; the males are usually only abundant in spring and autumn (Barker & Hebert, 1986). The production of males seems to be induced by adverse conditions such as low temperatures or crowding with subsequent accumulation of excretory products and depletion of food (U.S.Environmental Protection Agency, 1993). Under such unfavourable conditions, sexual (resting) eggs (embryos), which are contained in a case called an ephippium, may be produced by the females, and are cast off with the next moult. *D. magna* and *C. dubia* produce only by cyclic parthenogenesis, in which males contribute to the genetic makeup of the young during the sexual stage of reproduction. Other species such as *D. pulex* may reproduce either by cyclic or by obligate parthenogenesis, in which zygotes develop within the ephippium by ameoitic parthenogenesis with no genetic contribution from the males (Rand, 1995). The life history of daphnids comprises four distinct stages: egg, juvenile, adolescent and adult (Pennak, 1978). The life span of daphnids is highly variable, depending on the species and environmental conditions; in general the life span increases as temperature decreases due to a lowered metabolic activity. The average life span of *D. magna* is about 40 d at 25°C

and 56 d at 20°C, the average life span of *C. dubia* is about 30 d at 25°C and 50 d at 20°C (U.S. Environmental Protection Agency, 1993).

The eggs are released into the brood chamber and hatch there, the juveniles, which are similar to the adults, are released after about 2 d when the female moults (casts of the exoskeleton or carapace). *D. magna* and *D. pulex* mature in about 6 to 10 d and have three to five juvenile instars, each juvenile instar ending with a moult; *C. dubia* matures in 3 to 5 d. At the end of each adult instar, four events take place: release of young from the brood chamber to the outside, moulting, increase in size, and release of a new clutch of eggs into the brood chamber (Pennak, 1978). The adolescent stage involves one instar, this is when the first clutch of eggs reaches maturation in the ovary. One instar lasts about 2 d (*D. magna*) or 1 d (*C. dubia*) under good conditions, lasting longer when conditions are poor.

Cladocerans are useful test organisms for laboratory and field toxicity testing because they are ecologically important in their role as primary consumers in freshwater aquatic systems, consuming algae and bacteria and being consumed by a wide range of invertebrates and fish. They are broadly distributed in freshwater bodies and are present throughout a wide range of habitats. They are also easily and efficiently cultured in the laboratory, and their reproductive strategy of facultative parthenogenesis allows rapid production of large numbers of genetically identical test organisms. Their short life-cycle makes it possible to carry out relatively short full or partial life-cycle exposure tests, including the most sensitive stages. The small size of the daphnids requires only small volumes of test and dilution water. Thirdly this group of organisms has been found to be extremely sensitive to a wide range of environmental chemicals. However, cladocerans are not always the most sensitive organisms, with other crustaceans, insects and even some fishes sometimes exhibiting

higher sensitivities to certain chemicals. The organochlorine insecticide endosulfan for example is much more toxic in acute toxicity tests to insects than to cladocerans, with 24h LC_{50} values of 72 μg L^{-1} to the caddisfly larva *Limnephilus* spp. compared to 607 μg L^{-1} to *D. magna* (Ernst *et al.*, 1991). This has to be taken into account therefore when establishing exposure guidelines.

Most work has traditionally been carried out with the species *Daphnia magna*, which is common throughout northern Europe. This species has been intensely studied regarding its physiology and ecology, as well as in its role as a test organism in ecotoxicological research. An extensive amount of data exists describing the acute and chronic sensitivity of *D. magna* to a wide range of chemicals and conditions, as well as its physiology and ecology (McMahon, 1965; Winner & Farrell, 1976; Buikema *et al.*, 1980; Cowgill *et al.*, 1985; Lampert, 1977; Lampert, 1987; Day & Maguire, 1990; Baird *et al.*, 1989a; Barata & Baird, 1998). Standard methods have been developed for acute tests for *Daphnia* toxicity assessment over 24 or 48 h (HMSO, 1983; O.E.C.D., 1981; APHA-AWWA-WEF, 1995), and chronic reproduction bioassays over 14 or 21 days exist (O.E.C.D., 1981; U.S. Environmental Protection Agency, 1982).

The popularity of *D. magna* as a test organism in ecotoxicology is based on the fact that it is one of the largest species of cladocerans, facilitating culture and handling, as well as being so well studied and understood.

On the other hand, *D. magna* is a highly specialised zooplankter, and although it is a Palaearctic species, it is generally restricted to ponds and temporary pools (Koivisto, 1995; Lahr, 2000). Therefore many researchers have questioned its ecological

relevance as a representative species for toxicity testing, certainly in countries where it is not indigenous.

In the United States, much of freshwater acute and chronic toxicity testing is being carried out employing the cladoceran *Ceriodaphnia dubia*, which is endemic throughout the Americas, typically preferring subtropical climates (Gillooly & Dodson, 2000). *C. dubia* is a pelagic species, with an average body length of 0.8 to 0.9 mm considerably smaller than *D. magna* and with a shorter generation time, but morphologically very similar. *C. dubia* is thought to be synonymous with *C. affinis*, and the designation *C. dubia* has taxonimic preference (Berner, 1986). This species occurs in permanent as well as temporary water bodies (Hickey, 1989), even in larger streams, lakes and reservoirs (Mokry & Hoagland, 1990). *C. dubia* has also been reported from Asia (Fernando, 1980a; Fernando, 1994), and is indigenous in Australia and New Zealand, where it is being used for laboratory toxicity testing (Sunderam *et al.*, 1994; Patra, 1999; Bailey *et al.*, 2000; Woods *et al.*, 2002; Hickey, 1989). *C. dubia* has also the advantage that, being a subtropical organism, it is ideally maintained at temperatures of around 25°C, which leads to more rapid and earlier production of neonates, speeding up the life-cycle and facilitating toxicity testing (Cowgill *et al.*, 1985).

The short-term chronic 7-day life-cycle test with *C. dubia* was introduced in 1984 by Mount & Norberg and measures both lethality and reproductive impairment (Mount & Norberg, 1984). It has been refined in subsequent years by improving culturing methods and reproducibility (Cowgill *et al.*, 1985; Cowgill, 1987; DeGraeve & Cooney, 1987; DeGraeve *et al.*, 1992; Cooney *et al.*, 1992). The use of *C. dubia* for acute and chronic toxicity tests is now incorporated in guidelines from the U.S. Environmental Protection Agency (U.S. Environmental Protection Agency, 1993;

U.S. Environmental Protection Agency, 1994) and Environment Canada (Environment Canada, 1992).

3.1.3 Sensitivity of aquatic zooplankton to toxic substances

Many studies indicate that zooplankton are extremely sensitive to a wide range of pesticides and metals, in several cases more so than phytoplankton, other invertebrates or fish, and therefore might be crucial bioindicators of the overall impact of pesticides and metals in aquatic ecosystems. Cladocerans specifically have been found to be among the most sensitive components of the zooplankton. They have been shown to be significantly more sensitive to pyrethroids and organophosphate insecticides than algae (Bailey *et al.*, 1996), as well as other aquatic invertebrates such as coelenterates, plathelminthes, annelids, rotifers and molluscs (Mian & Mulla, 1992). Crustaceans and insects are also significantly more sensitive to pyrethroids than many fish species used in toxicity testing (Hill, 1989). Comparing a range of different insecticides, including organophosphates, pyrethroids, carbamates and benzoyl urea compounds, Lahr found cladocerans, together with fairy shrimp and certain aquatic insects, to exhibit the highest acute and chronic sensitivities, compared with other zooplankton in tropic-arid ponds (Lahr, 2000). Cladocerans are also among the most metal-sensitive zooplankters (Beisinger & Christensen, 1972; Canton & Sloof, 1982).

As mentioned in Chapter 2, the performance of laboratory organisms and their response to toxic chemicals is influenced by a variety of environmental factors, such as temperature, water chemistry and quality of food, as well as the age and size of test organisms. Care has to be taken therefore when comparing LC_{50} values from different studies.

The age and size of test organisms is of great importance, as many studies have shown juveniles to be significantly more sensitive than adults. This has been shown to be true

for cladocerans (Buhl *et al.*, 1993; Gerritsen *et al.*, 1998) as well as for marine copepods (Forget *et al.*, 1998). Most acute toxicity tests are therefore determined using juvenile organisms (neonate cladocerans, nauplii of copepods, first instars for insects). Acute toxicity tests are normally carried out without food or any other additives in the exposure solution, as the presence of food has also been shown to greatly affect toxicity. Taylor et al. (1998) found that the presence of algal food greatly reduced the acute toxicity of aqueous cadmium to *D. magna.* This is now thought to be due to a general increase in fitness of test organisms when fed, though a certain amount of cadmium adsorption onto the algal surfaces may have also slightly decreased the cadmium concentration in solution, leading to elevated LC_{50} values. At sublethal cadmium levels however, the presence of food increased toxicity, leading to lower EC_{50} values, because of increased cadmium ingestion due to high amounts of the metal being sorbed onto algal cell surfaces (Taylor *et al.*, 1998).

A considerable amount of recent studies also point out that cladoceran species other than *D. magna*, such as *C. dubia* and others, might be equally as sensitive or more sensitive to many toxic substances than *D. magna.*

In a relatively early study, Cowgill *et al.* found *C. dubia* and *D. magna* to be similarly sensitive to four organic benchmark chemicals, with responses of the two species within the same order of magnitude (Cowgill *et al.*, 1985). However, in these 48 h static acute toxicity tests, *C. dubia* was more sensitive to all four chemicals than was *D. magna*, showing a slightly higher sensitivity at 20°C and a pronounced one at 24°C.

In a later paper Cowgill & Milazzo present results from another comparative study on the relative sensitivity of *C. dubia* and *D. magna* to seven organic chemicals (solvents). The endpoint of survival at 48h (LC_{50}) is again very similar for both

species, within the same order of magnitude, with each species slightly more sensitive to some of the chemicals, although 48h LC_{50}s vary somewhat from those reported in the earlier study. Interestingly, they also present a comparative study of 48h LC_{50}s for the two species in the presence and absence of food (microalgae). Without food, *C. dubia* is significantly more sensitive to the four organic chemicals than *D. magna.* When food is added, sensitivity of *C. dubia* to all the compounds is greatly reduced, relatively more so than that of *D. magna. D. magna* even appears more sensitive to ethanol when food is present, although slightly less sensitive to the other three chemicals (Cowgill & Milazzo, 1991).

In a study on the relative toxicity of a range of synthetic pyrethroids to *D. magna* and *C. dubia*, Mokry & Hoagland also found *C. dubia* to exhibit an equal or higher sensitivity to that of *D. magna* (Mokry & Hoagland, 1990). They report 48h LC_{50} values for *C. dubia* of 0.55μg L^{-1} for permethrin, compared to 1.25μg L^{-1} for *D. magna,* and of between 0.07 – 0.30μg L^{-1} (0.32 – 1.04μg L^{-1}, *D. magna*) for four new generation pyrethroids.

Hickey studied relative sensitivities of four species of cladocerans from New Zealand, *D. carinata, Simocephalus vetulus, C. dubia* and *C.* cf. *pulchella*, and compared them with *D. magna* for their acute and chronic sensitivity to toxicants (Hickey, 1989). In acute toxicity tests with 5 widely different reference chemicals, including a metal, a PCP and an organic biocide as well as 2 effluent samples, he found *C. dubia* to be more sensitive than *D. magna* by up to a factor of 4. In chronic tests on 4 of the toxicants however responses of the 2 species were within 1 order of magnitude, with *C. dubia* slightly more sensitive to all chemicals except the biocide. Generally he found *C. dubia* to exhibit a similar sensitivity to the littoral cladoceran *S. vetulus*, with both species more sensitive than *D. magna* or *D. carinata.* He concludes that

threshold response levels varied with the nature of the chemical toxicant and the test species, suggesting that therefore for acute tests using different cladoceran species, a 2-fold (and up to 5-fold) difference in their threshold concentration sensitivity should be anticipated given comparable EC_{50} values. Concerning the usefulness of the different cladoceran species for toxicity testing, he recommends *C. dubia* as a routine test organisms because of its good laboratory growth as well as higher sensitivity, stating that both *C.* cf. *pulchella* as well as *S. vetulus* showed poor laboratory performance in his studies.

Elnabarawy *et al.* (1986) conducted a series of acute and chronic toxicity tests with the 3 cladoceran species *D. magna, D. pulex* and *Ceriodaphnia reticulata*, determining their relative sensitivities to several organic and inorganic chemicals. Their results showed the *Ceriodaphnia* to be equally or more sensitive than the *Daphnia* species, with chronic sensitivities within the same order of magnitude and acute sensitivity within one or two orders of magnitude. 48h EC_{50}s for cadmium were reported to be 184 (159 - 208) $\mu g\ L^{-1}$ for *C. dubia,* 178 (159 - 208) $\mu g\ L^{-1}$ for *D. magna* and 319 (288 - 362) $\mu g\ L^{-1}$ for *D. pulex.* They also suggested the *Ceriodaphnia* 7-d bioassay as an alternative short-term toxicity test.

In a different study employing two clones of *D. magna* as well as *D. pulex,* Lilius *et al.* (1995) found no significant differences in the overall sensitivities of the two different species and clones to 30 organic and inorganic reference chemicals.

Calculations of the 24 and 48h LC_{50} values for the three different species of cladocerans, *C. dubia, C. cornuta* and *S. vetulus*, were carried out to determine differences in their basic sensitivities to the test compounds, and establish

concentrations for the experiments on sublethal effects. It also allowed comparisons of their relative sensitivities with those of other organisms.

As described above, cladocerans form an important nutritional link between heterotrophs and autotrophs. A contaminant-induced decline in the population size of these important grazers could therefore have consequences on energy transfer through the trophic chain (Tidou *et al.*, 1992). Reduced algal grazing can also lead to algal blooms which has been found in studies on contaminant effects in freshwater mesocosms (Jak *et al.*, 1996). Differences in sensitivity to contaminants between organisms may affect trophic interactions not through simple direct toxicity, but through indirect effects, so called "knock-on" effects along the food chain, which can lead to changes in the whole structure of an aquatic community.

3.2 OBJECTIVES

The aim of this study was to determine the acute toxicity of 3 pesticides and one metal in their aqueous solutions to the standard test organism *C. dubia* and the two tropical cladocerans *C. cornuta* and *S. vetulus*. The pesticides studied were the organophosphate insecticide chlorpyrifos, the synthetic pyrethroid deltamethrin, and the triazine herbizide atrazine, as pure compounds. Cadmium, in its aqueous form, was employed for comparison, as a toxic compound with a very different mode of action.

Differences in their acute sensitivities were observed, and where possible sensitivity was determined after 24 and 48 hours, in the presence and absence of food. Acute, static tests were chosen as a suitable exposure method due to the small size of the test organisms, and their natural occurrence in still ponds. Data on acute sensitivities was used to establish concentrations for later sublethal exposure regimes.

3.3 MATERIALS & METHODS

Toxicity tests were conducted with a range of concentrations that were adapted from lethality values for *D. magna* and other cladoceran species found in the literature. Concentrations were calculated on a logarithmic scale. Acute toxicity tests were carried out using neonates less than 24 hours old. Up to 25 organisms of each species were exposed to a range of concentrations, in 3, 4 or 5 replicates with 5 animals each. For deltamethrin, the concentrations were 0 (control), 0.03, 0.06, 0.125, 0.25, 0.5 and 1.0 $\mu g\ L^{-1}$. For atrazine, the concentrations were 0, 0.5, 1.4, 3.8, 10.8 and 30 $mg\ L^{-1}$, which is the maximum solubility concentration. Chlorpyrifos concentrations were 0, 0.05, 0.09, 0.16, 0.3, 0.5 and 1.0 $\mu g\ L^{-1}$, and the cadmium concentration range was 0, 1.0, 2.2, 4.6, 10.0, 21.54, 46.4 and 100 $\mu g\ L^{-1}$. Chemical solutions were made up as described in Chapter 2.

Neonates of *C. dubia* were exposed in 60 mL narrow rimmed bottles with 50 mL of chemical solution, made up in moderate hard ASTM. *C. cornuta* neonates were also exposed in 60 mL narrow rimmed bottles with 50 mL chemical solution, but in hard ASTM. Neonates of *S. vetulus* were tested in 60 mL wide rimmed bottles with 50 mL volume, in hard ASTM. Toxicity tests were static, over 48 hours, at a temperature of 25+/-1°C. In the exposures where food was given, a solution of the microalga *Chlorella vulgaris* was added to the exposure solution at a concentration of between 1-2 $x10^6$ cells mL^{-1}.

Immobilisation was used as a sign of mortality. Immobilisation was defined as a lack of movement or response to gentle agitation of the test solution after 10 seconds (Parrish, 1985). The number of mortalities at each concentration was recorded at 24 and at 48 hours. From the results, 24 and 48h LC_{50} values were calculated using Probit Analysis (Finney, 1971), using a statistical package developed by Nogueira

(1996). For *S. vetulus*, LC_{50}s for deltamethrin without food were only established for 8 and 24h, as animals did not survive without food for 48h.

3.4 RESULTS

Acute toxicity values for the three pesticides can be found in Tables 3.1- 3.3. Survivorship curves for the three species can be found in Figures 3.1-3.3. The shapes of the curves vary between species and between compounds, indicating that responses differed in intensity.

Acute toxicity values for deltamethrin are given in table 3.1. For deltamethrin, 24 and 48h toxicity values without food for *C. cornuta* (0.07 $\mu g\ L^{-1}$ and 0.03 $\mu g\ L^{-1}$) were lower than those for *C. dubia* (0.23 $\mu g\ L^{-1}$ and 0.09 $\mu g\ L^{-1}$), however, as the 95% confidence limits for 48 hours overlapped, only the 24h results are significantly different. The toxicity values for *S. vetulus* without food could only be calculated for 8 and 24h, as the neonates did not survive well enough without food for 48 hours in the controls. The LC_{50} value for 8h without food was 0.3 $\mu g\ L^{-1}$, and for 24h without food it was below 0.1 $\mu g\ L^{-1}$, however from the variable results in this experiment it was not possible to calculate an exact and comparable 24h LC_{50} value for this compound.

When food was present, *C. cornuta* again appeared more sensitive to deltamethrin than *C. dubia* and *S. vetulus*. 24 and 48h LC_{50} values for *C. cornuta* in the presence of food are 0.14 $\mu g\ L^{-1}$ and 0.05 $\mu g\ L^{-1}$, respectively, compared with 0.49 $\mu g\ L^{-1}$ and 0.38 $\mu g\ L^{-1}$ for *C. dubia*, and 0.5 $\mu g\ L^{-1}$ and 0.35 $\mu g\ L^{-1}$ for *S. vetulus*. This time there was an overlap in the 95% confidence limits for the 24h values, indicating that only the sensitivity at 48 hours was significantly lower. Generally, *C. cornuta* was always equally as sensitive or more sensitive than the other two species, showing

significantly lower LC_{50} values than *C. dubia* and *S. vetulus* without food at 24 hours, and with food at 48 hours. Acute toxicity values were always within one order of magnitude.

The presence of food decreased the sensitivity of the three cladocerans, increasing LC_{50} values. This was only significant for *C. dubia* though, with 24h LC_{50} values of 0.23 $\mu g\ L^{-1}$ without food and 0.49 $\mu g\ L^{-1}$ with food, and 48h LC_{50} values of 0.09 $\mu g\ L^{-1}$ without food and 0.38 $\mu g\ L^{-1}$ with food. For *C. cornuta*, 95% confidence limits overlapped, indicating no significant effect of food on toxicity.

The acute toxicity values for atrazine were only possible to establish for one species, *S. vetulus* (see table 3.2). For *C. dubia* and *C. cornuta*, 24h LC_{50} values with and without food, as well as 48h LC_{50} values with food, were above the maximum solubility level of the compound of 30 $mg\ L^{-1}$. 48h LC_{50} values without food for *C. dubia* were found around 15 $mg\ L^{-1}$, but not definable for *C. cornuta*. 24h LC_{50} values for *S. vetulus* were 27.12 $mg\ L^{-1}$ without food, and 24.2 $mg\ L^{-1}$ with food, significantly lower when there was food present. The acute toxicity values for *S. vetulus* at 48 hours were less than 20 $mg\ L^{-1}$ both in the presence and absence of food, however they could not be established exactly from the toxicity tests. *S. vetulus* was significantly more sensitive to atrazine than the other two species.

Acute toxicity values for chlorpyrifos can be found in Table 3.3. The 24 and 48h LC_{50} values without food were lower for *S. vetulus* (0.16 $\mu g\ L^{-1}$ and 0.03 $\mu g\ L^{-1}$) and *C. dubia* (0.13 $\mu g\ L^{-1}$ and 0.07 $\mu g\ L^{-1}$) than for *C. cornuta* (0.28 $\mu g\ L^{-1}$ and 0.16 $\mu g\ L^{-1}$). In the 24h acute toxicity test, both *C. dubia* and *S. vetulus* were significantly more sensitive than *C. cornuta*, whereas at 48 hours only *C. dubia* exhibited significantly lower sensitivity than *C. cornuta*. When food was present, the same pattern was observed, with *S. vetulus* being more sensitive and showing lower 24 and 48h LC_{50}

values (0.16 μg L^{-1} and 0.09 μg L^{-1}) than *C. dubia* (0.23 μg L^{-1} and 0.13 μg L^{-1}) and *C. cornuta* (0.28 μg L^{-1} and 0.16 μg L^{-1}). However, only *S. vetulus* was significantly more sensitive than *C. cornuta* when food was present, as 95% confidence limits for *C. dubia* and *C. cornuta* overlapped, indicating no difference in sensitivity. The presence or absence of food had no effect on toxicity of chlorpyrifos to both *C. cornuta* and *S. vetulus*. For *C. dubia*, a very slightly significant effect could be found at both 24 and 48 hours, with LC_{50} values varying between 0.13 μg L^{-1} and 0.23 μg L^{-1} (without and with food), and between 0.07 μg L^{-1} and 0.13 μg L^{-1} (without and with food), indicating that at both times toxicity was slightly reduced (by about half) when food was present.

Acute toxicity values for cadmium were only determined for *C. dubia*, without food. The 24h LC_{50} value was 68.8μg L^{-1} Cd^{2+} (with 95% confidence limits of 9.6<LC50<217.9), and the 48h LC_{50} was 12.71 μg L^{-1} Cd^{2+} (3.59<LC_{50}<46.41).

Table 3.1: Acute 24 and 48 hour deltamethrin toxicity values for the three cladoceran species tested, with and without food

Species tested	24h LC_{50} with 95% C.I. ($\mu g\ L^{-1}$)		48h LC_{50} with 95% C.I. ($\mu g\ L^{-1}$)	
	+ food	- food	+ food	- food
Ceriodaphnia dubia	0.49 ($0.48<LC_{50}<0.5$)	0.23 ($0.16<LC_{50}<0.35$)	0.38 ($0.25<LC_{50}<0.5$)	0.09 ($0.03<LC_{50}<0.18$)
Ceriodaphnia cornuta	0.14 ($0.03<LC_{50}<0.64$)	0.07 ($0.06<LC_{50}<0.08$)	0.05 ($0.03<LC_{50}<0.07$)	0.03 ($0.01<LC_{50}<0.05$)
Simocephalus vetulus	0.5 ($0.01<LC_{50}<5.5$)	8h LC_{50}: 0.3 ($0.17<LC_{50}<0.56$)	0.35 ($0.23<LC_{50}<0.56$)	24h LC_{50}: <0.1

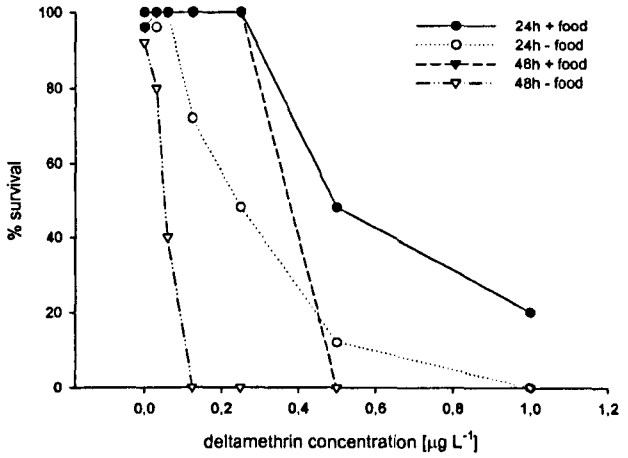
Table 3.2: Acute 24 and 48 hour atrazine toxicity values for the three cladoceran species tested, with and without food

Species tested	24h LC_{50} with 95% C.I. ($mg\ L^{-1}$)		48h LC_{50} with 95% C.I. ($mg\ L^{-1}$)	
	+ food	- food	+ food	- food
Ceriodaphnia dubia	>30	>30	>30	15 ($10<LC_{50}<30$)
Ceriodaphnia cornuta	>30	>30	>30	n.d.
Simocephalus vetulus	24.27 ($23.77<LC_{50}<24.93$)	27.12 ($27.07<LC_{50}<27.56$)	<20	<20

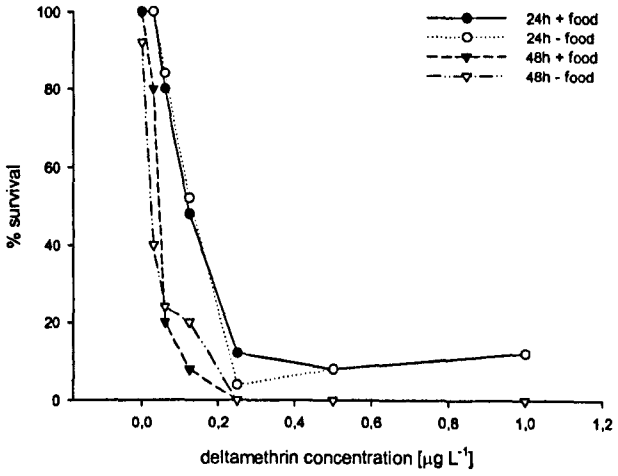
Table 3.3: Acute 24 and 48 hour chlorpyrifos toxicity values for the three cladoceran species tested, with and without food

Species tested	24h LC_{50} with 95% C.I. ($\mu g\ L^{-1}$)		48h LC_{50} with 95% C.I. ($\mu g\ L^{-1}$)	
	+ food	- food	+ food	- food
Ceriodaphnia dubia	0.23 ($0.16<LC_{50}<0.3$)	0.13 ($0.09<LC_{50}<0.16$)	0.13 ($0.09<LC_{50}<0.16$)	0.07 ($0.05<LC_{50}<0.09$)
Ceriodaphnia cornuta	0.28 ($0.26<LC_{50}<0.3$)	0.28 ($0.28<LC_{50}<0.28$)	0.16 ($0.13<LC_{50}<0.187$)	0.16 ($0.12<LC_{50}<0.2$)
Simocephalus vetulus	0.16 ($0.14<LC_{50}<0.18$)	0.16 ($0.13<LC_{50}<0.18$)	0.09 ($0.08<LC_{50}<0.09$)	0.03 ($0.001<LC_{50}<0.18$)

C.dubia



C .cornuta



S. vetulus

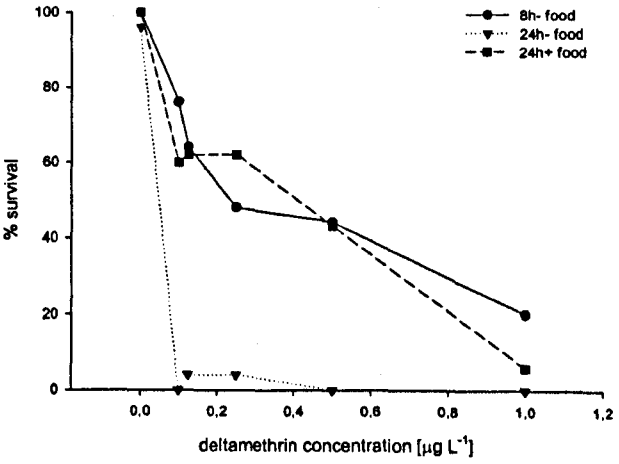
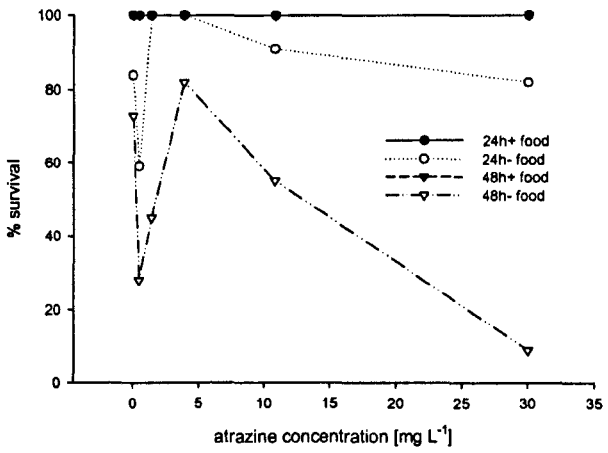
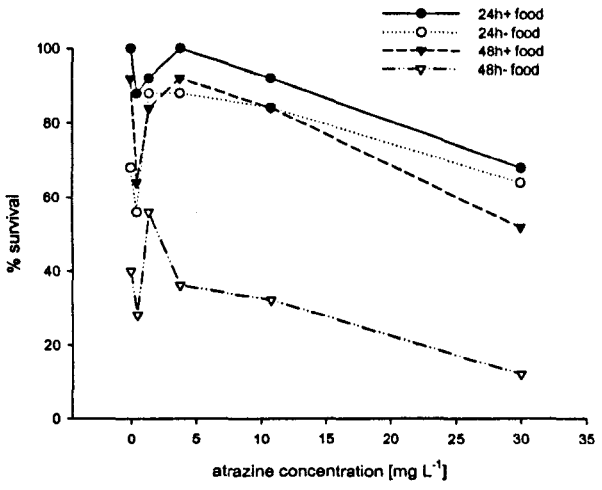


Figure 3.1 Mortality pattern of *C. dubia*, *C. cornuta* and *S. vetulus* exposed to different deltamethrin concentrations over a 48 hour period, with and without food. Group size 25

C. dubia



C. cornuta



S. vetulus

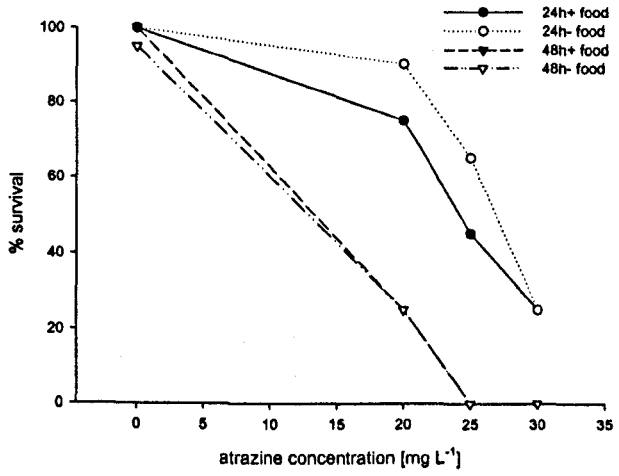
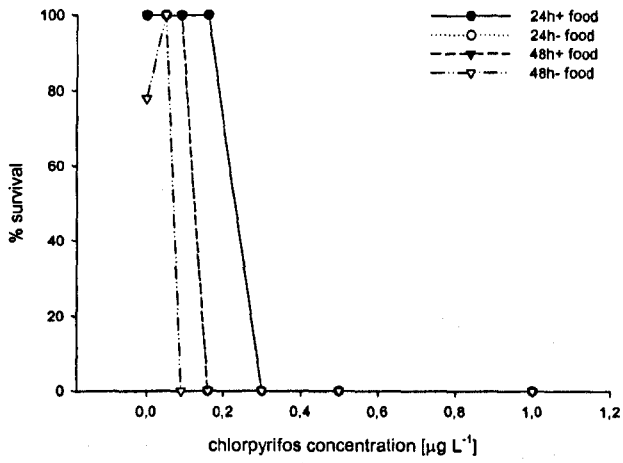
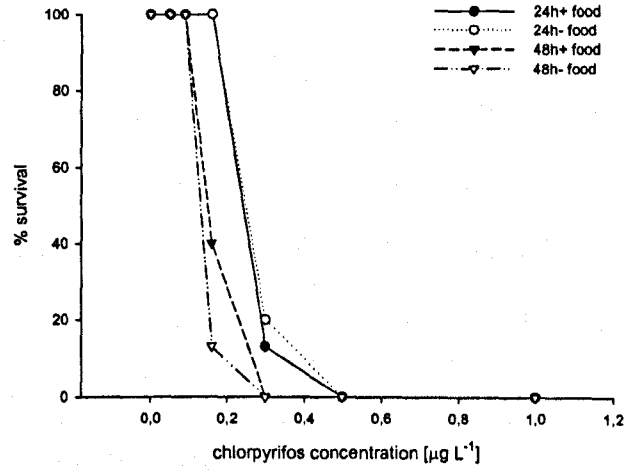


Figure 3.2 Mortality pattern of *C. dubia*, *C. cornuta* and *S. vetulus* exposed to different atrazine concentrations over a 48 hour period, with and without food. Group size 25

C. dubia



C. cornuta



S. vetulus

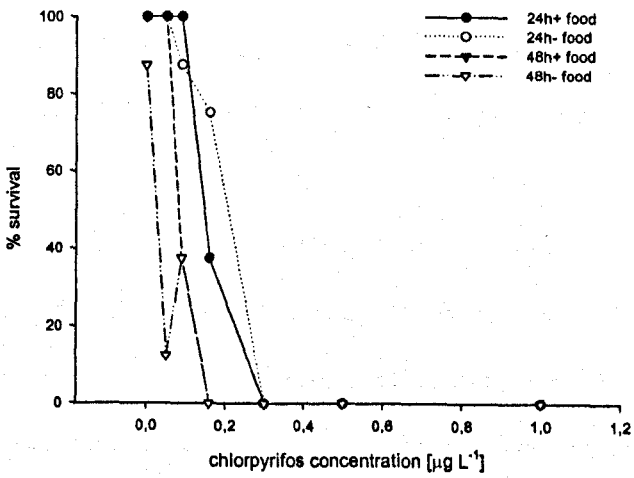


Figure 3.3 Mortality pattern of *C. dubia*, *C. cornuta* and *S. vetulus* exposed to different chlorpyrifos concentrations over a 48 hour period, with and without food. Group size 25

3.5 DISCUSSION

It is very difficult to make comparisons between acute toxicity results found in the literature and those established in this study due to the wide variation of test conditions, exposure regimes and ages of test organisms. As described in Chapter 1, differences in temperature, water hardness, pH, feeding regimes, age of test organisms and many other parameters can greatly influence toxicity. For metal toxicity, the water chemistry and pH are of especially great importance, as they can affect metal speciation and bioavailability. Schubauer-Berigan & Dierkes (1993) for example found that the toxicity of aqueous cadmium to *C. dubia* increased significantly (>4 fold) when the pH increased from 6-6.5 to 8-8.5. Water hardness, salinity and temperature have been found to greatly modify the toxicity of potassium dichromate to *D. magna*. Increasing water hardness from 80 – 800 mg L^{-1} $CaCO_3$ led to a 17 fold increase in toxicity at 7°C, and to a 21 fold increase at 28°C. At low water hardness, an increase in temperature from 7 to 28°C was found to make potassium chromate 12 times more toxic (Persoone *et al.*, 1989).

Pesticide toxicity is also strongly affected by temperature. Another important factor is whether the pesticide is being applied as the pure active ingredient, or as a formulation, since pesticide formulations often contain various other chemicals, such as solvents, which can modify toxicity.

The 3 cladoceran species in the current study exhibited similar acute sensitivities to the 3 compounds. All the 24 and 48h lethality values were within one order of magnitude, corresponding with findings reported from other studies. However, there are significant differences in their individual sensitivities, and it is interesting that the different species appear to vary in their sensitivity to different compounds.

C. cornuta has the highest susceptibility to the synthetic pyrethroid deltamethrin, exhibiting a significantly higher sensitivity at 24 hours without food, and at 48 hours with food, than *C. dubia.* Values for *S. vetulus* were not possible to calculate exactly, but appear to be within a similar range to those of *C. dubia.* 24 and 48h LC_{50} values without food of between 0.07 μg L^{-1} and 0.23 μg L^{-1} (24 hours) and between 0.03 μg L^{-1} and 0.09 μg L^{-1} (48 hours) correspond with values reported from other studies. Day *et al.* (1990) found deltamethrin acute toxicity values for young *D. magna*, tested without food and at 20 +/- 1°C, of 0.27 - 0.29 μg L^{-1} (24h LC_{50}) and of 0.05 – 0.07 μg L^{-1} (48h LC_{50}) (Day & Maguire, 1990). However, Lahr (2000) reports 48h LC_{50} values for *D. magna* of 0.8 μg L^{-1}, significantly higher than those determined in this study.

Compared to other aquatic invertebrates, the cladocerans seem to be sensitive to deltamethrin. Mian & Mulla (1992) report in a large comparative study deltamethrin 96h LC_{50} values for the crab *Uca pugilator* of about 0.56 μg L^{-1}, and for the shrimp *Penaeus duoarum* of 1.5 μg L^{-1}. Acute deltamethrin toxicity values for different insect species were also quite variable, but generally similar with one slightly higher than for the cladocerans: 24h LC_{50} values for the ephemeropteran larvae *Baetis parvus* reported to be 1.1 μg L^{-1}, for the trichopteran larvae *Hydropsyche californica* of 0.4 μg L^{-1}, and for larvae of *Culex* and *Aedes* spp. of between 0.02 – 0.4 μg L^{-1} (Mian & Mulla, 1992). Unfortunately not much detail on test conditions is provided, so it is difficult to assess whether these values are comparable to the ones obtained in this study. In a study on the sensitivities of invertebrates from temporary ponds in the Sahel, Lahr (2000) found two local species to be extremely sensitive to deltamethrin. He determined LC_{50} values for the fairy shrimp *Streptocephalus sudanicus* of 0.035 μg L^{-1} (24 hours) and 0.018 μg L^{-1} (48 hours); acute toxicity values for the notonectid

backswimmer *Anisops sardeus* were 0.013 μg L^{-1} (24 hours) and 0.012 μg L^{-1} (48 hours) (Lahr, 2000). However these toxicity tests were carried out at the slightly higher temperature of 27°C, using a commercial formulation (Decis®).

The triazine herbicide atrazine was considerably less toxic to the three cladocerans. For *C. dubia* and *C. cornuta*, all values except the 48h LC_{50} without food, which was around 15 mg L^{-1} for *C. dubia*, were above the maximum solubility concentration of 30 mg L^{-1}. *S. vetulus* however was significantly more susceptible to atrazine, with a 24h LC_{50} of around 27 mg L^{-1} and a 48h LC_{50} of less than 20 mg L^{-1}. Lethal toxicity data on atrazine and other herbicides is rare, as herbicides are generally not very acutely toxic to animals, being designed to control weeds. In a study on the risk assessment of agrochemicals in European estuaries, Steen *et al.* (1999) cite lowest acute toxicity values for atrazine of 19 μg L^{-1} (algae), 94 μg L^{-1} (crustacea), and 220 μg L^{-1} (fish), but they do not provide more detail on individual species and exposure regimes (Steen *et al.*, 1999). Kuivila & Foe (1995) determined 48h LC_{50} values of 69 mg L^{-1} for *D. magna.* For the marine copepod *Tigriopus brevicornis*, Forget *et al.* report 96h LC_{50} values for atrazine of 120.9 μg L^{-1} for the nauplius, and 153.2 μg L^{-1} for the adult. Generally it can be concluded that acute toxicity of atrazine to cladocerans is low, and sublethal effects at low concentrations are more important to investigate. Recently, the potentially endocrine disrupting effects of atrazine have received increasing attention. Dodson *et al.* (1999) found that low levels of the herbicide (0.5 – 10 μg L^{-1}) led to an increase in the production of males in the cladoceran *D. pulicaria* (Dodson *et al.*, 1999).

In contrast, the organophosphate insecticide chlorpyrifos was highly acutely toxic to the 3 cladocerans. Here, *C. dubia* and *S. vetulus* were markedly more sensitive than *C. cornuta*. At 24 as well as at 48 hours without food, both *C. dubia* and *S. vetulus* were

significantly more sensitive than *C. cornuta*. The acute toxicity values of 0.13 – 0.16 μg L^{-1} (24h LC_{50} *C. dubia* and *S. vetulus*) to 0.28 μg L^{-1} (24h LC50 *C. cornuta*), and 0.03 – 0.07 μg L^{-1} (48h LC_{50} *C. dubia* and *S. vetulus*) to 0.16 μg L^{-1} (48h LC50 *C. cornuta*) correspond reasonably well with acute toxicity values reported from different studies. 96h LC_{50} values for *C. dubia* of 0.08 – 0.13 μg L^{-1} (Kuivila & Foe, 1995) and 0.06 (0.04 – 0.07) μg L^{-1} (Bailey *et al.*, 1996) have been reported, and Woods *et al.* (2002) determined 48h LC_{50} values for *C. dubia* of 0.048 (0.032 – 0.072) μg L^{-1} (Woods *et al.*, 2002). Acute toxicity values for *D. magna* indicate a lower sensitivity to chlorpyrifos of this species, with 48h LC_{50} values of 1.3 μg L^{-1} cited (Lahr, 2000). Interestingly, Lahr (2000) reports significantly higher acute toxicity values for the tropical pond species *S. sudanicus* and *A. sardeus*, 24h LC_{50}s of 8.25 μgL^{-1} and 1.58 μg L^{-1}, respectively, and 48h LC_{50}s of 3.48 μg L^{-1} and 0.9 μg L^{-1} for the two species. Again, these toxicity tests were carried out at 27°C, and using the commercial formulation Dursban®.

From these references it appears that the three cladoceran species investigated in this study show extremely high acute sensitivity to chlorpyrifos.

The acute toxicity of cadmium was only determined for *C. dubia*. The 48h LC_{50} without food value of 12.71 μg L^{-1} (3.59 – 46.41) is consistent with values established by Robinson (1998): 48h LC_{50} for *C. dubia* of 17.38 μg L^{-1} (8.35 – 26.53). However, acute toxicity values found in this study are significantly lower than those reported by other researchers for *C. dubia*. Bitton *et al.* (1996) report a 48h LC_{50} for cadmium of 54 μg L^{-1} (+/- 3 μg L^{-1}), and Elnabarawy *et al.* (1986) cite 48h LC_{50}s of 184 μg L^{-1} (*C. reticulata*) and 178 μg L^{-1} (*D. magna*) (Bitton *et al.*, 1996; Elnabarawy *et al.*, 1986). Again the difference in these values could be due to differences in the test conditions, or the use of different clones which can sometimes vary considerably in their

susceptibility, particularly to metals (Baird *et al.*, 1990). The acute toxicity values established in this study support the generally accepted theory that crustaceans are among the most vulnerable freshwater organisms when exposed to cadmium (Sloof *et al.*, 1983; Robinson, 1998).

Comparing the overall sensitivity of the three species, it is interesting to note that there are significant differences in their sensitivities to the different compounds. *C. cornuta* is significantly more sensitive to the pyrethroid deltamethrin than *C. dubia* and *S. vetulus*, who are similarly sensitive (Table 3.1). Contrastingly, *S. vetulus* is by far the most sensitive species in exposure tests with the herbicide atrazine (Table 3.2). The third compound, the organophosphate chlorpyrifos, induces significantly higher acute toxicity in *C. dubia* and *S. vetulus*, with *C. cornuta* being significantly less susceptible (Table 3.3). These findings correspond with results from other studies which report that although sensitivity of different cladoceran species is usually within one order of magnitude, important differences in individual sensitivity need to be considered, especially in relation to different ecologically relevant exposure conditions (Elnabarawy *et al.*, 1986; Hickey, 1989; Lahr, 2000).

The role played by the algal food is an interesting one, as food has been found to modify toxicity in different ways. Algal cells as well as other particulate organic matter can reduce aqueous toxicity of certain toxic chemicals, especially those with a positive surface charge, such as cadmium Cd^{2+} and di-tallow dimethyl ammonium chloride DTDMAC, which strongly adsorb onto the negatively charged particles (Allen *et al.*, 1995; Taylor *et al.*, 1998). This reduction of toxicity is especially pronounced at high aqueous toxicant concentrations, when aqueous toxicity is more important than ingestion of the toxic substance, as feeding is inhibited. At these high concentrations, binding of toxic molecules to the algal particles will reduce aqueous

toxicant concentrations and therefore acute toxicity to the animals. At sublethal toxicant concentrations however, toxic molecules adsorbed to algal cells will in fact be more bioavailable to the animal, which still feeds at these levels, so that the presence of algal cells increases the toxicity (Taylor *et al.*, 1998).

In this study, the effect of food on acute toxicity seems variable. Acute toxicity appears to be affected by the presence of algal food only in *C. dubia*. Only this species shows a significant difference in 24 and 48h LC_{50} values to both the pyrethroid deltamethrin (Table 3.1) and the organophosphate, chlorpyrifos (Table 3.3). For both compounds, at 24 and at 48 hours, toxicity is significantly reduced in the presence of algal food cells. For the herbicide atrazine (Table 3.2), results are inconclusive due to the low acute toxicity of the compound that made LC_{50} values impossible to calculate, however, at 48 hours, toxicity is also notably reduced when food is present, as the 48h LC_{50} without food is 15 mg L^{-1}, whereas with food it is still above the solubility limit.

Both *C. cornuta* and *S. vetulus* exhibit no significant difference in their acute sensitivity to any of the three compounds with food present of absent. The fact that *C. dubia* seems to be more affected in its susceptibility to toxins by the presence or absence of food than the other two species is interesting, and corresponds somewhat with results from a study by Cowgill & Milazzo (1991). In their study on comparative sensitivities of *D. magna* and *C. dubia* to a range of different organic chemicals, they found the latter significantly more sensitive to all the compounds when no food was added. However, when food (algal cells) was added to the test solutions, acute sensitivity of *C. dubia* was markedly and significantly reduced, notably more than in the case of *D. magna*. This could indicate that *C. dubia* is more susceptible to a

shortage of food due to its different metabolism and an inability to compensate for increased energetic demands due to toxicity.

Other researchers have also found variable effects of food on toxicity. Barry (1996) found that effects of sublethal levels of endosulfan on a range of life-cycle parameters in *D. carinata* were more pronounced at higher food levels ($1x10^5$ cells mL^{-1} as opposed to $5x10^4$ cells mL^{-1}) (Barry, 1996). However in an earlier study Barry *et al.* (1995) found that the sublethal toxicity endosulfan to *D. carinata* was decreased in the presence of algal food compared to no food present (Barry *et al.*, 1995) They suggest that increased toxicity at higher food levels may have been due to an increase in metabolic rate or a trade-off between different life-cycle parameters.

Rose *et al.* (2001) studied the effects that an inhibition in feeding of *C.* cf. *dubia*, caused by the presence of fish kairomones, had on the toxicity of three insecticides with different modes of action (3,4-dichloraniline, fenoxycarb and chlorpyrifos). In this case, algal cells were present in the test solutions, but animals did not feed due to the kairomones. They found that the reduction in feeding (due to the presence of the hormones) decreased the sublethal and lethal toxicity of fenoxycarb, whereas the toxicity of 3,4-dichloraniline was unchanged. The toxicity of the organophosphate chlorpyrifos, however, was increased both in terms of lethality as well as sublethal life-cycle parameters such as brood size and growth rate when animals did not feed (Rose *et al.*, 2001). They suggest that the increased energy demands caused by the organophosphate poisoning, which leads to inhibition of acetylcholinesterase and in this way to increased and prolonged stimulation of cholinergic nerve junctions, lead to higher mortality and reduced fitness when feeding is inhibited.

Regarding the potential of the different chemicals to adsorb onto algal cells, the K_{OW} and K_{OC} values as stated in Table 1.2 suggest that deltamethrin would show the strongest tendency for adsorption, and atrazine the weakest one, with chlorpyrifos intermediate.

Regarding the need for developing bioassays with local species, different opinions exist. *C. dubia* has been shown to be a useful alternative toxicity test species representative of the freshwater zooplankton communities in North America, Australia and New Zealand. However, this species is not common in tropical areas. For the development of laboratory and especially *in situ* toxicity tests in tropical countries, it would be valuable to have an indigenous species that is comparable to the standard test organisms in its sensitivity and laboratory performance.

Investigations into isolating such locally relevant test species have been carried out by several researchers, mainly looking at cladocerans and fish (Hickey, 1989; Mokry & Hoagland, 1990; Sunderam *et al.*, 1992; Sunderam *et al.*, 1994; Davies *et al.*, 1994). Hickey (1989) found the local New Zealand species *C. dubia* and *S. vetulus* to be more sensitive than *D. magna* to chemical pollutants under local conditions.

Sunderam *et al.* in a comparative study on the sensitivity of Australian native and introduced fish in 1992 posed the question: "To what extent is it necessary to determine the toxicity of native species in local waters in order to derive water quality criteria". They concluded that, in the case of endosulfan, it was not strictly necessary, as sensitivities were quite similar. This finding was supported by Davies *et al.* (1994), who compared acute and chronic sensitivities to a range of pesticides of 2 Australian freshwater fish species and a crustacean with that of a standard fish test species native to northern cold waters. They found no significant differences in overall sensitivities

between the different fish species. However, both these studies only examined short-term responses of fish to pesticide exposure.

In a later study in 1994, Sunderam *et al.* obtained acute and chronic toxicity values for endosulfan toxicity to the cladocerans *C. dubia* and *M. macleayi* that were lower than those for *D. magna*, suggesting that considering local species performance might be of value.

Castillo (2000) also suggests that for suitably determining risk to environments which wildly differ from the temperate ones for which traditional toxicity tests have been developed, it is necessary to develop acute and chronic toxicity bioassays with local organisms, which are ecologically relevant (Castillo, 2000). Lahr (2000) supports this idea. His work on the effects of different pesticides on aquatic invertebrates in temporary ponds in the Sahel (West Africa) show that indigenous species can differ greatly in their sensitivity to toxicants and environmental parameters compared to standard temperate test organisms. He found very little similarities in the sensitivities of *D. magna* compared to a local species, the fairy shrimp *Streptocephalus sudanicus*, although both belong to the order Branchiopoda. He concludes that the use of locally important species is imperative when trying to investigate the effects of pesticides on ecosystems in tropical or otherwise non-temperate areas, and that the use of standard temperate species alone can lead to unacceptably high uncertainty in regional risk assessment (Lahr, 2000).

This study supports the importance of choosing local species and test conditions more representative of those in the environments investigated when developing bioassays for tropical conditions.

CHAPTER 4

FEEDING INHIBITION IN CLADOCERANS AS A SUBLETHAL ENDPOINT MEASURING EFFECTS OF CHEMICAL EXPOSURE

4.1 INTRODUCTION

4.1.1 Chronic and sublethal effects toxicity testing

For assessing the risks of chemicals to aquatic organisms, populations and communities, acute toxicity tests measuring mortality are not always adequately sensitive, and the fact that a chemical does not have direct lethal effects on aquatic organisms does not necessarily mean that it is not toxic to these species (Rand, 1995). Except for acute spills and accidents or the immediate vicinity of a chemical release site, pollutants in the aquatic environment are usually diluted and dispersed and thus present at low concentrations, which do not always induce acute mortality. These low, sublethal concentrations may nonetheless have a profound effect on the future survival of the organisms.

Chronic toxicity tests allow assessment of the possible adverse effects of a chemical under conditions of long-term exposure at sublethal concentrations. There are many designs available for chronic tests, some including the exposure of the test organism for an entire reproductive life cycle (full chronic tests), or for only several sensitive life stages (partial chronic tests). The duration of a chronic toxicity test varies with the species tested, for example, 21 d for *Daphnia magna*, and 275 – 300 d for the fathead

minnow *Pimephales promelas* (Rand, 1995). The data obtained in chronic toxicity tests allows the estimation of the Maximum Acceptable Toxicant Concentration (MATC) of a chemical: an estimated threshold concentration in a range between the highest concentration tested at which no significant deleterious effect was observed (NOEC), and the lowest concentration tested at which some significant harmful effect was observed (LOEC) (Mount & Stephan, 1967). The use of NOECs and LOECs has been criticised recently, with regression-based approaches advocated instead, but they are still being used for a means of simple comparison (Klüttgen *et al.*, 1996; Moore & Caux, 1997).

Although chronic tests are invaluable at providing information about effects at low concentrations, they are costly and difficult to carry out due to their duration, and potentially have more scope for error. Short-term sublethal tests provide an alternative, and various sublethal effects may be studied in the laboratory by a variety of procedures. Three classes of sublethal effects can be distinguished: biochemical/ physiological, histological, and behavioural (Shehan *el al.*, 1984). Biochemical and physiological effects tests look at effects on proteins, such as enzymes and stress proteins, hematology, and respiration. Histological effects studies investigate modifications in the functions of tissues and organs.

Behaviour is an integrated response, and chemically induced effects on behaviour may indicate effects at a molecular and physiological level, making behavioural endpoints sensitive sublethal effects indicators. In aquatic organisms, important behavioural endpoints are for example swimming and locomotion, prey-predator interactions, aggression, as well as feeding and foraging behaviour (Rand 1995).

Chemicals in aquatic organisms can also have adverse effects on the immune system (immunotoxic), produce cancerous tissue changes (carcinogenic), or lead to DNA alterations (genotoxic).

Modern toxicity bioassays strive to combine sensitive, ecologically relevant test species and endpoints with a simple design that is easy to validate and replicate, as well as a short test duration, providing relevance as well as cost-effectiveness. For those reasons, short-term sublethal toxicity tests continue to be developed and improved.

4.1.2 Feeding in cladocerans

4.1.2.1 Filter feeding and particle selection

Cladocerans, especially the larger *Daphnia* spp., are highly efficient filter feeders, grazing on phytoplankton, including blue-green algae, and bacteria as well as fungi, protozoans and organic detritus in the water column, and can also feed directly on bottom sediments at low algal densities (Hebert, 1978). Some littoral species (such as *Simocephalus*) can stir up food particles by scraping the bottom with their thoracic appendages and by producing water currents, so that they can filter the floating particles from the water (Lampert, 1987).

Cladocerans possess the most highly developed filtering apparatus within the Branchiopoda (Lampert, 1987), consisting of highly specialised thoracic appendages for the collection of food particles. The trunk limbs, which have been reduced to five, form a suction-and-pressure pump together with the carapace. The beating of the appendages creates a continuous stream of water, which is forced through the filter apparatus, and filter-like screens on the third and fourth pair of appendages retain particles from the water. These are then transported to the food groove, moved

towards the mouth, processed by the mandibles and swallowed. The food groove can be purged of excess or unsuitable particles by a rejectory movement of the post-abdominal claws (Lampert, 1987).

Daphnids filter relatively unselectively over a range of sizes of food particles. Unlike copepods they do not discriminate between individual particles on a chemosensory basis. The filtering rates (appendage beat rate) are high at low food concentrations and decreases at high particle densities (Lampert 1987). It is also dependent on the size of the animals, with smaller species showing higher rates. The maximum size of particles ingested also depends on the animal's size, with larger animals ingesting larger particles (DeMott, 1982). Larger daphnids have also been found to be more successful than smaller zooplankton species at controlling algal blooms due to their ability to digest certain species of digestion-resistant green algae (Tessier *et al.*, 2001).

The main mechanism for selection when feeding is by post abdominal claw rejection, when unsuitable particles such as filamentous algae are rejected together with ingested food in the food groove. This is a very inefficient mode of selectivity, as it also leads to an interruption in feeding (DeMott, 1982). Another method for rejection of particles is the partial closure of the carapace, or a decrease in the beats of the thoracic limbs (Gilbert & Durand, 1990).

Despite the widely accepted view of the unselective feeding mode of the cladocerans, Lampert (1982) suggested that daphnids exhibit some chemosensory rejection of some toxic cells, such as cyanobacteria. Waterborne chemicals have been found to act both as a stimulus as well as cause rejection (Haney *et al.*, 1995). Differences in surface properties of particles may also affect their capture efficiency, as Gerritsen & Porter (1982) showed that the retention of very small particles (<0.1 μm) by the filters

of *D. magna* was much higher when these particles had a neutral charge than when they were negatively charged (Gerritsen & Porter, 1982).

Two different terms are used to describe the quantitative uptake of food particles by daphnia. The ingestion rate (= feeding rate) measures the amount of food that passes the mouth and enters the gut: its dimension is mass per individual per time unit. The mass of the food can be expressed as cells, dry weight, carbon, or any other biomass parameter. The filtering rate (= filtration rate, grazing rate) describes the volume of water theoretically filtered, i.e. the volume of water that contained as many particles as have been removed by the animal. The dimensions are volume of water per individual per time unit (mL ind^{-1} h^{-1}) (Lampert, 1987).

4.1.2.2 Factors affecting baseline feeding rate

The equation used to quantify feeding rates in daphnids gives an indication as to which factors will affect baseline feeding during the bioassay. The equation is a simplified version of Gauld's equation as given in Allen *et al.*, (1995):

$$F = \frac{V * (C_0 - C_t)}{T * N} \qquad (4.1)$$

where: F = feeding rate of a single animal (cells ind^{-1} h^{-1})

V = volume of suspension (mL)

C_0 = initial cell concentration (cells mL^{-1})

C_t = final cell concentration (cells mL^{-1})

t = time animals were allowed to feed (h)

N = number of animals per replicate

From the equation it is apparent that food concentration, exposure volume, time spent feeding, and the number of animals per replicate are the main factors affecting feeding rates in the bioassay. Exposure volume, time spent feeding and group size all affect the food concentration, making this the most important factor in the equation.

The effect of increasing food concentrations on filtering and ingestion rates in daphnids has been studied by many researchers. Generally, the ingestion (feeding) rate increases with rising food concentration until an "incipient limiting concentration/ level" (ILC/ ILL) (McMahon, 1965) is reached. Above the ILC the ingestion rate remains constant. The filtering rate however is constant from zero until the ILC, above which it decreases. Therefore, daphnids exhibit the maximum filtering rate below the ILC, and the maximum ingestion rate at or above the ILC. Rejection rates remain low and constant below the ILC, but increase above the ILC to remove excess food from the food groove with the abdominal claw (Porter *et al.*, 1982). This type of response is termed the functional feeding response, and has been described for different species of *Daphnia* (Chow-Fraser & Sprules, 1992; Gerritsen & Porter, 1982; Porter *et al.*, 1983). Different mathematical models have been used to describe the shape of the functional response curve. In this study, a non-linear model was applied to the ingestion rate data to give a Holling type 2 response/ Ivlev curve/ Michaelis Menten curve, e.g.(Porter *et al.*, 1982). This model is characterised by a curvilinear increase in ingestion rate with food concentration (see Figure 4.1). Further definitions to the type 1 and type 3 functional response curves can be found in Chow-Fraser & Sprules (1992).

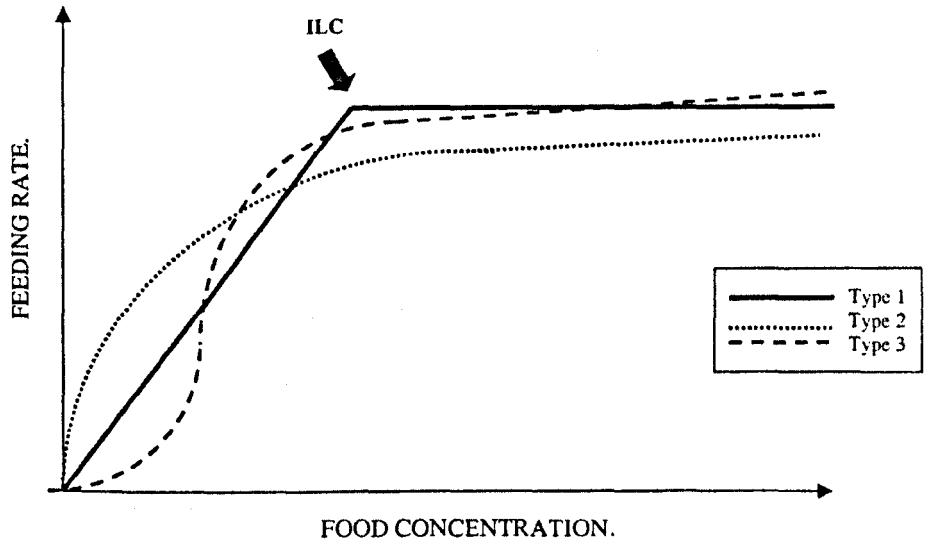


Figure 4.1 Diagrammatic representation of the Type 1, 2 and 3 mathematical models used to describe the functional feeding response of filter feeders (adapted from Chow-Fraser & Sprules, 1992)

Other important factors that influence the feeding rate are group size, as crowding has been shown to depress feeding rates in daphnids (Horn, 1981; Helgen, 1987), as well as in other filter feeders such as copepods (Frost, 1972; Frost, 1975) and the anostracan *Artemia* (Reeve, 1963). This can be due to inhibitory chemicals released by the daphnids themselves (Matveev, 1993), or by mechanical interference between animals (Peters, 1984). However, the density at which crowding-induced feeding inhibition occurs is highly species-dependent.

Other factors that could affect baseline feeding rates are temperature, body size, levels of dissolved oxygen, pH, and diel rhythms.

Daphnids display a bimodal diel feeding pattern with low, variable feeding rates during the day and high, more constant rates at night. This effect seems to be induced by light, although endogenous factors are thought to be involved as well (Starkweather, 1978). Vertical migration occurs among many species of cladocerans, and is thought to be a way of avoiding predatory pressure, especially by fish, which is higher during the day (Lampert, 1993; Fisken, 1997). The animals hide in the deeper, murkier layers of the lake during the day and ascend to the nutrient rich surface layers at night, where feeding increases (Matsumura-Tundisi *et al.*, 1984; Makino *et al.*, 1996). In a natural system, feeding rates of *Daphnia* were found to increase considerably around sunset and were measured to be 10 times higher at night than during the day (Haney, 1985).

Body size (age) of test organisms, as well as abiotic factors such as temperature, pH and dissolved oxygen, all influence the feeding rate; however, they are controlled in the laboratory bioassay and not thought to influence variability. Body size is known to influence feeding, with feeding rates generally increasing exponentially with an increase in size. In the bioassay, all animals are selected to be of the same age and

subsequent size to reduce the effect of body size on feeding. Temperature can have an effect on feeding rate, but the optimal temperature for maximum feeding depends on the species. Generally, feeding rate will increase with increasing temperature due to an acceleration of the metabolic rate, resulting in an increased energy requirement. However once the "optimum" temperature is exceeded, feeding declines rapidly (McMahon & Rigler, 1965; Lampert, 1987). Low dissolved oxygen levels have been found to reduce the filtering rate of daphnids, although this group of animals is relatively resistant to low oxygen levels. Effects of pH on feeding are controversial, but extreme pH ranges will lead to a decrease in feeding (Lampert, 1987).

4.1.3 Effects of reduced feeding on individuals, populations and communities

Contaminant-induced feeding inhibition, also referred to as toxic anorexia, is a useful sublethal endpoint for assessing adverse effects of pollutants on ecosystems as it is often more sensitive, by orders of magnitude, than survival, and can be assessed rapidly (Allen *et al.*, 1995). It is also of high ecological importance, as a reduction in feeding will affect the individual animal in its life-history performance, and has the potential to cause adverse consequences at the population and community level.

Reduced feeding and food uptake directly affect the energy budget of an animal. A very simple individual energy budget is given by:

$$\mathbf{A = P + R} \qquad \textbf{(4.2)}$$

Where: A = assimilation, P = production (i.e. growth and reproduction), R = respiratory heat loss (Baird *et al.*, 1990)

The amount of food consumed (energy) directly affects assimilation, and bioenergetics models integrate energy input, which affects all other parameters (Juchelka & Snell, 1994). The acquisition and allocation of food determines all individual life-history traits, such as development and growth rate, fecundity and survival, potentially affecting population dynamics and community structure (Maltby, 1994). These parameters determine the population growth, estimated by the intrinsic rate of natural increase (*r*), which describes long-term persistence of a population (Van Leeuwen *et al.*, 1985).

Daphnids are very sensitive to changes in food levels, and a reduction in energy input can change energy allocation patterns towards such ecologically relevant parameters such as growth and reproduction.

Resource allocation has been previously studied in *D. magna*. Bradley *et al.* (1991) found that under conditions of starvation, reproduction in *D. magna* declined but continued for a short period, and clutch sizes were reduced. Instead there was an increase in the investment per offspring, with fewer, larger neonates produced, a phenomenon also observed by Enserink (1995). It is thought that this strategy helps to maintain fitness through improving offspring survival when food is limited.

Food levels have also been found to cause a trade-off between growth and reproduction in *D. magna*. Whereas some females reproduced at an earlier age and smaller size, producing smaller neonates, others reproduced later, giving growth priority over reproduction (Enserink, 1995). Other studies have also shown a reduction in growth in daphnids exposed to low concentrations of toxic substances. Baird *et al.* (1990) found reduced growth in *D. magna* exposed to sublethal concentrations of DCA and cadmium, resulting in reduced reproductive investment in terms of egg mass and clutch size. It was suggested that this reduction in reproductive

output reflected a reduction in energy input, caused by an inhibition of feeding due to toxicant exposure.

Klüttgen *et al.* (1996) also found strong effects of sublethal concentrations of DCA on life-cycle parameters such as growth and reproduction in *D. magna* and *C. dubia*. Reproduction in terms of number of offspring was adversely affected by exposure to low levels of DCA in both species. However, interestingly, different life-cycle parameters were differently affected in these two closely related species. Low concentrations of DCA increased the age at first reproduction in *Daphnia*, but not in *Ceriodaphnia*. Growth and filtration rates in *Daphnia* were also reported to be inhibited by this chemical in this study, whereas there was no significant inhibition in *Ceriodaphnia* (Klüttgen *et al.*, 1996). Baird *et al.* (1990) however did not find any inhibition effect of DCA on growth or filtration rates in *D. magna*.

These results suggest that different cladoceran species have different life-history strategies, and important parameters might be differently affected by toxicant stressors. This could lead to different population dynamics and competitive performances, even between closely related species.

Changes in growth and reproduction of individuals in turn can lead to reductions in population numbers and mean population body size, which in turn affect the structure and dynamics of the populations and communities (Baird *et al.*, 1990). *Daphnia* species form an important part of the freshwater zooplankton. They are especially efficient grazers, controlling phytoplankton populations (Gliwicz, 1975; Lynch & Shapiro, 1981; Lampert *et al.*, 1986), and are also important as prey for insects, predatory zooplankton, and fish (Ramcharan *et al.*, 1992; Hart, 1986), thus being an important organism in terms of energy transfer within the aquatic food web.

Reduced body size due to pollutant exposure will reduce vulnerability to predation, and smaller, stressed cladocerans are also less effective grazers, increasing the potential for phytoplankton blooms (Porter, 1976; Gliwicz, 1975; Lynch, 1978; Tessier *et al.*, 2001). Day & Kaushik (1987) found that exposure of a pond community to the synthetic pyrethroid fenvalerate led to changes in community structure due to a decrease in zooplankton filtration rates. Larger bodied daphnids (*D. galeata* and *D. mendotae*), which have higher filtration rates, disappeared after 3 weeks, leading to an increase in numbers of rotifers in the absence of competition. The smaller rotifers exerted a reduced grazing pressure, resulting in an algal bloom. Jak (1997) observed a similar trend in ponds treated with different metals and 3,4DCA. Numbers of the more sensitive *Daphnia* species decreased, leading to an increase in rotifer and copepod populations, and a rise in phytoplankton biomass due to reduced grazing pressure.

These studies show that reductions in cladoceran feeding due to sublethal concentrations of toxicants can potentially change the structure and function of aquatic freshwater communities (Gliwicz & Sieniawska, 1986).

4.1.4 Feeding inhibition as a sublethal endpoint in toxicity tests

Several studies have shown that contaminant-induced feeding inhibition is a sensitive sublethal endpoint, generally occurring at concentrations much below those that affect survival (Fernandez-Casalderrey *et al.*, 1993; Fernandez-Casalderrey *et al.*, 1994; Allen *et al.*, 1995; Taylor *et al.*, 1998; Hartgers *et al.*, 1999).

Many chemicals have been found to negatively affect feeding rates and feeding behaviour in different aquatic organisms. The largest proportion of studies investigating the effects of toxic substances on feeding has been dealing with fish. A review can be found in (McWilliam, 2001). In invertebrates, contaminant-induced

feeding inhibition has been reported from a wide variety of organisms from different trophic levels that were exposed to a range of different substances. Feeding inhibition has been studied in the mussel *Mytilus edulis* (organic pesticides) (Donkin, 1997), the amphipods *Gammarus pulex* and *Hyalella azteca* (metals and organic pesticides) (Crane, 1995; Hatch & Burton, 1999), and the rotifer *Brachionus calyciflorus* (metals and organic chemicals) (Fernandez-Casalderrey *et al.*, 1992; Ferrando & Andreu, 1993; Janssen *et al.*, 1993). Feeding inhibition has also been studied with different cladocerans and chemicals: *D. magna* (Ferrando & Andreu, 1993; Fernandez-Casalderrey *et al.*, 1993; Fernandez-Casalderrey *et al.*, 1994; Allen *et al.*, 1995; Taylor *et al.*, 1998; Villaroel *et al.*, 1998; Villarroel *et al.*, 1999; Hartgers *et al.*, 1999), *D. pulicaria* (Lampert, 1987), and *Ceriodaphnia* spp. (Day *et al.*, 1987; Bitton *et al.*, 1996; Day & Kaushik, 1987).

These studies demonstrate that feeding inhibition measured during direct exposure to contaminants is a sensitive endpoint. Bioassays which measure feeding inhibition during exposure to toxicants have already been developed using *C. dubia*. The CerioFast bioassay (feeding activity suppression test) (Bitton *et al.*, 1995; Bitton *et al.*, 1996; Jung & Bitton, 1997) measures feeding rates of *C. dubia* feeding on yeast cells, and the CAUST bioassay (*Ceriodaphnia* algal uptake suppression test) (Lee *et al.*, 1997) involves *C. dubia* feeding on the alga *Scenedesmus subspicatus*. Both bioassays were developed for short-term toxicity screening, taking only 1 h to perform. Due to their short duration though they are not particularly sensitive, and their feeding EC_{50} values are often higher than 48h LC_{50} values. Therefore they are not really a useful alternative to 24h feeding bioassays for measuring sublethal effects.

A laboratory bioassay which measures feeding inhibition over a slightly longer period of time, which would increase the sensitivity, would be a useful tool for assessing the effects of low doses of environmental contaminants, singly and in combinations, on an ecologically relevant parameter.

4.2 OBJECTIVES

The aims of this chapter were the development of a laboratory-based bioassay with the cladoceran *C. dubia,* measuring feeding inhibition as a sublethal endpoint. The *C. dubia* bioassay was then modified for the two tropical cladoceran species *C. cornuta* and *S. vetulus*. Functional feeding response curves for the three species were determined to calculate the optimum food level for the bioassay.

Feeding rates of the three species were measured during exposure to the four chemicals, and the median effective concentrations that induced inhibition of feeding (IC_{50}) were calculated for all compounds and for the two species *C. dubia* and *S. vetulus*. The IC_{50} describes that concentration of the test compound which leads to an inhibition of feeding rates by 50% compared to the controls.

4.3 MATERIALS & METHODS

4.3.1 Animals

All animals used for the feeding experiments were young adult females just after they had moulted and released their first brood (1st adult instar). Adult animals were chosen because due to their size their feeding rates were higher and less variable than in juveniles. The fact that the young females had just released their first brood and subsequently moulted meant that it would be between 24 and 48 h until the next moult, increasing the probability to complete the 24 h bioassay without moulting of

the animals (see 4.3.3). To guarantee that animals used for experiments were of the same age, neonates were removed from the breeding cultures every day, within 24 h of release from the mothers. If possible, neonates for experiments were removed within 6 or 12 h of release, to produce test animals of a closer age group.

Ages of the different species at the start of a test varied depending on their respective developmental rates. *C. dubia* reached their first adult instar and released the first brood at 3 to 4 d, *C. cornuta* at 3 to 4 d. *S. vetulus*, being the largest species, took about 6 to 7 d to reach maturity (see Chapter 2).

All animals were acclimated to the test conditions, and experiments were conducted at a constant temperature in an incubator (25°C +/- 1°C). All feeding experiments were carried out in the dark, as feeding rates have been shown to be higher and more uniform in the dark (Haney, 1985).

4.3.2 Acclimation

All animals were acclimated to experimental conditions using the same methodology. *C. dubia* and *C. cornuta* were acclimated in 175 mL narrow rimmed glass jars containing 150 mL ASTM, with 20 animals per jar. *S. vetulus* were acclimated in 250 mL wide rimmed jars containing 200 mL ASTM and 10 to 20 animals. *C. cornuta* and *S. vetulus* were kept in hard ASTM, *C. dubia* in moderate hard ASTM (see Chapter 2). Conditions were kept as uniform as possible for all test animals, to minimise the effects of environmental variation on the development of the animals, maximising phenotypic consistency. Overcrowding of animals must be avoided as it can lead to poor growth and altered moulting behaviour.

Animals were fed daily at a concentration of 1-2 $x10^6$ cells mL^{-1} as this was the food level used during the experiments, and seaweed extract was added at a concentration

of 5 mL L^{-1} to guarantee healthy development. Animals were kept under these conditions until they had released their first brood/ moulted.

4.3.3 Moulting

Moulting of animals has been found to lead to a decrease of feeding rates, probably due to a cessation of feeding during ecdysis (McWilliam, 2001). This reduction in feeding can increase variability in baseline feeding rates. Therefore the aim was to keep moulting and release of neonates to a minimum, by completing the bioassay within one moult cycle. One or two animals moulting per jar was considered to be the maximum tolerable number before compromising baseline feeding rates too greatly, so replicates where excessive moulting and release of broods occurred had to be discarded. Therefore it was vital to ensure animals were of the same age (see 4.3.1) and of good health (see 4.3.2).

4.3.4 Calculation of feeding rates

Cladoceran feeding rates were measured by determining the algal clearance rates, based on observed changes in the numbers of algal cells counted before and after the exposure period. Methods of sampling and determining of feeding rate are the same for all experiments unless stated otherwise. Algal cell concentrations were measured using a Coulter Multisizer, Model II (Coulter Electronics Ltd.), fitted with a 70 μm orifice tube. Coulter Counters have been shown to be a suitable method for measuring feeding rates through algal clearance (Kersting & Van der Leeuw, 1974), and have been successfully used for this purpose in several studies (Waddell, 1993; Taylor *et al.*, 1998; Barata & Baird, 2000).

Feeding rates were obtained by determining algal cell concentrations at the beginning and at the end of each experiment. Initial concentrations were measured at the start of

the experiment (t = 0) and at the end (t = 24), from blank samples that did not contain animals, to ensure algal concentrations did not change throughout the experiment due to growth or death of algae cells.

For measuring cell concentrations, animals were removed from the test jars, and survival, moults and numbers of neonates were noted down. The screw-topped jars were shaken vigorously, and settled cells were stirred up using a 3 mL plastic pipette, to guarantee even dispersal of cells. A 500 µL sample was withdrawn using a 1 mL micropipette, and added into an accuvette disposable sample container holding 19.5 mL of the electrolyte Isoton II (both Coulter Electronics Ltd). The accuvette was gently shaken to distribute cells evenly, making sure not to generate any air bubbles, which would also be counted by the Coulter Counter. It was then placed onto the sample platform of the Coulter Counter, making sure the orifice tube and foil were submerged in the sample. The count took approximately 25 seconds, stopping automatically after 500 µL of sample had passed the orifice tube and all cells contained in this volume were counted. Each sample was counted three times, and the average sum of these three counts was calculated. The number of cells mL^{-1} was determined by multiplying the average of three counts by 80.

The feeding rate was calculated using the equation 4.1 given in section 4.1.2.2.

4.3.5 Set-up of the bioassay

4.3.5.1 Determination of the functional response

The *Ceriodaphnia* feeding bioassay was adapted from the *D. magna* feeding bioassay described by McWilliam (2001). The most important factor for measuring the feeding rate is the concentration of algal cells. It needs to be high enough so that animals feed at their maximum feeding rate, guaranteeing a significant algal clearance rate over the

period of the bioassay. Algal food levels must not be depleted during the experiment to a concentration that could inhibit feeding, which could occur if initial concentrations of algae were too low. They must also not be so high that they could clog the filtering apparatus, leading to interruptions in feeding. Variability of feeding rates at different food concentrations was also assessed.

Seven different food concentrations were used to determine the functional response. For *C. dubia* and *C. cornuta*, concentrations were $1x10^5$, $5x10^5$, $1x10^6$, $2x10^6$, $3x10^6$, $5x10^6$, and $1x10^7$ cells mL^{-1}. For *S. vetulus*, cell concentrations were $2x10^5$, $5x10^5$, $1x10^6$, $2x10^6$, $5x10^6$, $1x10^7$, and $2x10^7$ cells mL^{-1}.

Experiments with *C. dubia* and *C. cornuta* were performed using 8 mL narrow rimmed glass bottles containing 7 mL of test solution, with 4 animals per jar. For *S. vetulus*, exposure bottles were 50 mL wide rimmed jars, containing 40 mL of test solution and 4 animals per jar. These relationships of animals to exposure volumes were found to allow maximum feeding without interference between animals. Five replicates were used per treatment. Each treatment (algal concentration) also included a blank, which was a jar containing the same test solution, but without animals.

Animals were allowed to feed for 24 h. This time period was both practical to carry out during the laboratory during working hours, and allowed a long enough period of feeding to see a significant reduction of algal cells.

For 7 different food concentrations, 140 neonates were acclimated to a food level of $1x10^6$ cells mL^{-1}, and fed at this concentration each day until the release of their first brood on day 4 or 5. Twenty animals were randomly assigned to each of the food levels used in the experiment and acclimated to this concentration for 2 h in 175 mL (*C. dubia* and *C. cornuta*) or 250 mL (*S. vetulus*) glass jars containing 150 mL of ASTM and the matching concentration of *C. vulgaris*.

After being left to feed for 24 h, daphnids were removed from the jars using a 3 mL plastic pipette and stored in 1.5 mL eppendorf tubes in the fridge until body length was determined. Algal concentrations were measured on the same day. Although McWilliam (2001) did not find significant differences between algal samples measured directly after the experiment or stored in the fridge for 24 h or frozen for up to one week, in this study, storing or freezing of samples led to more variable cell counts. Therefore all samples were processed immmediately after the termination of the experiment.

Algal counts were performed as described in 4.3.4. For $1x10^5$ cells mL^{-1}, 4 mL of sample were taken out and added to 16 mL of Isoton II, for $2x10^5$ and $5x10^5$ cells mL^{-1}, 2 mL of sample were added to 18 mL of Isoton II, for $1x10^6$ cells mL^{-1}, 1 mL was suspended in 19 mL isoton, for $2x10^6$ cells mL^{-1}, 0.5 mL was suspended in 19.5 mL isoton, and for $4x10^6$ cells mL^{-1}, 0.25 mL were added to 19.75 mL isoton. Feeding rates were calculated as in 4.3.4.

Body lengths of the 3 cladocerans were measured by placing animals that had been anaesthetised with CO_2 in ASTM mixed with carbonated water on a glass slide and measuring length using an Olympus dissecting microscope with a 1 cm graticule eye-piece, at a magnification of x 40. Body length in mm was measured from the tip of the head to the base of the tail spine.

Feeding rate data was fitted to an equation for the type II functional response using non-linear regression techniques (SigmaStat and SigmaPlot 2001). This equation is based on the Michaelis-Menten equation of enzyme kinetics, stating that substrate utilisation is hyperbolic with change in substrate concentration. The type II functional response was given as:

$$F = F_{max} * \frac{(C)}{K + (C)} \qquad (4.3)$$

where: F = feeding rate (cells ind^{-1} h^{-1})

F_{max} = maximum feeding rate (cells ind^{-1} h^{-1})

(C) = algae concentration (cells mL^{-1})

K = half saturation constant

This equation is adapted from Equation 4.1, allowing the calculation of the maximum feeding rate F_{max} and the half saturation constant K based on the feeding values of the individual species determined using Equation 4.1.

Standard operating procedure protocols (SOPs) were prepared for the contaminant-induced feeding inhibition bioassay for both *C. dubia* and *S. vetulus*, which are attached in Appendix 1.

4.3.5.2 Determination of feeding inhibition at sublethal exposure concentrations to the four chemicals

Feeding inhibition was measured directly, and tests lasted for 24 h. For each test concentration, 5 glass jars were filled with the appropriate solution (4 replicates and one blank to establish initial algal concentrations). *C. vulgaris* was added at the

concentration of $1x10^6$ cells mL^{-1}. *C. dubia* and *C. cornuta*: exposure vessels were 8 mL narrow rimmed glass jars containing 7 mL of test solution and 4 animals per jar. *S. vetulus*: exposure vessels were 50 mL wide rimmed glass jars containing 40 mL of test solution and 4 animals each. Controls were set up in the same way, containing hard or moderate hard ASTM and *C. vulgaris* at a concentration of $1x10^6$ cells mL^{-1} only. Animals were acclimated as in 5.3.5.1, and for each test concentration, 4 animals were randomly assigned to 4 of the jars, and allowed to feed for 24 h. Animals were then removed from the test jars, and samples were measured and feeding rates calculated as in 4.3.4.

4.3.5.2.1 Chemical concentrations

Stock solutions for all chemicals were prepared as described in Chapter 2 . Chemical analyses for the test substances were carried out as described in Chapter2. Nominal chemical concentrations used for establishing feeding responses were as follows:

Deltamethrin: 0.01; 0.017; 0.031; 0.053; 0.094; 0.164; 0.286; 0.5 $\mu g\ L^{-1}$.

Chlorpyrifos: 0.01; 0.017; 0.031; 0.053; 0.094; 0.164; 0.286; 0.5 $\mu g\ L^{-1}$.

Cadmium: 1.0; 1.53; 2.35; 3.61; 5.54; 8.5; 13.0; 20.0 $\mu g\ L^{-1}$.

Atrazine: 1.0; 1.63; 2.64; 4.3; 7.0; 11.35; 18.46; 30 $mg\ L^{-1}$.

4.3.6 Statistical analysis

Feeding inhibition was measured for *C. dubia* and *S. vetulus* exposed to a range of each of the four chemicals, and for *C. cornuta* only with deltamethrin, as feeding rates with this species were more varible than with the other two. After feeding rates had been calculated using Equation 4.1 (section 4.1.2.2), the relationship between feeding rate and toxicant concentration was fitted using a normal allosteric decay model (SigmaPlot and SigmaStat 2001). The equation used was:

$$F = F_{cont} * \frac{(k_t)^i}{(k_t)^i + (Tox)^i} \qquad (4.4)$$

where: F = feeding rate as a proportion of the control feeding rate

F_{cont} = the maximum feeding rate

k_t = 50% inhibition concentration (IC_{50})

Tox = measured concentration of the test substance

i = allosteric decay index

This model assumes a negative sigmoid relationship between the feeding rate and the concentration of the test compound. It was chosen because it closely matches the expected pattern, with an initial period of no response at low concentrations, followed by an accelerated negative response as concentrations increase (Allen *et al.*, 1995). Absolute feeding rate data from the 24 h exposure experiments were converted to proportions of control feeding rates by dividing by the mean of the control feeding rate, and converted to percentages (% of control feeding rates). Data was then fitted to the model using a nonlinear regression procedure (based on the Marquardt algorithm),

using SigmaPlot / SigmaStat 2001. Goodness-of-fit was estimated from the coefficient of determination (r^2). The fitted model was used to generate a feeding inhibition curve for each chemical compound, and to calculate a standard measure of feeding inhibition, k_i, which is defined as the concentration of the test substance that reduces feeding by 50% (IC_{50}). (This nonlinear method is more useful than the one-way ANOVA procedure often used to generate no-effect concentrations for nonlethal endpoints, because it is based on a realistic a priori model of the toxic response.)

4.4 RESULTS

4.4.1 Functional feeding response

Fitted values of the functional feeding response and average body lengths of the 3 species are given in Table 4.1.

All 3 species showed a type II functional response (Figures 4.2 – 4.4), which is characterised by a curvilinear increase in feeding rate with rising food concentrations. Feeding curves for *C. dubia* and *C. cornuta* showed a typical plateau phase, with feeding slowing down when the maximum feeding rate was reached. For *S. vetulus* however, this plateau was not reached even at very high food levels.

Table 4.1 Fitted values of the type II functional feeding response curves for the 3 species (see Figures 4.1 – 4.3). F_{max} = maximum feeding rate; K = half saturation constant (at half the maximum feeding rate); r^2 = the coefficient of determination, together with (significant) probability levels

Species	F_{max} (cells ind^{-1} h^{-1})	K (cells mL^{-1})	r^2	*P*	Average body length (mm)
C. dubia	1.55×10^5 (S.E. 0.1)	2.1×10^6 (S.E. 0.29)	0.94	< 0.001	0.82 ± 0.06
C. cornuta	6.32×10^4 (S.E. 0.79)	1.63×10^6 (S.E. 0.49)	0.84	< 0.002	0.43 ± 0.03
S. vetulus	1.05×10^6 (S.E. 0.25)	1.36×10^7 (S.E. 0.49)	0.85	< 0.01	1.25 ± 0.08

Feeding rate of *C. dubia* showed a good fit to the type II model, with the coefficient of determination, $r^2 = 0.94$, indicating that most of the variation in feeding rates could be explained by the model. F_{max}, the maximum feeding rate, was 1.55×10^5 cells ind^{-1} h^{-1}, and the half saturation constant (at half the maximum feeding rate), K, was 2.1×10^6 cells mL^{-1}. The average body length of *C. dubia* was 0.082 mm ± 0.15.

C. cornuta exhibited a slightly higher variation in feeding rates, with a value of $r^2 = 0.84$. The maximum feeding rate, F_{max}, was 6.32×10^4 cells ind^{-1} h^{-1}, and K was 1.63×10^6 cells mL^{-1}. Average body length was 0.43 mm ±0.03.

Feeding rates of *S. vetulus* were also more variable than in *C. dubia*, with $r^2 = 0.85$. F_{max} was 1.05×10^6 cells ind^{-1} h^{-1}, and K was 1.36×10^7 cells mL^{-1}, with an average body length of 0.43 mm ±0.03.

Variation in feeding rates increased in all 3 species with increasing food concentrations. This is obvious by the spread of the data around the fitted line at the higher food concentrations (and indicates an increasing deviation from the type II response).

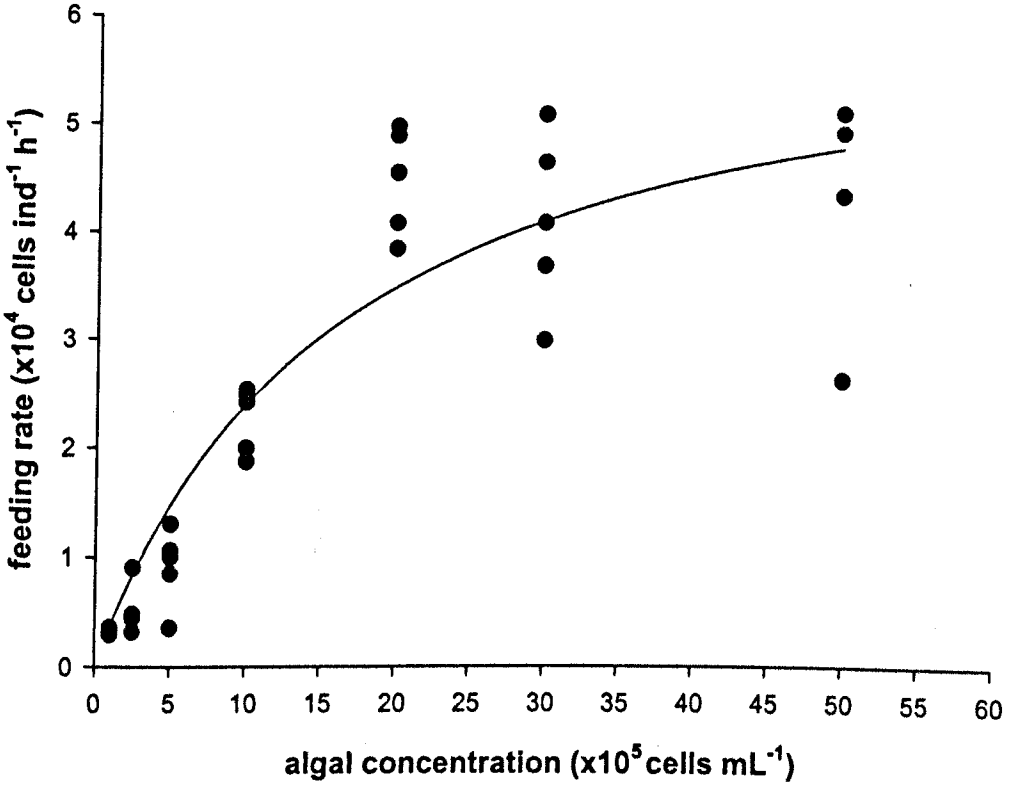


Figure 4.2 Responses of the feeding rate of young adults of *C. cornuta* (4 d old) to food concentration, with a fitted model as calculated from the type 2 equation. Group size = 4.

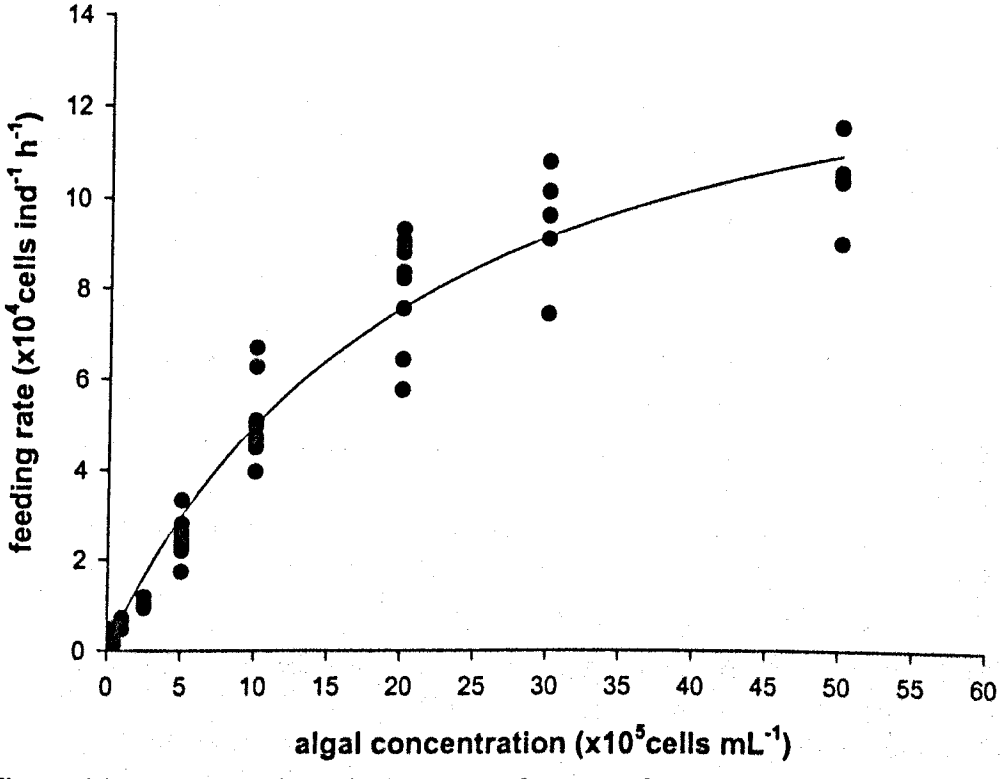


Figure 4.3 Responses of the feeding rate of young adults of *C. dubia* (5 d old) to food concentration, with a fitted model as calculated from the type 2 equation. Group size = 4.

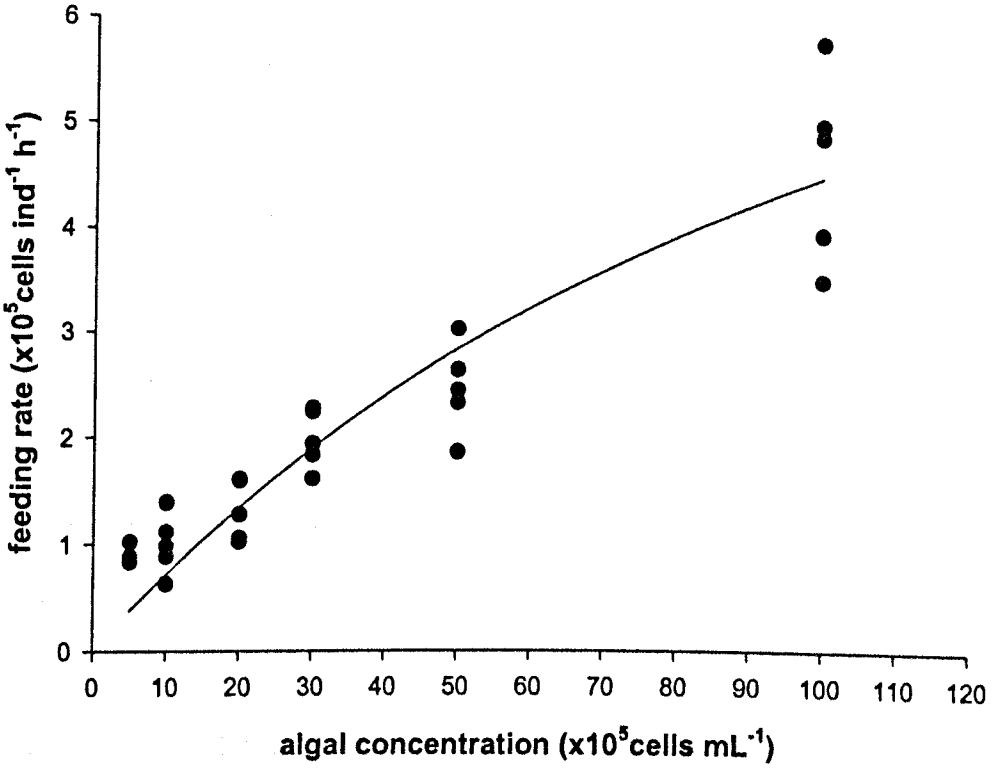


Figure 4.4 Responses of the feeding rate of young adults of *S. vetulus* (7 d old) to food concentration, with a fitted model as calculated from the type 2 equation. Group size = 4.

4.4.2 Feeding inhibition

The effects of each of the test chemicals on the feeding rates of the 3 species, together with the fitted allosteric decay model, are presented in Figures 4.5 – 4.11. The parameters on which each decay model is based are given in Table 4.2. In all cases feeding was reduced with increasing toxicant concentrations, except for the organophosphate chlorpyrifos. Here no inhibition in feeding was observed; animals fed normally until lethal concentrations were reached.

The data conformed well to the allosteric decay model, and in all cases the coefficient of determination was significant at $p < 0.05$.

Feeding data of *C. cornuta* fitted least well to the model and showed the highest variation, with a coefficient of determination value of $r^2 = 0.46$ (see Figure 4.5). The concentration at which deltamethrin inhibited feeding by 50%, k_t, or the deltamethrin 24h-feeding IC_{50}, was 0.23 µg L^{-1}, and the allosteric decay index, i, which describes the shape of the slope (curve), was 2.92. As the feeding inhibition response under exposure to toxic chemicals was so variable in this species, no other such experiments were carried out with *C. cornuta*, and instead efforts were concentrated on *C. dubia* and *S. vetulus*, who showed a more consistent feeding response.

Table 4.2 Fitted values of the allosteric decay equation for each of the compounds tested and for the 3 species

Species + Compound	k_t (= 24h IC_{50}) ($\mu g\ L^{-1}$)	*i*	r^2	*P*
***C. cornuta*:**				
Deltamethrin	0.23 (S.E. 0.05)	2.92	0.46	<0.05
S. vetulus:				
Deltamethrin	0.64 (S.E. 0.08)	1.06	0.72	<0.0001
Atrazine	13420 (S.E.1390)	2.11	0.75	<0.0001
Atrazine 2	8150 (S.E. 1178)	1.62	0.77	<0.0002
***C. dubia*:**				
Deltamethrin	0.19 (S.E. 0.01)	2.09	0.96	<0.0001
Atrazine	13230 (S.E. 510)	4.09	0.94	<0.0001
Chlorpyrifos	= 24h LC_{50} (0.23)	-	-	-
Cadmium	5.31 (S.E. 0.78)	0.63	0.77	<0.0001

k_t, the concentration at which feeding rate is inhibited by 50%- the IC_{50}; *i*, the allosteric decay index; and the coefficient of determination, r^2, together with significant probability levels

Feeding data of *S. vetulus* conformed well to the allosteric decay model. Deltamethrin inhibited feeding by 50% in this species at a concentration of 0.64 μg L^{-1}, the coefficient of determination was $r^2 = 0.72$, and the allosteric decay index i was 1.06 (Figure 4.6). For atrazine there were two, slightly different, sets of parameters from two different feeding inhibition studies, only one of them is shown in Figure 4.6. Here the atrazine 24h IC_{50} was 13.42 mg L^{-1}, with $r^2 = 0.75$ and $i = 2.11$. In the second atrazine feeding experiment, the IC_{50} was found to be notably lower at 8.15 mg L^{-1}, with $r^2 = 0.77$ and $i = 1.62$.

C. dubia feeding data showed the best fit to the model and the least amount of variation between replicates. Sublethal sensitivity to deltamethrin was slightly higher than in *S. vetulus*, comparable to that of *C. cornuta*, with a feeding IC_{50} of 0.19 μg L^{-1}, $r^2 = 0.96$, and $i = 2.09$ (Figure 4.10). Feeding sensitivity to atrazine was comparable to or lower than that of *S. vetulus*, the IC_{50} was 13.23 mg L^{-1}, with $r^2 = 0.94$ and $i = 4.09$ (Figure 4.8). Chlorpyrifos was the only compound (Figure 4.9) that did not inhibit feeding at sublethal concentrations. Feeding rates were consistent if somewhat variable around 100% (control) until lethal concentrations were reached (around 0.3-0.5 μg L^{-1}), when all animals were dead after 24 h.

Cadmium (Figure 4.11) at sublethal concentrations led to a pronounced decrease in feeding rates, but there was more variation in the data, (which did not fit the model as well as the data for deltamethrin and atrazine). The feeding IC_{50} for cadmium in *C. dubia* was determined at 5.31 μg L^{-1}, with a coefficient of determination of $r^2 = 0.77$, and an allosteric decay index $i = 0.63$.

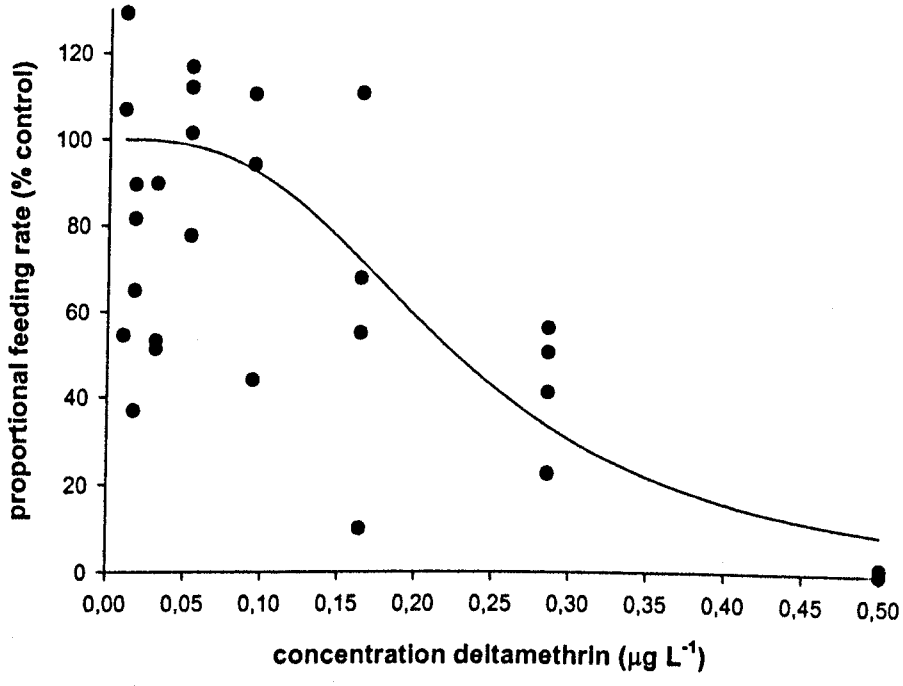


Figure 4.5 Feeding rate of *C. cornuta* as a function of concentration of deltamethrin. Absolute feeding rates were converted to proportions by dividing by the mean of the control rate, and fitted to an allosteric decay model, using the parameters given in Table 4.2.

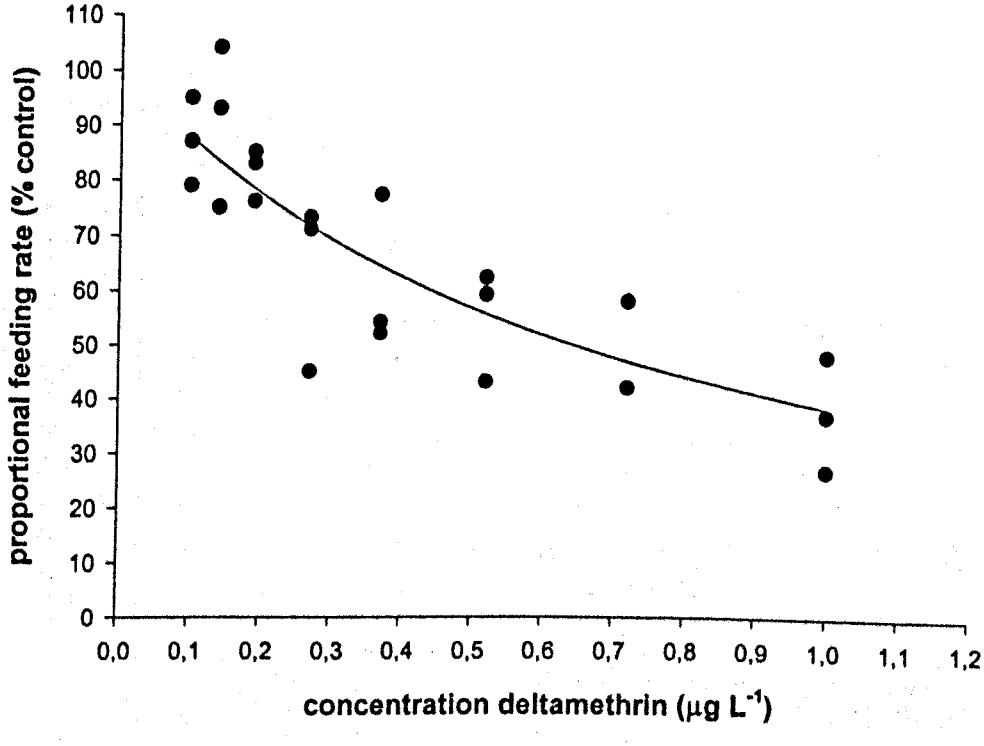


Figure 4.6 Feeding rate of *S. vetulus* as a function of concentration of deltamethrin. Absolute feeding rates were converted to proportions by dividing by the mean of the control rate, and fitted to an allosteric decay model, using the parameters given in Table 4.2.

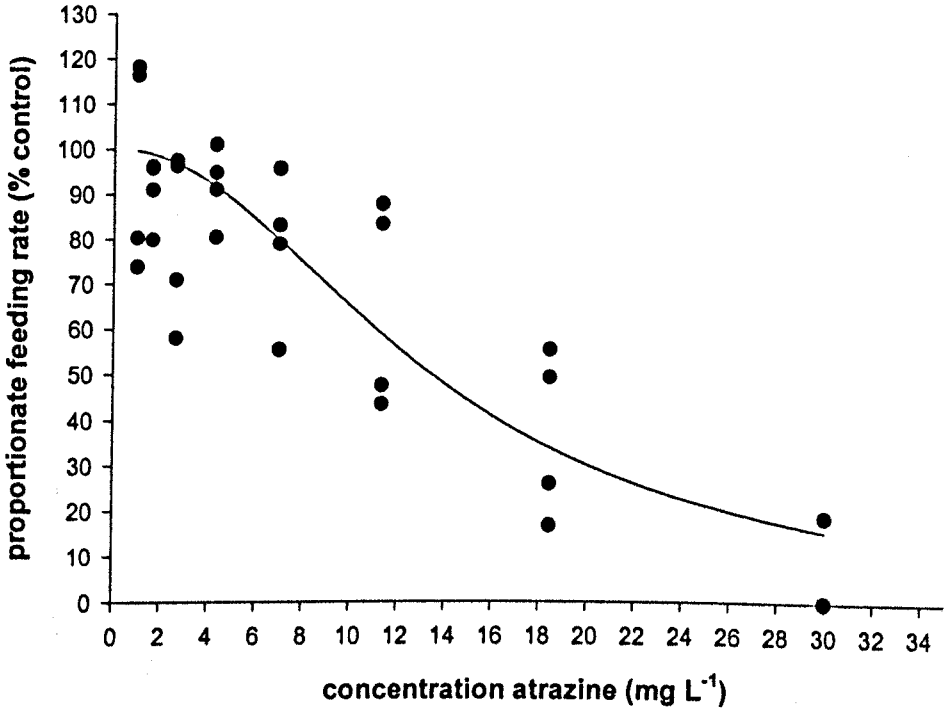


Figure 4.7 Feeding rate of *S. vetulus* as a function of concentration of atrazine. Absolute feeding rates were converted to proportions by dividing by the mean of the control rate, and fitted to an allosteric decay model, using the parameters given in Table 4.2.

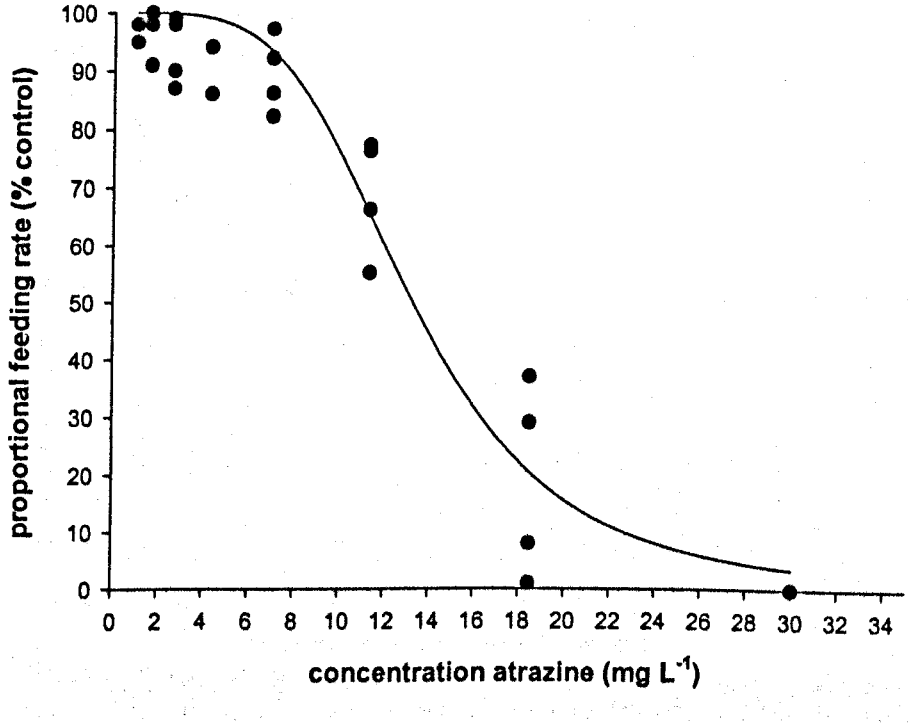


Figure 4.8 Feeding rate of *C. dubia* as a function of concentration of atrazine. Absolute feeding rates were converted to proportions by dividing by the mean of the control rate, and fitted to an allosteric decay model, using the parameters given in Table 4.2.

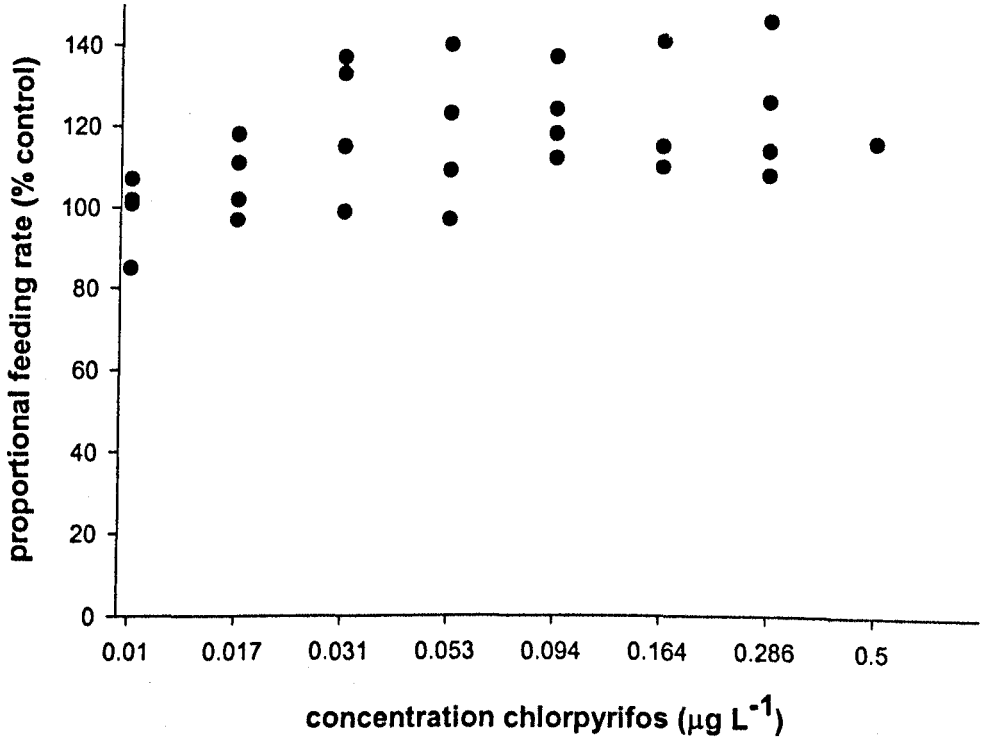


Figure 4.9 Feeding rate of *C. dubia* as a function of concentration of chlorpyrifos.

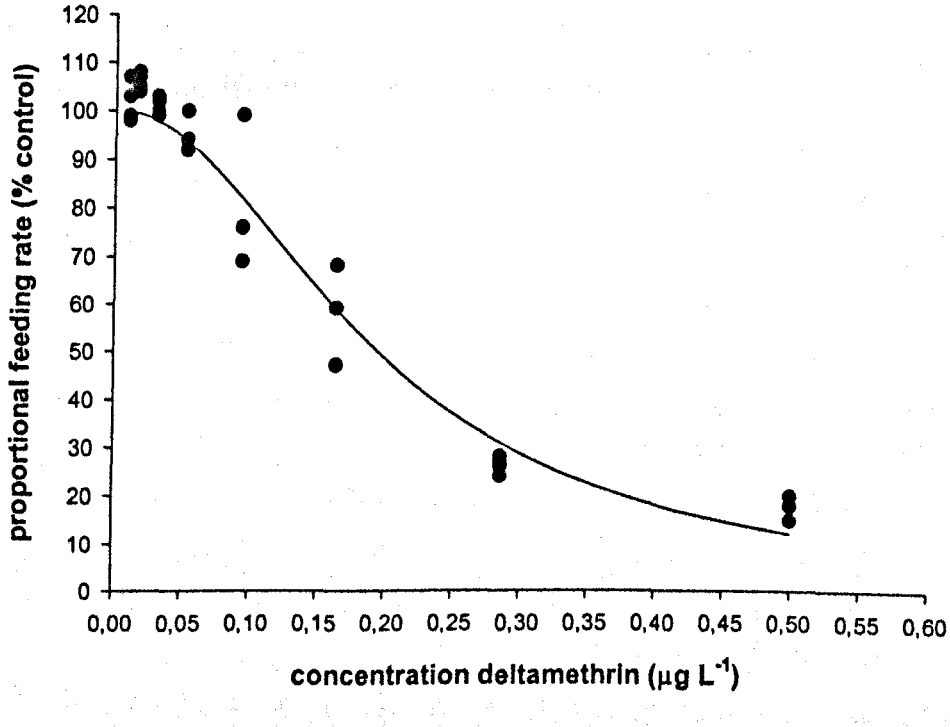


Figure 4.10 Feeding rate of *C. dubia* as a function of concentration of deltamethrin. Absolute feeding rates were converted to proportions by dividing by the mean of the control rate, and fitted to an allosteric decay model, using the parameters given in Table 4.2.

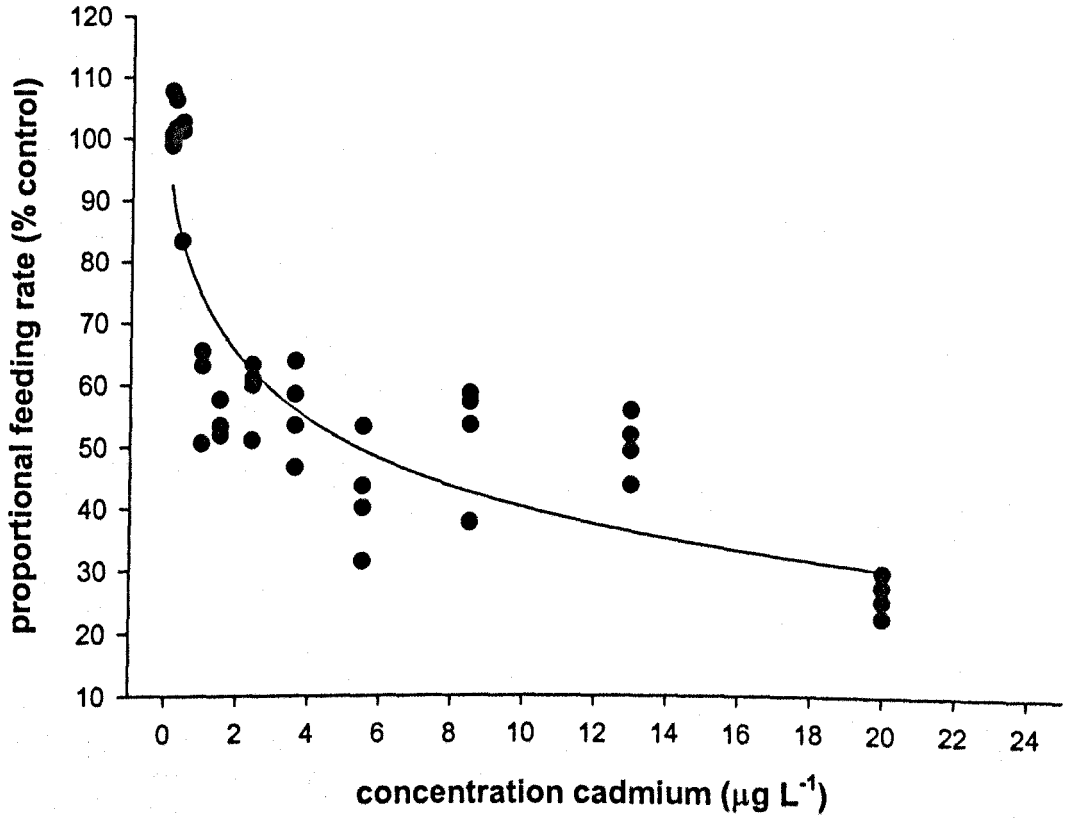


Figure 4.11 Feeding rate of *C. dubia* as a function of concentration of cadmium. Absolute feeding rates were converted to proportions by dividing by the mean of the control rate, and fitted to an allosteric decay model, using the parameters given in Table 4.2.

Table 4.3 24h LC_{50} values, in the presence and absence of food, and direct exposure feeding IC_{50} values (both with 95% confidence limits), with coefficient of determination (r^2) (indicating fitted model accuracy for feeding data) (See also lethality data in Tables 3.1-3.3)

Species + compound	24h LC_{50} ($\mu g\ L^{-1}$) + food	24h LC_{50} ($\mu g\ L^{-1}$) - food	24h Feeding IC_{50} ($\mu g\ L^{-1}$)	r^2 (n = 4)
S. vetulus:				
Deltamethrin	0.5 (0.01-5.5)	< 0.1	0.64 (0.56-0.72)	0.72
Atrazine	24270 (23770-24930)	27120 (27070-27560)	8150 (6972-9328)	0.77
Chlorpyrifos	0.16 (0.14-0.18)	0.16 (0.13-0.18)	no effect below lethal threshold	-
C. dubia:				
Deltamethrin	0.49 (0.48-0.5)	0.23 (0.16-0.35)	0.19 (0.18-0.2)	0.96
Atrazine	>30000	>30000	13230 (12720-13740)	0.94
Chlorpyrifos	0.23 (0.16-0.3)	0.13 (0.09-0.16)	no effect below lethal threshold	-
Cadmium	68.8 (9.6-217.9)	-	5.31 (4.53-6.09)	0.77

Table 4.3 shows the feeding IC_{50} values compared to 24h LC_{50} values. For most compounds, feeding IC_{50}s were significantly lower than the LC_{50}s. Chlorpyrifos was the only substance tested that did not have an effect on feeding rates at a level below the lethality threshold.

4.5 DISCUSSION

The feeding inhibition bioassay was found to work well using *C. dubia*, but less well using *S. vetulus*, and less well with *C. cornuta.* To guarantee a good performance of the bioassay, reliable and repeatable results must be produced. This was achieved with *C. dubia* and also with *S. vetulus*, by manipulating experimental parameters and in this way minimising variability in baseline feeding rates, and detecting significant differences in feeding rates. Feeding experiments with *C. cornuta* reveal a high variation in baseline feeding rates, because of overall low feeding rates due to the animals' small size and their inconsistent laboratory performance in terms of health and reproduction of culture populations.

Twenty-four hours proved to be a useful time period for running the bioassay, as it was practical to perform during normal laboratory working hours and produced a suitable reduction of algal cells to permit the detection of differences in feeding rates. Feeding bioassays have been carried out successfully over shorter time periods, such as the *D. magna* feeding bioassay (McWilliam, 2001), which involves feeding periods of test animals of 4 h. However, *D. magna* are a much larger species and therefore feeding rates are higher, allowing detection of significant reductions of algal cells after shorter feeding periods. Feeding bioassays employing smaller species such as *Ceriodaphnia* have been developed for short time periods as low as 1 h (Bitton *et al.*, 1995; Bitton *et al.*, 1996; Jung & Bitton, 1997; Lee *et al.*, 1997). These tests though

function by measuring direct feeding rates, counting the amount of food (stained yeast or microalgal cells) present in the gut of the animals. These short-term feeding bioassays have also been shown to be rather insensitive, producing feeding IC_{50} values which are often higher than 48h LC_{50}s. It has also been found that animals require a certain settling period after being handled, before feeding rates normalise. Peters & Downing (1984) suggested a time period of 1- 2 h to be necessary after transfer of animals for settling and stabilising of feeding rates in daphnids (Peters, 1984).

For measuring feeding rates of *Ceriodaphnia* and *Simocephalus* indirectly by determining the reduction of algal cells, a time period of 24 h produced good results. The numbers of replicates used in this study were chosen as a compromise between achieving the highest power possible and those numbers that were practically feasible. 4 to 5 replicates per treatment proved to be the maximum possible, as more replicates would have required excessively large numbers of test animals. Also the analysis of samples within 24 h after the experiment meant that there was a limit to the numbers of samples from one experiment that could be practically processed. For most experiments the numbers of replicates proved suitable, as subsequent statistical analysis of data consistently detected contaminant-induced effects on feeding rates.

4.5.1 Functional feeding response

All 3 daphnid species exhibited a type II functional response (Figures 4.2- 4.4), characterised by a curvilinear increase in ingestion rate with increasing food concentration. This type of response has been described for daphnids by other authors such as Porter *et al.* (1982).

The concentration of algal food cells used for the bioassay is of great importance, as it has to be high enough to ensure that the animals are feeding at a normal rate during

experiments. A depletion of food levels during the experiment could lead to a reduction in feeding rates as animals move down the functional response curve. Ideally a food concentration should be chosen from near the top of the functional response curve, where the curve gradient nears its plateau and begins to level out, since this is where feeding rates are at their maximum. However, feeding rates at the higher food concentrations (> 1 x 10^6/ 2 x 10^6 cells mL^{-1}) were increasingly variable and deviated more from the type II response. Such high algal densities can lead to increased clogging of the filtering limbs and subsequent post-abdominal claw rejections (Porter *et al.*, 1982), disrupting normal filtering behaviour and decreasing feeding rates, thus increasing variability.

A food concentration of 1 x 10^6 was hence chosen to be used for all experiments. No excessive food depletion occurred during a 24 h exposure period at this concentration, and feeding rates were consistent.

F_{max}, the maximum feeding rate (24 h), was related to size of animals. *C. cornuta*, the smallest species at an average length of 0.43 mm, showed the lowest maximal feeding rate of 6.32 x 10^4 cells ind^{-1} h^{-1}, compared with the maximal feeding rates of *C. dubia* (0.82 mm) of 1.55 x 10^5 cells ind^{-1} h^{-1}, and *S. vetulus* (1.25 mm) of 1.05 x 10^6 cells ind^{-1} h^{-1}. This corresponds with values reported by McWilliam (2001), who determined maximum feeding rates of *D. magna* (1.85 mm) of 1.48 x 10^6 cells ind^{-1} h^{-1}. However, K, the half saturation constant, describing the algal concentration where feeding rates were 50% of their maximum, was higher for the 3 tropical/ subtropical daphnids than that reported for *D. magna*. Feeding experiments with *D. magna* were carried out at a temperature of 20°C, and the K value was calculated as 1.12 x 10^6 cells mL^{-1}. The test temperature in this study was 25°C. K values were 1.63 x 10^6

cells mL^{-1} for *C. cornuta*, 2.1 x 10^6 cells mL^{-1} for *C. dubia*, and 1.36 x 10^7 cells mL^{-1} for *S. vetulus*, where no plateau phase was reached in the functional response curve. The increase in water temperature leads to higher metabolic rates, and a continuing increase in feeding rates at higher food concentrations (see Figures 4.2- 4.4). Also numbers of microalgae and bacteria can sometimes reach extremely high concentrations in warm, shallow tropical freshwater streams and ponds, possibly favouring an adaptation of tropical freshwater zooplankton species with higher filtration rates and an ability to avoid clogging of the filtering limbs.

4.5.2 Feeding inhibition

Feeding inhibition as an endpoint proved generally to be more sensitive than survival. For most test substances, the feeding IC_{50} value was significantly lower than the LC_{50} value, indicating that feeding inhibition was a more sensitive endpoint for measuring toxicity than lethality.

The IC_{50} of *S. vetulus* exposed to deltamethrin was slightly higher than the corresponding LC_{50}, however the calculation of the LC_{50} for this compound was not very precise due to variability in lethality data. Large confidence limits of the LC_{50} value indicate that the lethal concentration could be between 0.01 and 5.5 $\mu g\ L^{-1}$. Chlorpyrifos was the only compound tested which did not inhibit feeding at sublethal concentrations. Animals fed normally until the lethal threshold concentration of 0.2 – 0.3 $\mu g\ L^{-1}$. Some studies report an effect of different acetylcholinesterase-inhibitors (AChE) on feeding. The organophosphate pesticide pirimiphos-methyl reduced feeding rates of the amphipod *Gammarus pulex* at 0.6 $\mu g\ L^{-1}$ after a 6 d exposure period (McLoughlin *et al.*, 2000). Other AChE inhibitors have also been found to inhibit feeding in *C. dubia* (Bitton *et al.*, 1996) and *Daphnia* spp. (Lampert *et al.*,

1989; Fernandez-Casalderrey *et al.*, 1994). However, McWilliam (2001) found that pirimiphos-methyl did not reduce feeding rates in *D. magna* at sublethal concentrations, and no sublethal effects of organophosphate pesticides on feeding in *D. magna* were observed by C. Barata (pers. comm.). It is therefore possible that this group of chemicals does not always affect feeding rates in daphnids due to its special mode of action.

Feeding inhibition responses of *C. dubia* and *S. vetulus* fitted well to the allosteric decay model. The slope of the curve varied with the different compounds, with a higher allosteric decay index *i* indicating a steeper slope. Steepness of slope increased for both species from cadmium to deltamethrin to atrazine.

The cadmium feeding IC_{50} for *C. dubia* of 5.31 $\mu g\ L^{-1}$ corresponds well with values reported from other studies. Wong *et al.* (1989) observed feeding inhibition effects in the cladoceran *Moina macropopa* at cadmium concentrations of 5 $\mu g\ L^{-1}$ (Wong, 1989). Feeding IC_{50} values for *C. dubia* at concentrations of 54 $\mu g\ L^{-1}$ were reported by Bitton *et al.* 1996), however this value was obtained using the 1-h Ceriofast™ bioassay, which is relatively unsensitive.

Allen *et al* (1995) determined a feeding IC_{50} of cadmium for *D. magna* of 2.63 $\mu g\ L^{-1}$, and McWilliam (2001) calculated a cadmium IC_{50} for the same species at 1.3 $\mu g\ L^{-1}$. This could indicate a slightly higher sensitivity to cadmium of *D. magna* compared to the *C. dubia* feeding IC_{50} of 5.31 $\mu g\ L^{-1}$ found in this study. However both the studies with *D. magna* were carried out at the much lower algal concentration of 5×10^5 cells mL^{-1}, compared to an algal concentration of 1×10^6 cells mL^{-1} which was used in this study. As higher food concentrations have been found to reduce sensitivity of test animals to toxic chemicals by increasing fitness or potentially adsorbing toxic

molecules, the elevated food concentration could explain the lower cadmium sensitivity (Taylor *et al.*, 1998).

Regarding effects on cladoceran feeding rates caused by organic chemicals, very little data is reported in the literature. Day & Kaushik (1987) reported feeding inhibition in various *Daphnia* spp. and in *C. lacustris* exposed to the pyrethroid fenvalerate at concentrations of 0.05 $\mu g\ L^{-1}$ and 0.01 $\mu g\ L^{-1}$, respectively (Day & Kaushik, 1987). The herbicide atrazine has been found to have an effect on feeding in *D. pulicaria* at a concentration of 2 $mg\ L^{-1}$ (Lampert *et al.*, 1989). Allen *et al.* (1995) determined effects of 2 organic chemicals on feeding inhibition in *D. magna*, reporting 24h EC_{50} values of 2,330 $\mu g\ L^{-1}$ for 3,4 DCA, and of 387.4 $\mu g\ L^{-1}$ for di-tallow dimethyl ammonium chloride (DTDMAC).

In this study, IC_{50}s for atrazine were determined at 13.23 $mg\ L^{-1}$ (*C. dubia*), and 8.15 $mg\ L^{-1}$ (*S. vetulus*). Again, temperature regimes, exposure times and food concentrations varied greatly between the two studies, making comparisons very difficult.

In concluding it can be said that feeding inhibition was found to be a sensitive, reliable and repeatable endpoint for measuring sublethal toxicity effects. Since feeding rates in *C. cornuta* were found to be more variable, efforts were concentrated on the other 2 species. Chlorpyrifos was also excluded from further studies as it was not found to cause any feeding inhibition at sublethal levels.

The question of how different sublethal concentrations of the remaining 3 chemicals, cadmium, deltamethrin and atrazine, each with a different toxicological mode of action, will affect feeding when present in different combinations, will be investigated in Chapter 5.

CHAPTER 5

EFFECTS OF BINARY AND TERTIARY MIXTURES OF TWO PESTICIDES AND A METAL ON FEEDING OF TWO CLADOCERAN SPECIES

5.1 INTRODUCTION

Within the last two decades scientific and regulatory concern over the effects and risks of chemical mixtures regarding both human and environmental health has increased (U.S.Environmental Protection Agency, 1986; De March, 1987b; De March, 1987a; Carpy *et al.*, 2000). Tens of thousands of chemicals are being produced and released, entering the atmosphere, terrestrial and aquatic systems and all organisms within in various ways, and it is now generally recognised that effects of chemicals can no longer be considered as those of single compounds, but as more or less complex mixtures.

A growing number of studies are being carried out examining the effects of such mixtures (Vouk *el al.*, 1987). Recent attempts to study mixture toxicity have mainly focused on industrial chemicals (Broderius *et al.*, 1995; Newsted, 1995; Niederlehner *et al.*, 1998; Altenburger *et al.*, 2000) and metals (Kraak *et al.*, 1994; Jak *et al.*, 1996). Generally it is accepted that mixtures of chemicals with the same mode of action ("similar" chemicals) can be described by the simple concentration addition model (CA) (Anderson & Weber, 1975; Berenbaum, 1985; McCarty *et al.*, 1992; Broderius *et al.*, 1995; Altenburger *et al.*, 2000). In this type of joint action, the individual compounds act as dilutions of each other and the overall effective concentration (C_{eff})

can be calculated by adding up all the effective concentrations of the individual compounds. Those individual effective concentrations are expressed as fractions of the effect concentrations, or Toxic Units (TUs):

$$C_{eff} = \sum_{i=1}^{n} (c_i/ECx_i) \qquad (4.1)$$

Where: C_{eff} = overall effective concentration, n = number of mixture components, c_i = actual respective concentration of compound i in the mixture, ECx_i = effective concentration of compound i, i.e. the concentration at which the studied effect, e.g. 50% mortality among the test organisms, occurs if the organisms are only exposed to compound i (singly). (as already seen in Equation 1.1)

If, at a total concentration of the mixture provoking x% effect, the sum of toxic units equals one, concentration addition applies.

The concept of concentration addition has been found to be useful for mixtures of chemicals that act through narcosis (Könemann, 1981; Hermens *et al.*, 1984; Broderius & Kahl, 1985; Deneer *et al.*, 1988; Altenburger *et al.*, 2000). For mixtures of chemicals with more specific modes of action, few experimental studies have been carried out, with many studies reporting deviations from the model. If all the compounds in a mixture exert their toxicity through the same specific mode of action, the potency of the mixture is expected to correspond to CA. If the compounds however have different mechanisms of action, CA may no longer apply, as the components of the mixture can no longer be considered to act as dilutions of each other.

For such mixtures of dissimilar chemicals, the response addition model (also referred to as independent action) has been developed, predicting toxicity at less than simple

concentration-addition levels (Broderius, 1990; Dawson & Wilke, 1991; Broderius *et al.*, 1995; Backhaus *et al.*, 2000). This concept is based on the assumption that the compounds of a given mixture act on different physiological systems within the exposed organisms (Bliss, 1939). The mathematical formulation is as follows:

$$E(c_{mix}) = E(c_1 + \ldots + c_n) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \qquad (4.2)$$

Where: $E(c_{mix})$ = predicted effect (scaled from 0-1) of an n-compound mixture, c_i = concentration of the ith compound, and $E(c_i)$ = effect of that concentration if the compound is applied singly (as already seen in 1.2).

There is considerable disagreement about how to deal with mixtures composed of chemicals with dissimilar toxicological modes of action. Many researchers have pointed out that as organisms are structured entities with highly interconnected physiological processes, independent action (response addition) should be an unlikely type of joint action of complex effect levels, such as death, inhibition of growth or reproduction (Plackett & Hewlett, 1967; Broderius *et al.*, 1995). On the other hand, independent action has in some cases been found to be more suitable than concentration addition when predicting the toxicity of mixtures of dissimilar chemicals, especially in the field of pharmacology (Pöch, 1993). Such pharmacological studies, however, have usually investigated mixture effects of large numbers of industrial chemicals, using simple bacterial bioassays or in-vitro studies with molecular and biochemical endpoints (Backhaus *et al.*, 2000). Although the model of response addition has been shown to describe mixture toxicity of some complex mixtures (Hermens & Leeuwangh, 1982; Parrott & Sprague, 1993; Backhaus

et al., 2000), it is not universally accepted, and many exceptions have been reported. One of the main difficulties of this model is its implication that multiple toxicity effects cannot be expected when each of the mixture's components is present below its respective response threshold (Broderius, 1991). In contrast, chemical concentrations below their individual NOEC (no-observed-effect-concentration), at concentrations statistically estimated to elicit non-significant effects of only 1%, have been found to contribute to the overall toxicity of a mixture in predictions based on the concept of concentration-addition (Faust *et al.*, 2001). Generally it has been concluded that neither model unambiguously provides mechanistic explanations for the joint action of chemical agents in complex systems, such as whole cells, single organisms, or populations of organisms (Greco *et al.*, 1992).

Despite the above, concentration-addition is still considered to be a useful reference point for the description or prediction of joint actions of compounds, even if CA is not expected (Deneer, 2000). Many studies have found that even if compounds clearly differ in their modes of action, their joint action may be almost identical to that predicted by CA. It also has to be taken into account that compounds can have more than one mode of action. Several studies investigating the acute toxicity to fish of mixtures of chemicals with presumably different modes of action found good agreement with the joint toxicity predicted by CA (Eaton, 1973; Hermens & Leeuwangh, 1982; Hermens *et al.*, 1984). The toxicity of a mixture of pesticides with different modes of action to algae was also found to fit the CA predictions reasonably well (Altenburger *et al.*, 1996). However, not all mixture toxicity data fit these models (Broderius & Smith, 1979; Deneer *et al.*, 1988). Research from over three decades ago indicates that the simple concentration-addition and response-addition models are not always good predictors of the toxicity of mixtures of herbicides and insecticides

(Lichtenstein *et al.*, 1973; Macek, 1975). A considerable number of studies report significant deviations in the form of more-than-additive or less-than-additive toxicity from combinations of differently acting chemicals, especially metals and pesticides, which exert toxicity through specific modes of action. Tables 5.1 – 5.3 show an overview of studies on mixture toxicity of binary and tertiary mixtures of reactive chemicals such as metals and pesticides. The studies in Table 5.1 were found to correspond to CA although they include dissimilar chemicals. Table 5.2 and 5.3 present deviations from CA, with mixture effects being either less-than-additive (Table 5.2) or more-than-additive (Table 5.3).

Table 5.1. Binary mixtures of chemicals with dissimilar, specific modes of action, specifically pesticides and metals, conforming with concentration-addition (CA)

Compounds in mixture	Species tested	Endpoint	Reference
Selenium + Molybdenum/ Arsenic	*C. dubia*	Lethality (8d-LC_{50})+ Reproduction (3 broods)	(Naddy *et al.*, 1995)
Methyl-mercury + chlorpyrifos (OP)	*Hyalella azteca* (amphipod)	Lethality (96hLC_{50}) + Hg-Accumulation	(Steevens & Benson, 2001)
Methyl-parathion (OP) + Methoxychlor (OC)	*Chironomus tentans* (chironomid)	Lethality (96h LC_{50})	(Pape-Lindstrom & Lydy, 1997)
Methyl-parathion (OP) + carbofuran (carbamate)	*C. dubia*	Lethality (24, 48, 96h LC_{50})	(Norberg-King *et al.*, 1991)
Diazinon + chlorpyrifos (OPs)	*C. dubia*	Lethality (24, 48, 96h LC_{50})	(Bailey *et al.*, 1997)
Atrazine + carbofuran	*C. tentans*	Lethality + 10-d sediment tests	(Douglas *et al.*, 1993)
Atrazine + esfenvalerate (pyrethroid)	Zooplankton: *Bosmina*, copepods, *Lepomis macrochirus* (bluegill)	Total numbers of zooplankton + fish	(Fairchild *et al.*, 1994)
Atrazine + permethrin	*Anabaena inaequalis* + 2 fungi	Growth + photosynthesis activity	(Stratton, 1983)
Cu, Cd, + 3 surfactants/ azinphos +parathion (OPs)	*Gammarus italicus* (amphipod)	Lethality (24h LC_{50})	(Pantani *et al.*, 1990)
Garlon 4 + triclopyr (herbicides)	*Daphnia* + trout	Lethality (96h LC_{50})	(Servizi *et al.*, 1987)
Atrazine + bifenthrin (pyr.)	*Bosmina* spp. +	Lethality (96h LC_{50})	(Hoagland & Drenner, 1993)

Table 5.2 Binary and tertiary mixtures of chemicals with specific modes of action, specifically metals and pesticides, exhibiting less-than-additive toxicity

Compounds in mixture	Species tested	Endpoint	Reference
Molybdenum + arsenic	*C. dubia*	Lethality (96h LC_{50}) + Reproduction	(Naddy *et al.*, 1995)
Non-ionic surfactant (NP) + copper/ mercury	*Onchorhynchus mykiss* (rainbow trout))	Lethality (10d LC_{50})	(Calamari & Marchetti, 1973)
Methyl-mercury + dieldrin/ chlorpyrifos	*H. azteca*	Lethality (96h LC_{50})	(Steevens & Benson, 2001)
Diazinon (OP)+ ammonia	*C. dubia*	Lethality (48h LC_{50})	(Bailey *et al.*, 2001)
Profenofos (OP) + endosulfan (OC)	*C. dubia*	Lethality (48h LC_{50})	(Woods *et al.*, 2002)
Decylamine + atrazine	*Selenastrum capricornutum* (green microalgae)	Growth rate (EC_{50})	(Christensen *et al.*, 2001)
Atrazine + mevinphos (OP) / methoxychlor (OC)	*C. tentans*	Lethality (96h LC_{50})	(Pape-Lindstrom & Lydy, 1997)
Atrazine + malathion	*C. tentans*	Lethality (96h LC_{50})	(Belden & Lydy, 2000)
Fungicide + herbicide	*O. mykiss*	Lethality (96h LC_{50})	(Matthiessen *et al.*,1988)
Acephate + fenvalerat (OP + pyr.)	*Fundulus heteroclitus* (mummichog)	Lethality (96h LC_{50})	(Fulton & Scott, 1991)

Table 5.3 Binary and tertiary mixtures of similar and dissimilar chemicals with specific mode of action, specifically metals and pesticides, exhibiting more-than-additive toxicity (synergy)

Compounds in mixture	Species tested	Endpoint	Reference
Copper + cadmium	*Dreissena polymorpha* (zebra mussel)	Filtration rate	(Kraak *et al.*, 1994)
Cadmium + zinc	*Penaeus setiferus* (white shrimp)	Lethality (96h LC_{50})	(Vanegas *et al.*, 1997)
Binary mixtures: Copper/ cadmium + Zinc	Luminescent microbial biosensors	Inhibition of luminescence	(Preston *et al.*, 2000b)
Binary mixtures: Arsenic/ cadmium/ copper + dichlorvos/ malathion/ carbofuran	*Tigriopus brevicornis* (copepod)	Lethality (96h LC_{50}) + AChE-inhibition	(Forget *et al.*, 1999)
Binary mixtures: carbaryl, carbofuran, dichlorvos, fenitro-thion, phosalene (carbam.+ OPs)	*Callionymus lyra* (dragonet)	AChE-inhibition	(Bocquené *et al.*, 1995)
Binary mixtures: Copper, mercury + ABS/ LAS (anionic surfactants)	*O. mykiss*	Lethality (96h LC_{50})	(Calamari & Marchetti, 1973)
LAS + parathion/ other OPs	*P. promelas*	Lethality (96h LC_{50})	(Solon & Nair III, 1970)
Bin. + tert. Mixtures: Chlorpyrifos, pro-fenofos + endosulfan (OPs + OC)	*C. dubia*	Lethality (24h LC_{50})	(Woods *et al.*, 2002)
Atrazine + alachlor (herbicides)	*Rana pipiens* + *Bufo americanus* larvae (amphibians)	Lethality (96h LC_{50})	(Howe *et al.*, 1998)
Atrazine + parathion (OP)	Mosquito larvae	Lethality	(Lichtenstein *et al.*, 1973)
2,4-D (herbicide)+ parathion	Mice	AChE-inhibition	(Kuntz *et al.*, 1990)

Cont. Table 5.3

Compounds in mixture	Species tested	Endpoint	Reference
Prochloraz (fungicide) + λ-cyhalothrin (pyr.)	*Apis mellifera* (honeybee)	Detoxification products *in vitro* + *in vivo*	(Pilling *et al.*, 1995)
Piperonyl butoxide (oxidase inhibitor) + permethrin	*Apis mellifera*	Lethality (48h LC_{50})	(Hagler *et al.*, 1989)
Prochloraz + malathion	*Alectoris rufa* (red-legged partridge)	Lethality + P-450 activity	(Johnston *et al.*, 1989); (Johnston *et al.*, 1990)
Atrazine + chlorpyrifos/ methyl-parathion/ malathion	*C. tentans*	Lethality (96h LC_{50})	(Pape-Lindstrom & Lydy, 1997)
Atrazine + chlorpyrifos/ methyl-parathion/ diazinon	*C. tentans*	Lethality (96h LC_{50})	(Belden & Lydy, 2000)
Atrazine + chlorpyrifos Cyanazine + chlorpyrifos	*C. tentans*	Lethality (48h LC_{50}) + AChE-inhibition	(Jin-Clark *et al.*, 2002)
Quinalphos + phenthoate (OPs)	*Oreochromis mossambicus* (fish)	Lethality	(Durairaj & Selvarajan, 1995)
Malathion + dioxathion (OPs)	*O. mykiss*	Lethality	(Marking & Dawson, 1975)
Carbaryl + phenthoate	*Channa punctatus* (fish)	Lethality	(Sambasiva Rao *et al.*, 1985)
Binary + tertiary mixtures: Deltamethrin + carbaryl + MGK-264 (esterase inh.)	*Lymnaea acuminata* (snail)	Lethality	(Tripathi & Agarwal, 1997)
Bin. + tert. Mixtures: Dichlorvos + deltamethrin + PB	*Lymnaea acuminata*	Lethality + AChE-inhibition	(Tripathi & Agarwal, 1998)

As can be seen from Tables 5.2 and 5.3, there are numerous exceptions to the model of concentration addition in mixtures of pesticides and metals. Most studies have been conducted using acute lethality as a toxicity parameter. Several studies on the toxicity of mixtures of non-organic chemicals have shown that the degree of additivity may decrease when using a more sensitive endpoint (Eaton, 1973; Broderius & Smith, 1979). This phenomenon has also been reported by Hermens *et al.* (1984) and by Deneer *et al.* (1988), who found that additivity of a mixture of 9 – 14 different organic chemicals was significantly lower when reproductive inhibition was used as an endpoint instead of lethality (Hermens *et al.*, 1984; Deneer *et al.*, 1988).

In the present investigation we decided to test whether mixtures of 3 reactive chemicals with different toxic modes of action, the nonessential metal cadmium, the herbicide atrazine, and the pyrethroid insecticide deltamethrin, would act additively, less-than-additively, or more-than-additively in sublethal mixture concentrations, and whether the contaminant-induced inhibition of feeding in cladocerans would be a suitable endpoint to assess such mixture toxicity.

5.2 OBJECTIVES

The objectives of this study were the establishment of feeding curves of *C. dubia* and *S. vetulus* when exposed to binary mixtures and a tertiary mixture of the synthetic pyrethroid deltamethrin, the herbicide atrazine, and cadmium, at sublethal concentrations established from feeding inhibition curves with the individual compounds singly. From the results the effects of the 3 chemicals in combinations were judged to be either additive, less-than-additive, or more- than-additive. These results were then presented in a graphic model.

5.3 MATERIALS & METHODS

Feeding measurements under binary and tertiary mixture exposure were performed using the same experimental methods as the feeding experiments in Chapter 4.

Chemical stocks were made up as described in Chapter 2.

For assessing the effects of binary and tertiary mixtures of the 3 chemicals, cadmium, deltamethrin, and atrazine, a factorial design was chosen. This means that different combinations of relative component mixtures were tested, i.e. 2 to 3 single chemical concentrations were combined in different relative concentrations. This allows the assessment of the relative impacts of the different chemicals in the mixture.

Due to the low chemical concentrations employed and the small exposure volumes, it was not possible to determine the exact chemical concentrations in the exposure solutions, which would have been essential if one wanted to calculate the exact toxic units of the individual compounds in the mixture. Instead, mixtures were composed of solutions that were also tested individually, and the individual chemical effects were compared with those from the mixtures. This allowed the question of whether effects in the mixtures were only due to one chemical, or indeed a product of both chemicals in the mixture (additive), or if the effects were slightly less-than-additive (antagonistic) or more-than-additive (synergistic) to be addressed.

For *C. dubia*, 4 young females (6-7 d old) were exposed in the same design as before, in 7 mL glass bottles covered with aluminium foil and a lid to avoid evaporative loss. Per treatment there were 4 replicates, a total of 16 animals per treatment. Algal concentrations were 2×10^6 cells mL^{-1}. Animals were left to feed in the dark for a period of 24 h, after which mortalities and neonates present were noted, and the algal concentrations after 24 h determined using a Coulter Counter according to the method

described in Chapter 3. Feeding rates were determined according to Equation 4.4 (Chapter 4).

For *S. vetulus*, four 8-9 d old females were exposed in 40 mL glass bottles, and experiments conducted in accordance with the *C. dubia* experiments (Chapter 4). Three to 4 replicates were used per treatment depending on the numbers of young of one cohort available.

In the binary and tertiary mixture experiments, each chemical was tested individually in 2 or 3 different concentrations. These concentrations were aimed to be roughly the IC_{25}, IC_{50} and IC_{75} of that compound as established in the single exposure experiments in Chapter 4. However, conservative preparation of the individual solutions to avoid excessive toxicity (of the compounds) led to most individual solutions having relatively lower effective toxic values. Binary mixtures were prepared by using a 50:50 mixture of the individual solutions, so that exactly 50% of the effective concentration of each chemical was present in the mixture. For tertiary mixtures, the ratio was 33:33:33, so that each chemical would be present at 1/3 of its effective concentration.

In the case of *C. dubia*, for the mixtures of 7 mL, 3.5 mL of compound A was combined with 3.5 mL of compound B, and for tertiary mixtures 2.4 mL of each of the 3 solutions was used. For the *S. vetulus* experiments, binary mixtures of 40 mL consisted of 20 mL of compound A and 20 mL of compound B. Tertiary mixtures were only established for *C. dubia*, as test performance was better with this species.

The predicted effect (IC_x), which describes the relative inhibition of feeding (x %) as a percentage of the controls, was calculated using the response curves established from the single exposure experiments in Chapter 4. Contaminant-induced feeding inhibition does not follow a linear relationship. Rather it can be described with the

non-linear allosteric decay model used in Chapter 4. For each compound, a special curve defined by the median effective concentration (EC_{50}/IC_{50}) and the allosteric decay index *i* was established. Depending on the value of *i*, the curve was more or less steep, which in turn defined the individual IC values.

From the effective inhibition values determined for the individual compounds, a corresponding concentration was calculated using the response curve for the individual compound. For example, if the single effect from Solution A was 85% feeding relative to the controls (100%), a 15% inhibition effect was assumed, i.e. an IC_{15}. The corresponding concentration for that chemical was determined from the curve, and for the binary mixtures, that value was then halved, to represent the amount of that compound present in the binary mixture. That 1/2 concentration was then matched up with an IC value, which was not always half of the individual IC value due to the shape of the curve. The resulting IC values for compounds A and B were then added together to determine the predicted IC value (i.e. the expected inhibition effect compared to the control feeding) of the mixture. This predicted IC value was then compared to the value obtained from exposing the test animals to the binary mixture.

5.4 RESULTS

Results are presented in tables and graphs. The tables contain the relative IC values for the 3 compounds used to establish the relationships between observed and predicted effects in the graphs. Table 5.4 contains the values used to calculate the individual response curves for the 3 chemicals, as described in Chapter 4.

Table 5.4 IC_{50} values and allosteric decay indices *i* used for calculating predicted IC values from the response curves of the individual chemicals

	C. dubia		*S. vetulus*	
	IC_{50}	*i*	IC_{50}	*i*
Deltamethrin	0.19 $\mu g\ L^{-1}$	2.09	0.64 $\mu g\ L^{-1}$	1.06
Atrazine	13.23 $mg\ L^{-1}$	4.09	8.15 $mg\ L^{-1}$	1.62
Cadmium	5.31 $\mu g\ L^{-1}$	0.63	5.31 $\mu g\ L^{-1}$	0.63

Values for *S. vetulus* for cadmium were adopted from those for *C. dubia.*

The IC_x of a compound is the concentration of that compound that affects x% inhibition of feeding in the test organisms.

All feeding inhibition effects (IC values) are expressed as the mean of four replicates. Raw data is given in Appendix 2; indication of standard deviation in the graphs was omitted to avoid crowding. The diagonal line in the graphs represents concentration addition. If the mixtures corresponded strictly to additivity, the predicted and the observed values would be identical, and data would lie on the diagonal line. IC values that present a less-than-additive effect of the mixture lie below that line, and IC values that present a more-than-additive effect lie above the line.

Table 5.5 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 1: *C. dubia* exposed to binary mixtures of atrazine and cadmium

Compound	Feeding inhibition = %	Concentration	½ of concentration	Predicted IC-value
Cadmium	0	0	0	0
Cadmium	26	1.01 $\mu g\ L^{-1}$	0.51	18.5
Cadmium	53	6.43 $\mu g\ L^{-1}$	3.22	42
Atrazine	12	8.13 $mg\ L^{-1}$	4.07	1.5
Atrazine	17	8.98 $mg\ L^{-1}$	4.49	2
Atrazine	19	9.28 $mg\ L^{-1}$	4.64	2.5
Cd IC_0 + AT $IC_{1.5}$	1.5			20
Cd $IC_{18.5}$ + AT $IC_{1.5}$	20			34
Cd IC_0 + AT IC_2	2			20
Cd $IC_{18.5}$ + AT IC_2	20.5			46
Cd IC_{42} + AT $IC_{2.5}$	44.5			69

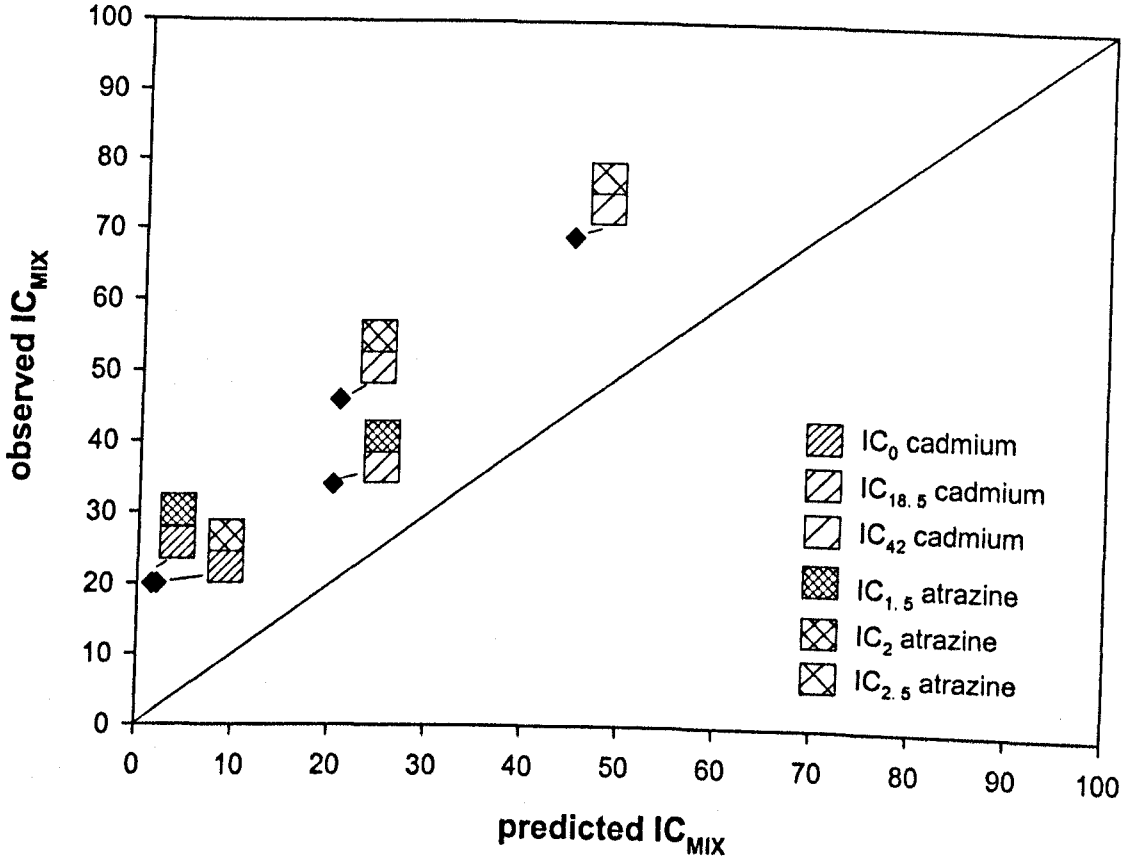


Figure 5.1 Observed versus predicted feeding inhibition expressed as IC_x values in *C. dubia* exposed to mixtures of cadmium and atrazine, Experiment 1. Group size = 16
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.6 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 1, as plotted in Figure 5.1

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
1 Cd IC_0 + 4 AT $IC_{1.5}$	1.5	20
2 Cd $IC_{18.5}$ + 4 AT $IC_{1.5}$	20	34
1 Cd IC_0 + 5 AT IC_2	2	20
2 Cd $IC_{18.5}$ + 5 AT IC_2	20.5	46
3 Cd IC_{42} + 6 AT $IC_{2.5}$	44.5	69

Table 5.7 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 2: *C. dubia* exposed to binary mixtures of atrazine and cadmium

Compound	Feeding inhibition = %	Concentration	½ of concen-tration	Predicted IC-value
Cadmium	11	0.19 $\mu g\ L^{-1}$	0.096	7.5
Cadmium	31	1.49 $\mu g\ L^{-1}$	0.745	22.5
Cadmium	43	3.39 $\mu g\ L^{-1}$	1.7	33
Atrazine	23	9.84 mg L^{-1}	4.92	2
Atrazine	29	10.63 mg L^{-1}	5.32	3
Atrazine	36	11.49 mg L^{-1}	5.75	4
Cd $IC_{7.5}$ + AT IC_2	9.5			17
Cd $IC_{22.5}$ + AT IC_2	24.5			40
Cd $IC_{7.5}$ + AT IC_3	10.5			22
Cd $IC_{22.5}$ + AT IC_3	25.5			35
Cd IC_{33} + AT IC_3	37			45

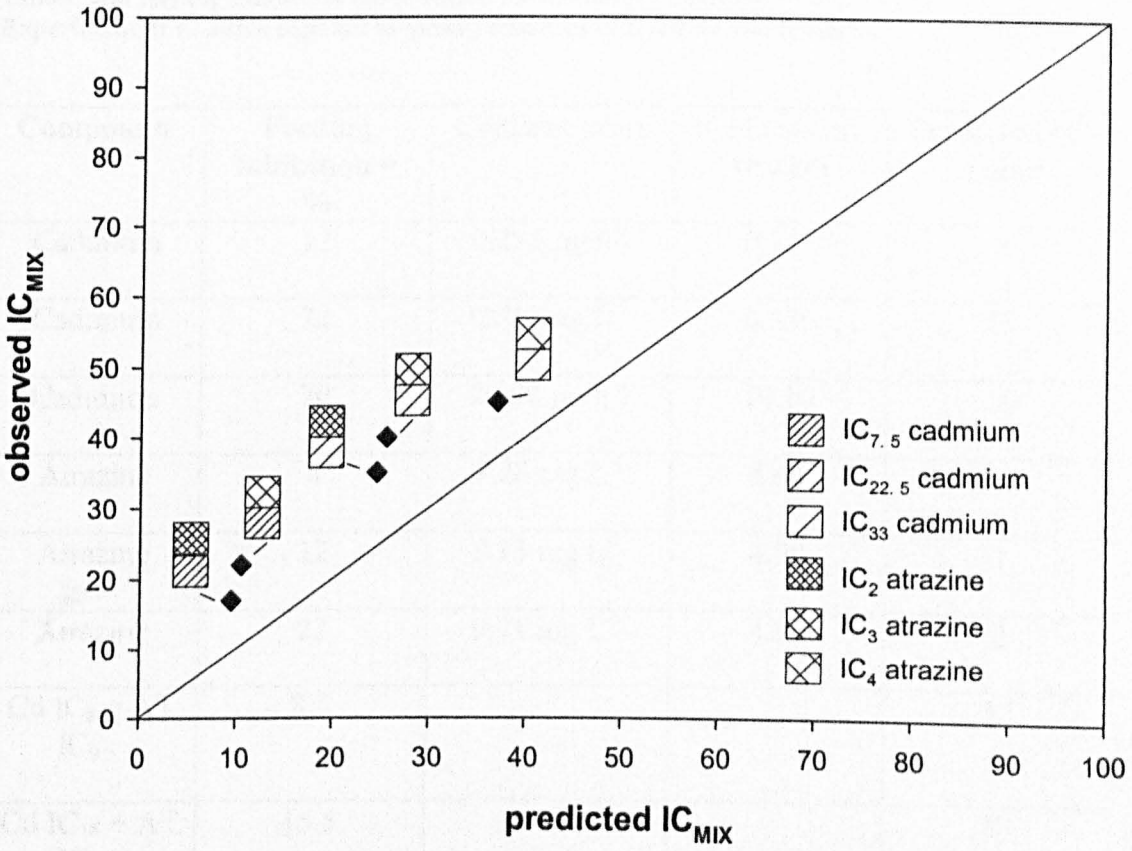


Figure 5.2 Observed versus predicted feeding inhibition expressed as IC_x values in *C. dubia* exposed to mixtures of cadmium and atrazine, Experiment 2. Group size = 16
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.8 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 2, as plotted in Figure 5.2

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
Cd $IC_{7.5}$ + AT IC_2	9.5	17
Cd $IC_{22.5}$ + AT IC_2	24.5	35
Cd $IC_{7.5}$ + AT IC_3	10.5	22
Cd $IC_{22.5}$ + AT IC_3	25.5	40
Cd IC_{33} + AT IC_3	37	45

Table 5.9 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 3: *C. dubia* exposed to binary mixtures of atrazine and cadmium

Compound	Feeding inhibition = %	Concentration	½ of concentration	Predicted IC-value
Cadmium	12	0.225 $\mu g\ L^{-1}$	0.113	8
Cadmium	22	0.712 $\mu g\ L^{-1}$	0.356	15
Cadmium	70	20.38 $\mu g\ L^{-1}$	10.19	60
Atrazine	8	7.28 mg L^{-1}	3.64	0.5
Atrazine	12	8.13 mg L^{-1}	4.06	1
Atrazine	22	9.71 mg L^{-1}	4.86	2
Cd IC_8 + AT $IC_{0.5}$	8.5			12
Cd IC_{15} + AT $IC_{0.5}$	15.5			37
Cd IC_8 + AT IC_1	9			12
Cd IC_{15} + AT IC_1	16			39
Cd IC_{60} + AT IC_2	62			53

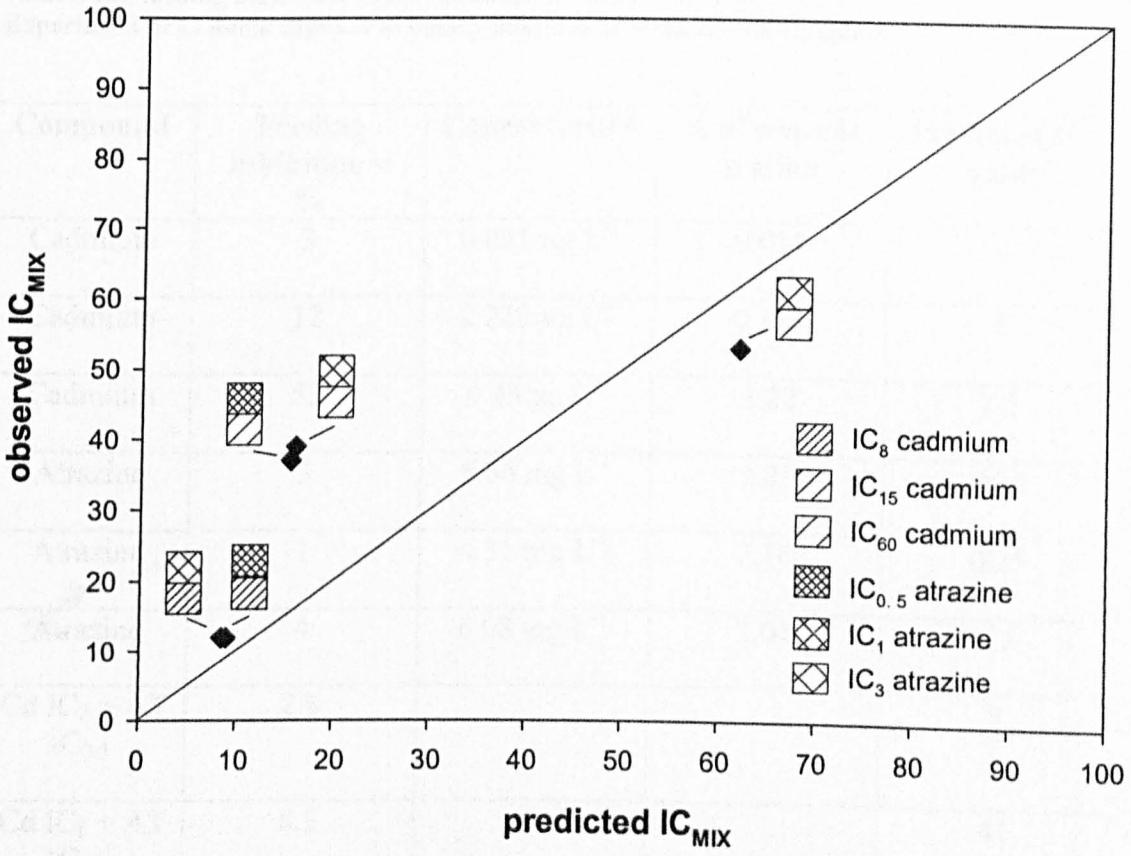


Figure 5.3 Observed versus predicted feeding inhibition expressed as IC_x values in *C. dubia* exposed to mixtures of cadmium and atrazine, Experiment 3. Group size = 16
♦indicates data point. Boxes refer to mixture components (see legend)

Table 5.10 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 3, as plotted in Figure 5.3

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
Cd IC_8 + AT $IC_{0.5}$	8.5	12
Cd IC_{15} + AT $IC_{0.5}$	15.5	37
Cd IC_8 + AT IC_1	9	12
Cd IC_{15} + AT IC_1	16	39
Cd IC_{60} + AT IC_2	62	53

Table 5.11 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 4: *C. dubia* exposed to binary mixtures of atrazine and cadmium

Compound	Feeding inhibition = %	Concentration	½ of concentration	Predicted IC-value
Cadmium	3	0.021 $\mu g\ L^{-1}$	0.011	2
Cadmium	12	0.225 $\mu g\ L^{-1}$	0.113	8
Cadmium	53	6.43 $\mu g\ L^{-1}$	3.22	42
Atrazine	3	5.66 $mg\ L^{-1}$	2.83	0.25
Atrazine	1	4.31 $mg\ L^{-1}$	2.16	0.25
Atrazine	4	6.08 $mg\ L^{-1}$	3.04	0.5
Cd IC_2 + AT $IC_{0.5}$	2.5			9
Cd IC_8 + AT $IC_{0.5}$	8.5			41
Cd IC_2 + AT $IC_{0.25}$	2.25			41
Cd IC_8 + AT $IC_{0.25}$	8.25			74
Cd IC_{42} + AT $IC_{0.5}$	42.5			84

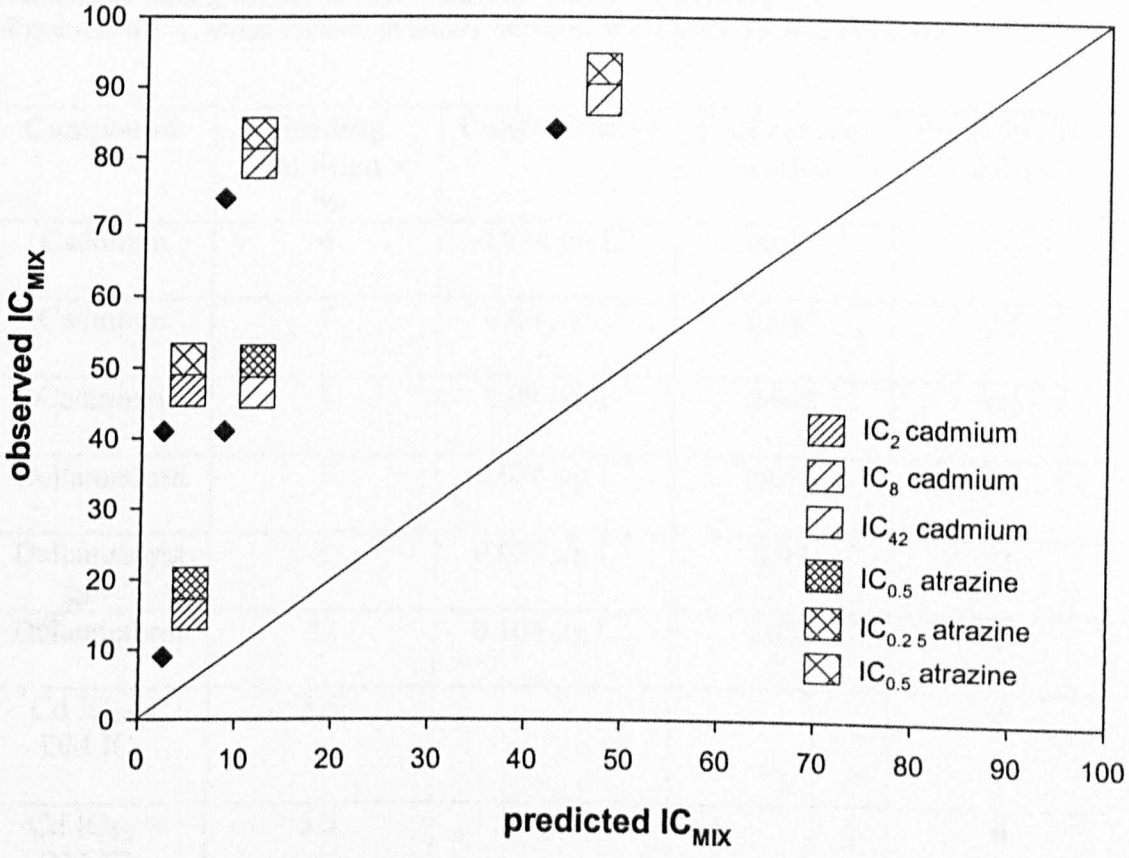


Figure 5.4 Observed versus predicted feeding inhibition expressed as IC_x values in *C. dubia* exposed to mixtures of cadmium and atrazine, Experiment 4.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.12 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 4, as plotted in Figure 5.12

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
Cd IC_2 + AT $IC_{0.5}$	2.5	9
Cd IC_8 + AT $IC_{0.5}$	8.5	41
Cd IC_2 + AT $IC_{0.25}$	2.25	41
Cd IC_8 + AT $IC_{0.25}$	8.25	74
Cd IC_{42} + AT $IC_{0.5}$	42.5	84

Table 5.13 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 5: *C. dubia* exposed to binary mixtures of deltamethrin and cadmium

Compound	Feeding inhibition = %	Concentration	½ of concentration	Predicted IC-value
Cadmium	4	0.034 $\mu g\ L^{-1}$	0.017	2.5
Cadmium	7	0.09 $\mu g\ L^{-1}$	0.045	4.5
Cadmium	7	0.09 $\mu g\ L^{-1}$	0.045	4.5
Deltamethrin	3	0.036 $\mu g\ L^{-1}$	0.018	1
Deltamethrin	8	0.059 $\mu g\ L^{-1}$	0.03	2
Deltamethrin	22	0.104 $\mu g\ L^{-1}$	0.052	6
Cd $IC_{2.5}$ + DM IC_1	3.5			6
Cd $IC_{4.5}$ + DM IC_1	5.5			6
Cd $IC_{2.5}$ + DM IC_2	4.5			5
Cd $IC_{4.5}$ + DM IC_2	6.5			7
Cd $IC_{4.5}$ + DM IC_6	10.5			18

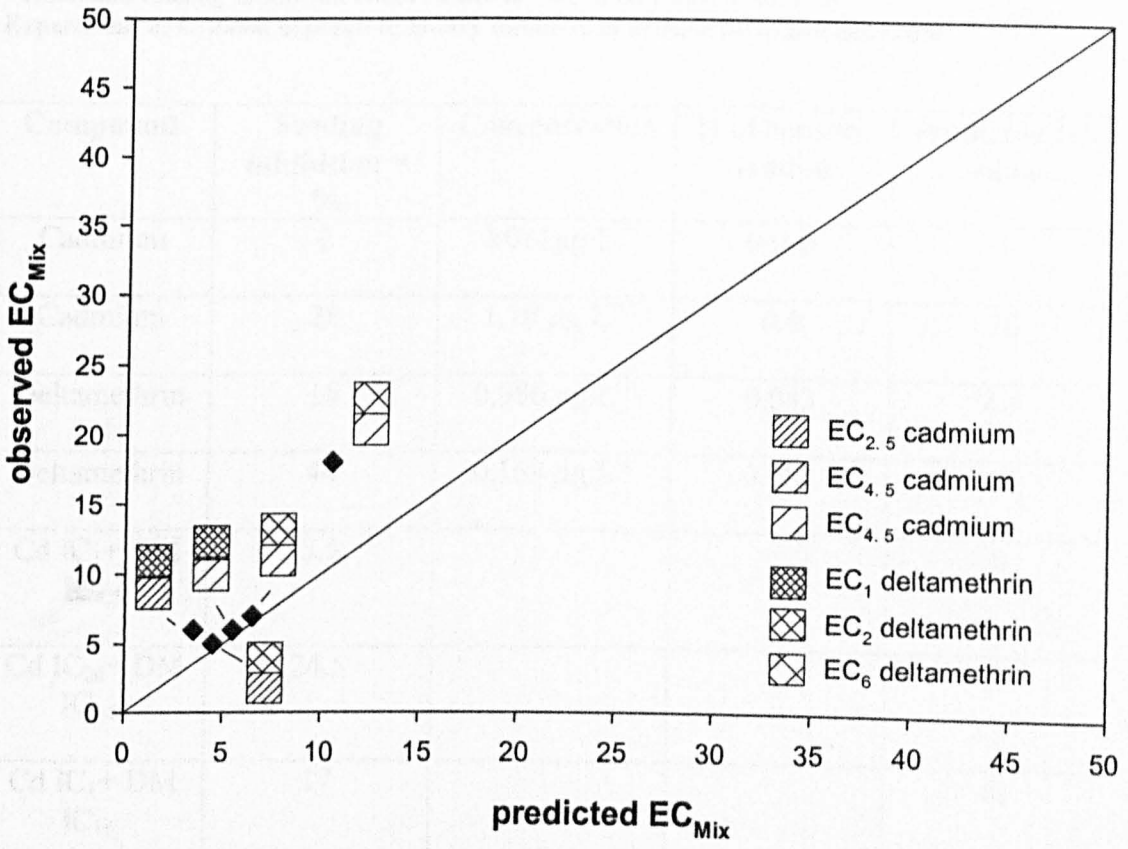


Figure 5.5 Observed versus predicted feeding inhibition expressed as IC_x values in *C. dubia* exposed to mixtures of cadmium and deltamethrin, Experiment 5.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.14 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 5, as plotted in Figure 5.5

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
Cd $IC_{2.5}$ + DM IC_1	3.5	6
Cd $IC_{4.5}$ + DM IC_1	5.5	6
Cd $IC_{2.5}$ + DM IC_2	4.5	5
Cd $IC_{4.5}$ + DM IC_2	6.5	7
Cd $IC_{4.5}$ + DM IC_6	10.5	18

Table 5.15 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 6: *C. dubia* exposed to binary mixtures of deltamethrin and cadmium

Compound	Feeding inhibition = %	Concentration	½ of concentration	Predicted IC-value
Cadmium	2	0.011 $\mu g\ L^{-1}$	0.005	1
Cadmium	28	1.19 $\mu g\ L^{-1}$	0.6	20
Deltamethrin	16	0.086 $\mu g\ L^{-1}$	0.043	4.5
Deltamethrin	44	0.169 $\mu g\ L^{-1}$	0.085	16
Cd IC_1+ DM $IC_{4.5}$	5.5			8
Cd IC_{20}+ DM $IC_{4.5}$	24.5			47
Cd IC_1+ DM IC_{16}	17			61
Cd IC_{20}+ DM IC_{16}	36			71

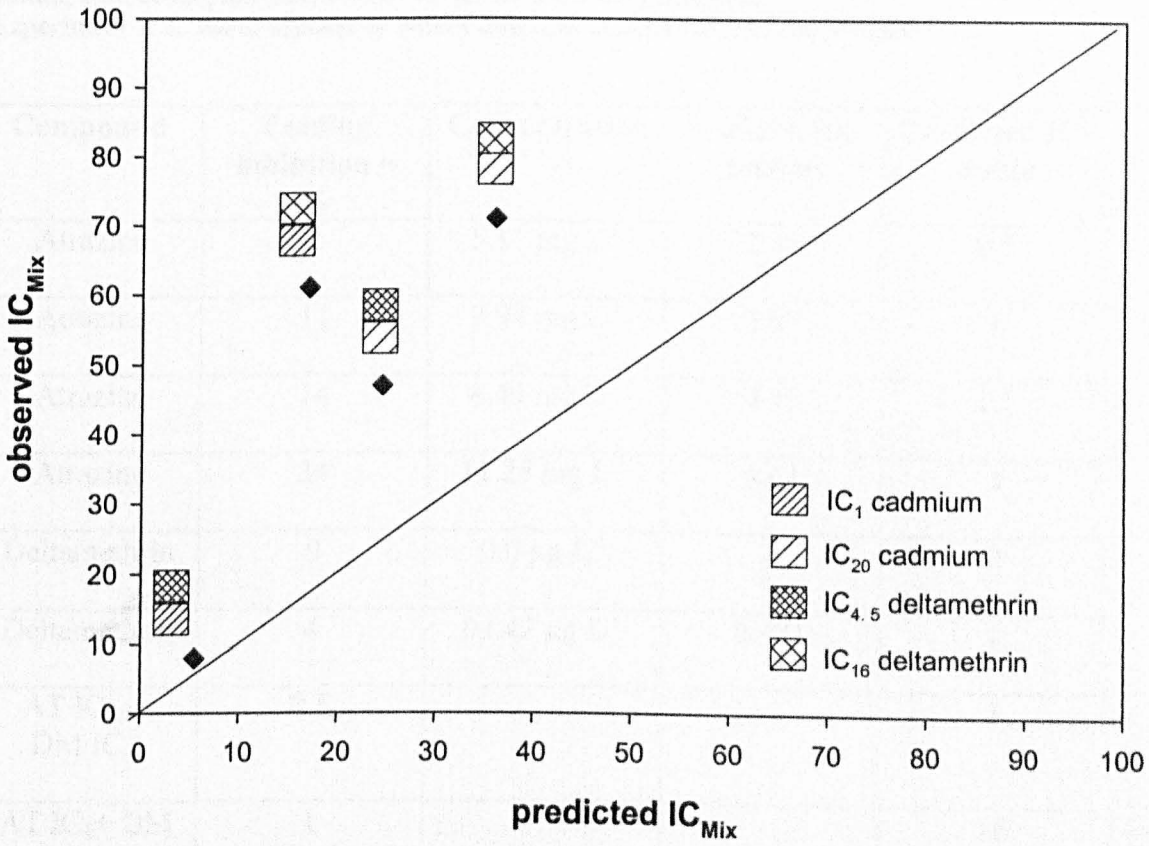


Figure 5.6 Observed versus predicted feeding inhibition expressed as IC_x values in *C. dubia* exposed to mixtures of cadmium and deltamethrin, Experiment 6.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.16 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 6, as plotted in Figure 5.6

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
Cd IC_1+ DM $IC_{4.5}$	5.5	8
Cd IC_{20}+ DM $IC_{4.5}$	24.5	47
Cd IC_1+ DM IC_{16}	17	61
Cd IC_{20}+ DM IC_{16}	36	71

Table 5.17 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 7: *C. dubia* exposed to binary mixtures of deltamethrin and atrazine

Compound	Feeding inhibition = %	Concentration	½ of concentration	Predicted IC-value
Atrazine	2	5.11 mg L^{-1}	2.56	0.5
Atrazine	11	7.94 mg L^{-1}	3.97	1
Atrazine	14	8.49 mg L^{-1}	4.35	1.5
Atrazine	34	11.25 mg L^{-1}	5.63	3
Deltamethrin	0	0.0 μg L^{-1}	0	0
Deltamethrin	4	0.042 μg L^{-1}	0.021	1
AT $IC_{0.5}$+ DM IC_0	0.5			3
AT IC_1+ DM IC_0	1			16
AT $IC_{1.5}$+ DM IC_1	2.5			20
AT IC_3+ DM IC_1	4			42

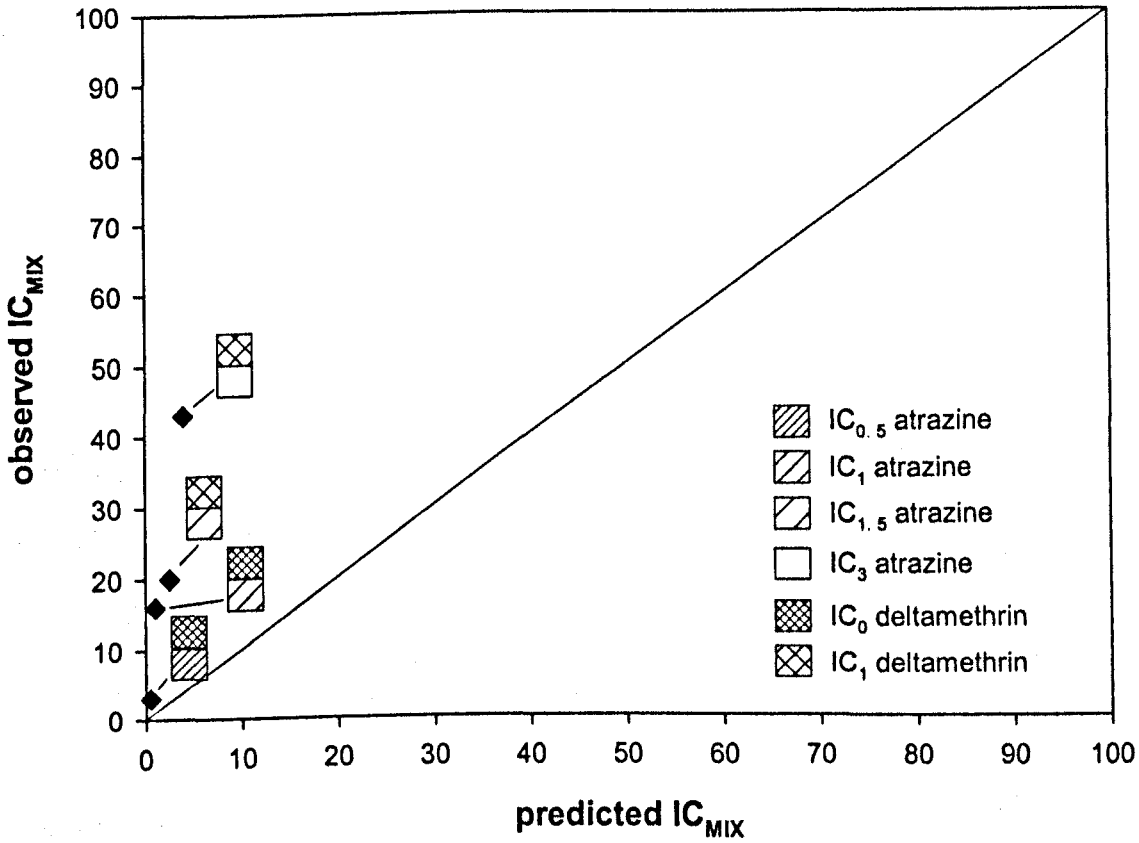


Figure 5.7 Observed versus predicted feeding inhibition expressed as IC_x values in *C. dubia* exposed to mixtures of atrazine and deltamethrin, Experiment 7
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.18 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 7, as plotted in Figure 5.7

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
AT $IC_{0.5}$+ DM IC_0	0.5	3
AT IC_1+ DM IC_0	1	16
AT $IC_{1.5}$+ DM IC_1	2.5	20
AT IC_3+ DM IC_1	4	42

Table 5.19 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 8: *C. dubia* exposed to binary mixtures of deltamethrin and atrazine

Compound	Feeding inhibition = %	Concentration	½ of concentration	Predicted IC-value
Atrazine	7	7.03 mg L^{-1}	3.51	0.5
Atrazine	13	8.31 mg L^{-1}	4.16	1
Atrazine	15	8.66 mg L^{-1}	4.33	1.5
Deltamethrin	0	0.0 μg L^{-1}	0	0
Deltamethrin	9	0.063 μg L^{-1}	0.032	3
Deltamethrin	66	0.26 μg L^{-1}	0.13	32
AT $IC_{0.5}$ + DM IC_0	0.5			28
AT IC_1 + DM IC_0	1			46
AT $IC_{0.5}$ + DM IC_3	3.5			78
AT IC_1 + DM IC_3	4			82
AT $IC_{1.5}$ + DM IC_{32}	33.5			87

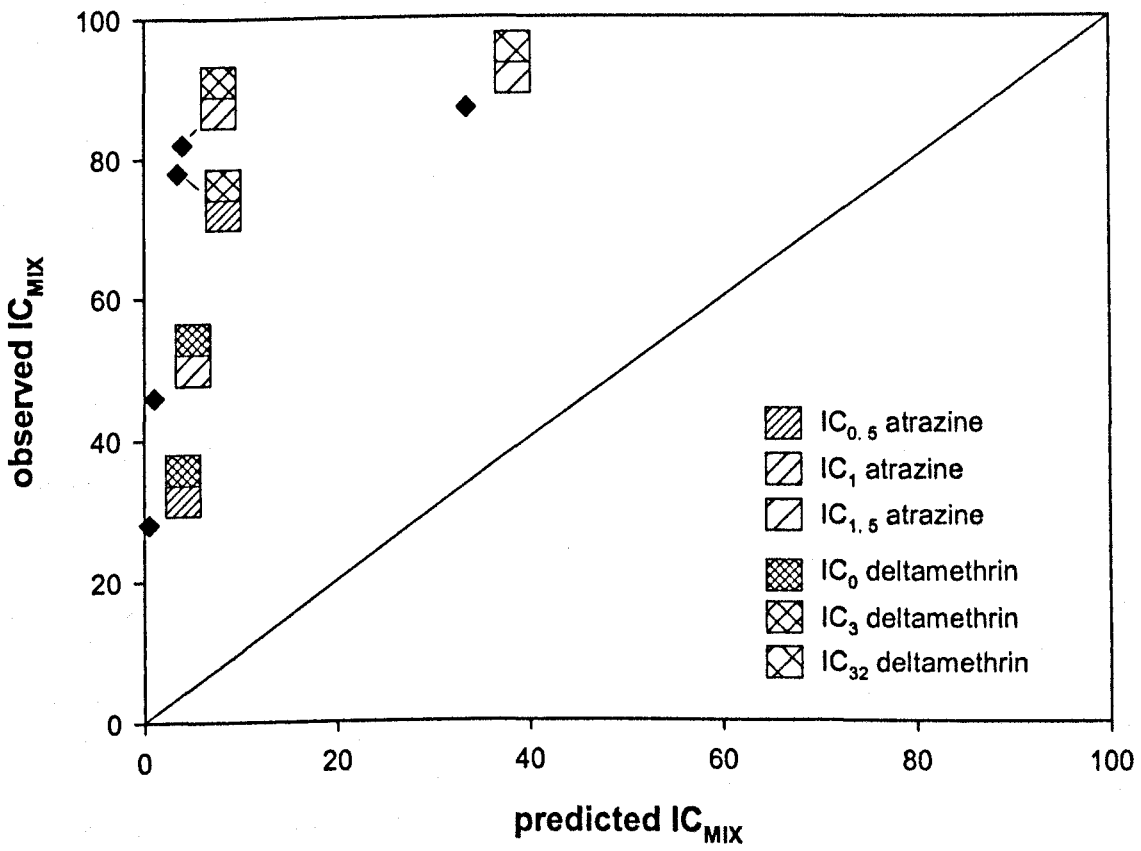


Figure 5.8 Observed versus predicted feeding inhibition expressed as IC_x values in *C. dubia* exposed to mixtures of atrazine and deltamethrin, Experiment 8.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.20 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 8, as plotted in Figure 5.8

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
AT $IC_{0.5}$ + DM IC_0	0.5	28
AT IC_1 + DM IC_0	1	46
AT $IC_{0.5}$ + DM IC_3	3.5	78
AT IC_1 + DM IC_3	4	82
AT $IC_{1.5}$ + DM IC_{32}	33.5	87

Table 5.21 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 9: *C. dubia* exposed to binary mixtures of deltamethrin and atrazine

Compound	Feeding inhibition = %	Concentration	½ of concentration	Predicted IC-value
Atrazine	1	4.31 mg L^{-1}	2.16	0.25
Atrazine	5	6.44 mg L^{-1}	3.22	0.5
Atrazine	11	7.94 mg L^{-1}	3.97	1
Deltamethrin	12	0.073 μg L^{-1}	0.037	3.5
Deltamethrin	42	0.163 μg L^{-1}	0.082	15
Deltamethrin	81	0.38 μg L^{-1}	0.19	50
AT $IC_{0.25}$ + DM $IC_{3.5}$	3.75			30
AT $IC_{0.5}$ + DM $IC_{3.5}$	4			54
AT $IC_{0.25}$ + DM IC_{15}	15.25			75
AT $IC_{0.5}$ + DM IC_{15}	15.5			88
AT IC_1 + DM IC_{50}	51			100

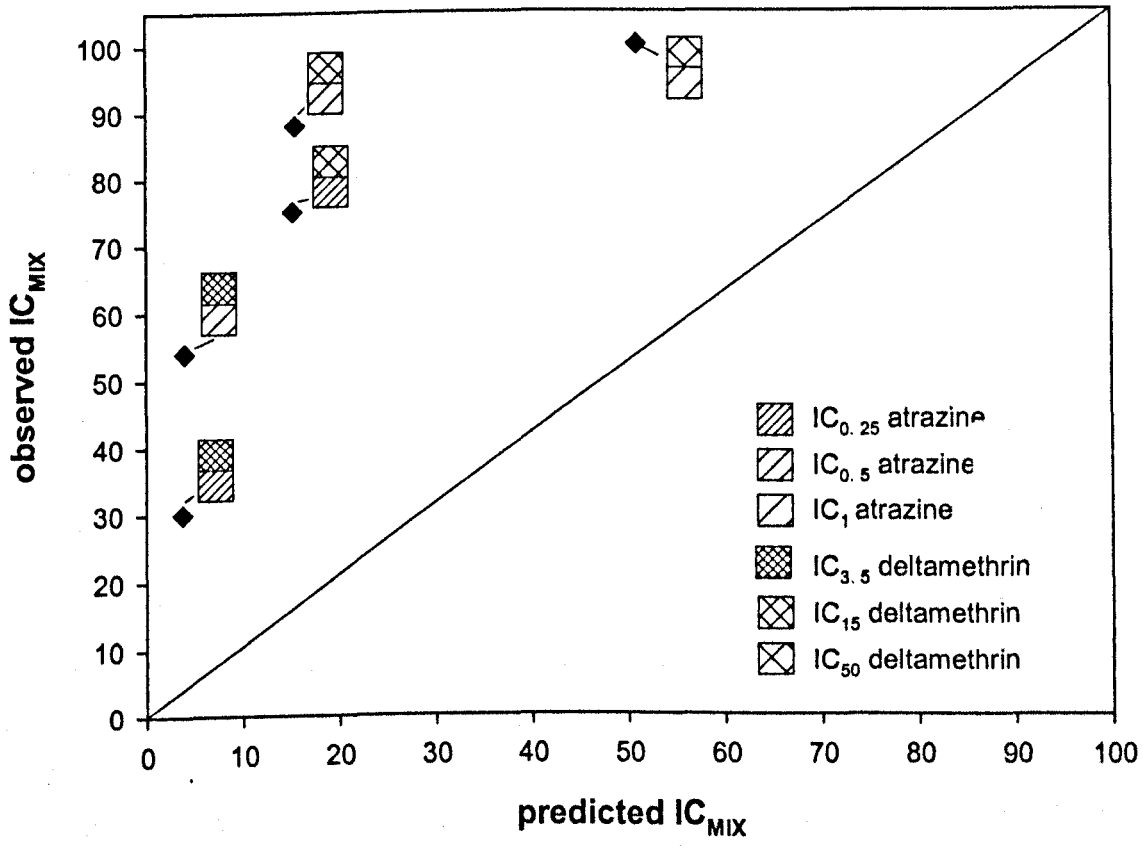


Figure 5.9 Observed versus predicted feeding inhibition expressed as IC_x values in *C. dubia* exposed to mixtures of atrazine and deltamethrin, Experiment 9.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.22 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 9, as plotted in Figure 5.9

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
AT $IC_{0.25}$ + DM $IC_{3.5}$	3.75	30
AT $IC_{0.5}$ + DM $IC_{3.5}$	4	54
AT $IC_{0.25}$ + DM IC_{15}	15.25	75
AT $IC_{0.5}$ + DM IC_{15}	15.5	88
AT IC_1 + DM IC_{50}	51	100

Table 5.23 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 10: *C. dubia* exposed to binary mixtures of deltamethrin and atrazine

Compound	Feeding inhibition = %	Concentration	½ of concentration	predicted IC-value
Atrazine	18	9.13 mg L^{-1}	4.57	1.5
Atrazine	22	9.71 mg L^{-1}	4.86	2
Atrazine	26	10.25 mg L^{-1}	5.13	3
Deltamethrin	18	0.092 µg L^{-1}	0.046	5
Deltamethrin	41	0.16 µg L^{-1}	0.08	14
Deltamethrin	59	0.226 µg L^{-1}	0.113	25
AT $IC_{1.5}$+ DM IC_5	6.5			25
AT IC_2 + DM IC_5	7			25
AT $IC_{1.5}$ + DM IC_{14}	15.5			26
AT IC_2 + DM IC_{14}	16			38
AT IC_3 + DM IC_{25}	28			47

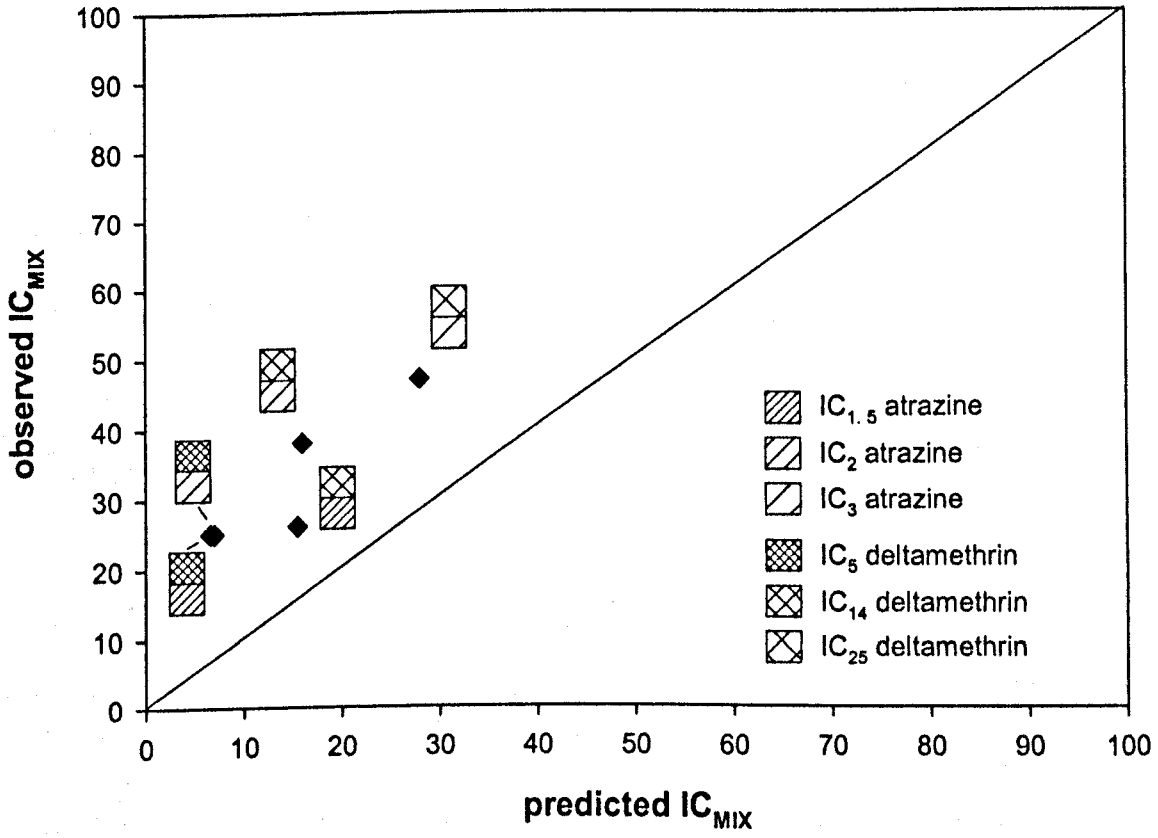


Figure 5.10 Observed versus predicted feeding inhibition expressed as IC_x values in *C. dubia* exposed to mixtures of atrazine and deltamethrin, Experiment 10.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.24 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 10, as plotted in Figure 5.10

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
AT $IC_{1.5}$+ DM IC_5	6.5	25
AT IC_2 + DM IC_5	7	25
AT $IC_{1.5}$ + DM IC_{14}	15.5	26
AT IC_2 + DM IC_{14}	16	38
AT IC_3 + DM IC_{25}	28	47

Table 5.25 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 11: *S.vetulus* exposed to binary mixtures of cadmium and atrazine

Compound	Feeding inhibition = %	Concentration	½ of concentration	Predicted IC-value
Cadmium	55	7.31 $\mu g\ L^{-1}$	3.66	44
Cadmium	65	14.19 $\mu g\ L^{-1}$	7.10	45.5
Cadmium	86	94.73 $\mu g\ L^{-1}$	47.37	80
Atrazine	18	3.2 mg L^{-1}	1.6	6.5
Atrazine	57	9.7 mg L^{-1}	4.85	30
Atrazine	69	13.36 mg L^{-1}	6.68	42
Cd IC_{44}+ AT $IC_{6.5}$	50.5			18
Cd $IC_{45.5}$ + AT $IC_{6.5}$	52			71
Cd IC_{44} + AT IC_{30}	74			70
Cd $IC_{45.5}$ + AT IC_{30}	75.5			73
Cd IC_{80} + AT IC_{42}	>100			85

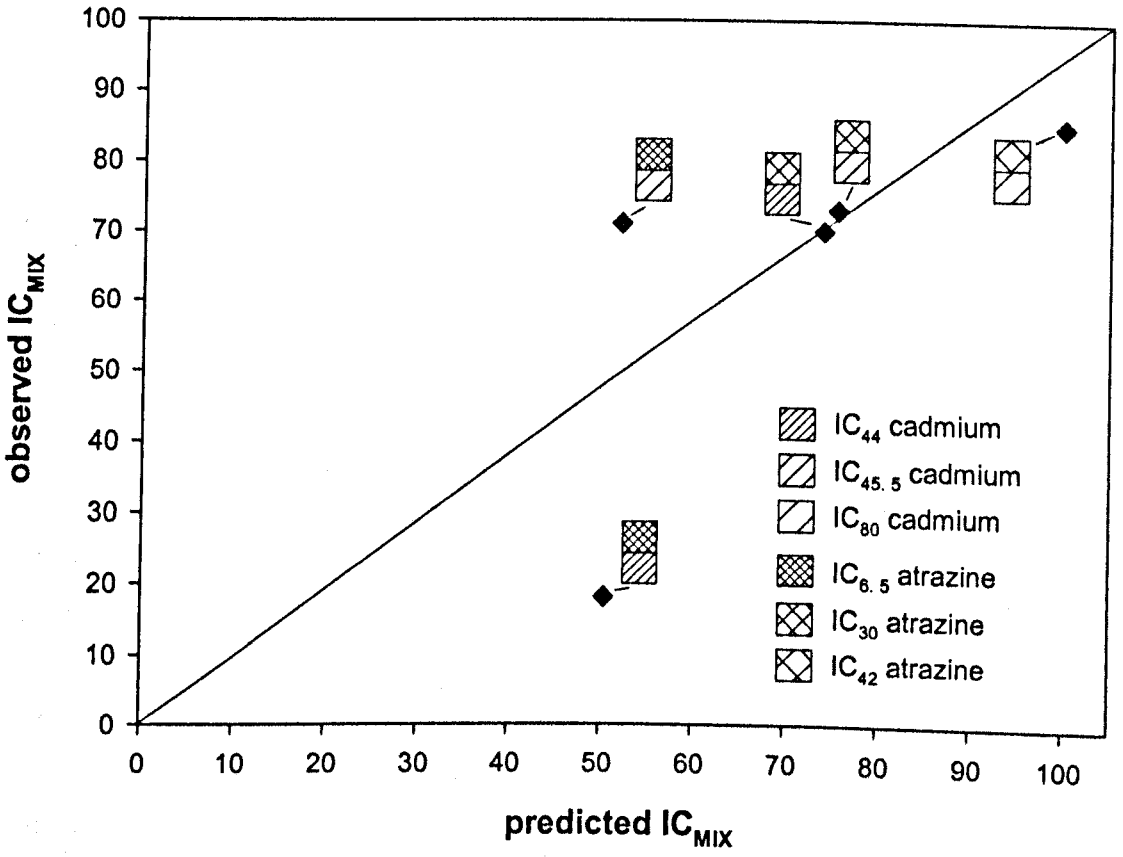


Figure 5.11 Observed versus predicted feeding inhibition expressed as IC_x values in *S. vetulus* exposed to mixtures of cadmium and atrazine, Experiment 11.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.26 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 11, as plotted in Figure 5.11

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
Cd IC_{44}+ AT $IC_{6.5}$	50.5	18
Cd $IC_{45.5}$ + AT $IC_{6.5}$	52	71
Cd IC_{44} + AT IC_{30}	74	70
Cd $IC_{45.5}$ + AT IC_{30}	75.5	73
Cd IC_{80} + AT IC_{42}	>100	85

Table 5.27 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 12: *S.vetulus* exposed to binary mixtures of cadmium and deltamethrin

Compound	Feeding inhibition = %	Concentration	½ of concentration	Predicted IC-value
Cadmium	24	0.85 $\mu g\ L^{-1}$	0.425	17
Cadmium	39	2.61 $\mu g\ L^{-1}$	1.31	29
Cadmium	67	16.34 $\mu g\ L^{-1}$	8.17	56
Deltamethrin	46	0.55 $\mu g\ L^{-1}$	0.275	29
Deltamethrin	71	1.49 $\mu g\ L^{-1}$	0.745	54
Deltamethrin	83	2.86 $\mu g\ L^{-1}$	1.43	70
Cd IC_{17}+ AT IC_{29}	46			64
Cd IC_{29} + AT IC_{29}	58			88
Cd IC_{17} + AT IC_{54}	71			95
Cd IC_{29} + AT IC_{54}	83			97
Cd IC_{56} + AT IC_{70}	>100			95

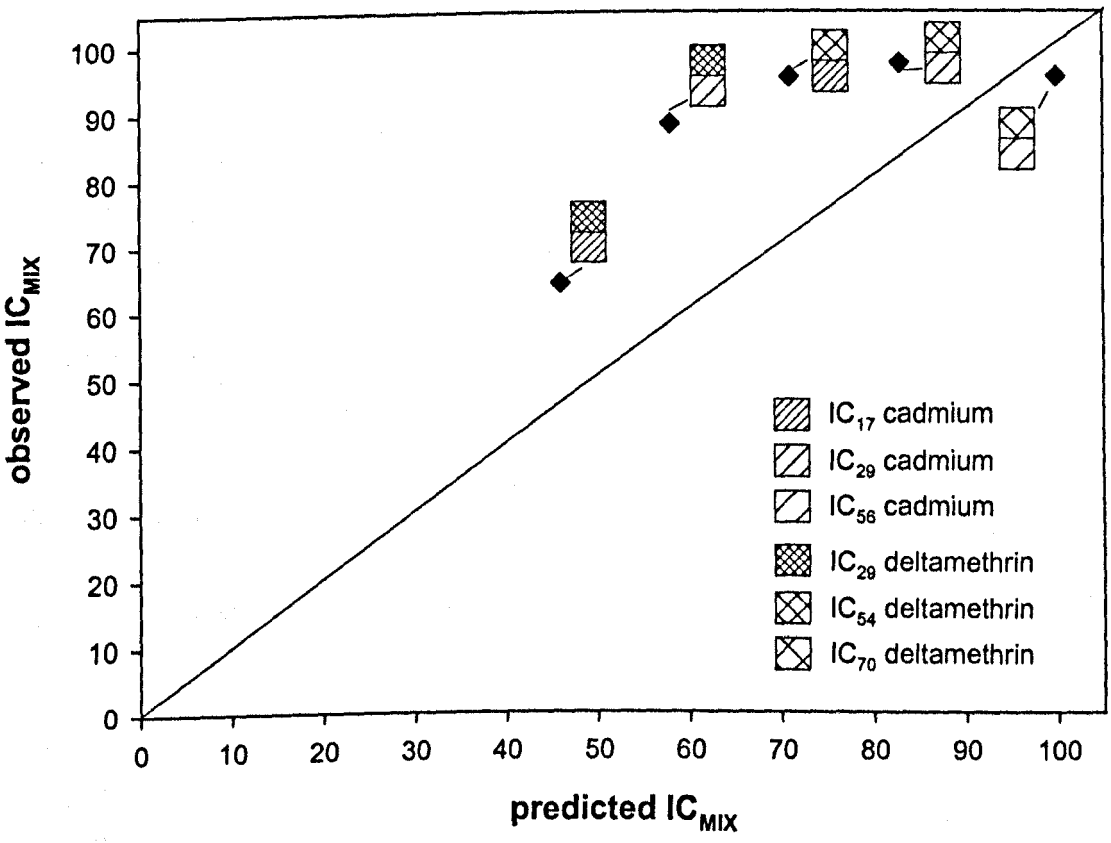


Figure 5.12 Observed versus predicted feeding inhibition expressed as IC_x values in *S. vetulus* exposed to mixtures of cadmium and deltamethrin, Experiment 12.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.28 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 12, as plotted in Figure 5.12

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
Cd IC_{17}+ AT IC_{29}	46	64
Cd IC_{29} + AT IC_{29}	58	88
Cd IC_{17} + AT IC_{54}	71	95
Cd IC_{29} + AT IC_{54}	83	97
Cd IC_{56} + AT IC_{70}	>100	95

Table 5.29 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 13: *S. vetulus* exposed to binary mixtures of deltamethrin and atrazine

Compound	Feeding inhibition = %	Concentration	½ of concentration	Predicted IC-value
Atrazine	7	1.76 mg L^{-1}	0.88	2
Atrazine	9	2.07 mg L^{-1}	1.04	3
Deltamethrin	10	0.08 μg L^{-1}	0.04	5
Deltamethrin	28	0.26 μg L^{-1}	0.13	15
AT IC_2 + DM IC_5	7			24
AT IC_3 + DM IC_{15}	18			49

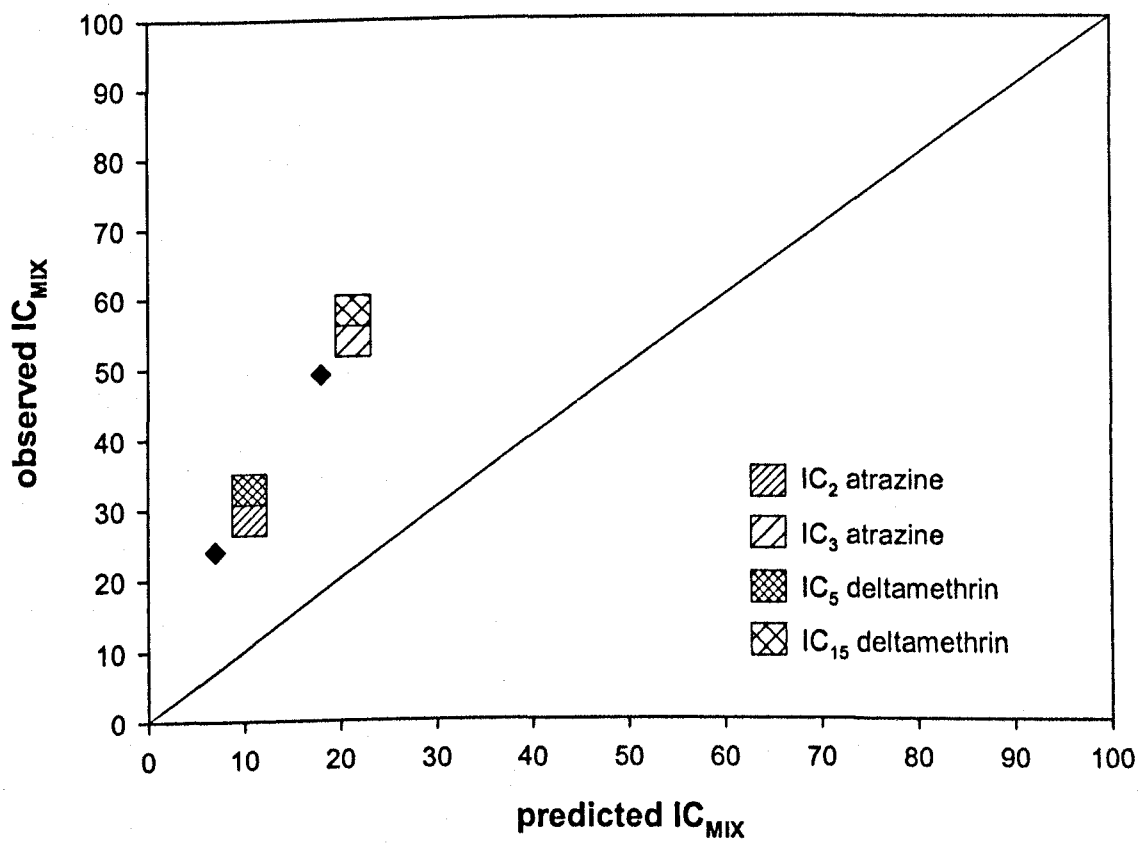


Figure 5.13 Observed versus predicted feeding inhibition expressed as IC_x values in *S. vetulus* exposed to mixtures of atrazine and deltamethrin, Experiment 13.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.30 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 13, as plotted in Figure 5.13

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
AT IC_2 + DM IC_5	7	24
AT IC_3 + DM IC_{15}	18	49

Table 5.31 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 14: *S. vetulus* exposed to binary mixtures of deltamethrin and atrazine

Compound	Feeding inhibition = %	Concentration	½ of concentration	Predicted IC-value
Atrazine	8	1.81 mg L^{-1}	0.905	3
Atrazine	13	2.52 mg L^{-1}	1.26	4.5
Atrazine	33	5.27 mg L^{-1}	2.64	14
Deltamethrin	14	0.115 μg L^{-1}	0.058	7
Deltamethrin	25	0.227 μg L^{-1}	0.114	14
Deltamethrin	36	0.372 μg L^{-1}	0.186	21
AT IC_3+ DM IC_7	10			12
AT $IC_{4.5}$ + DM IC_7	11.5			17
AT IC_3 + DM IC_{14}	17			32
AT $IC_{4.5}$ + DM IC_{14}	18.5			26
AT IC_{14} + DM IC_{21}	35			77

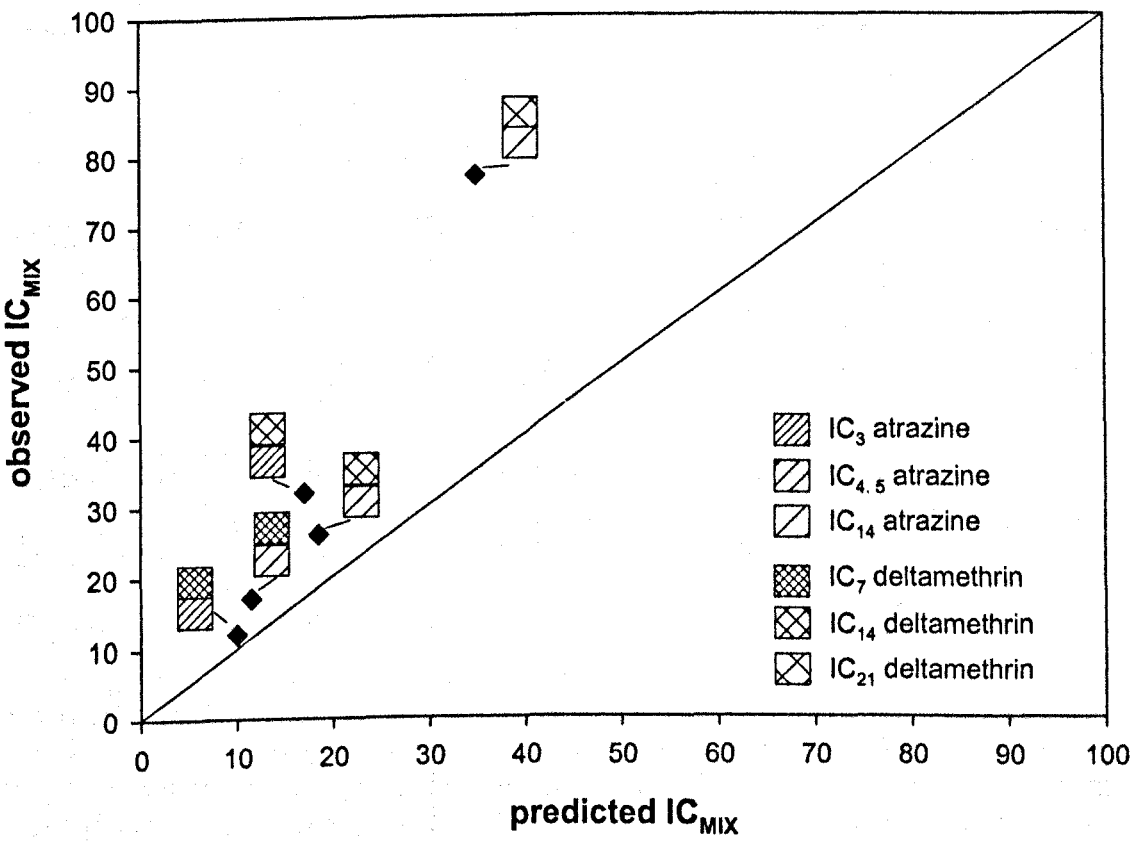


Figure 5.14 Observed versus predicted feeding inhibition expressed as IC_x values in *S. vetulus* exposed to mixtures of atrazine and deltamethrin, Experiment 14.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.32 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 14, as plotted in Figure 5.14

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
AT IC_3+ DM IC_7	10	12
AT $IC_{4.5}$ + DM IC_7	11.5	17
AT IC_3 + DM IC_{14}	17	32
AT $IC_{4.5}$ + DM IC_{14}	18.5	26
AT IC_{14} + DM IC_{21}	35	77

Table 5.33 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 15: *S. vetulus* exposed to binary mixtures of deltamethrin and atrazine

Compound	Feeding inhibition = %	Concentration	½ of concentration	predicted IC-value
Atrazine	25	4.14 mg L^{-1}	2.07	10
Atrazine	29	4.69 mg L^{-1}	2.35	12
Atrazine	68	12.98 mg L^{-1}	6.49	41
Deltamethrin	21	0.183 μg L^{-1}	0.092	11
Deltamethrin	32	0.314 μg L^{-1}	0.157	18
Deltamethrin	51	0.665 μg L^{-1}	0.333	33
AT IC_{10}+ DM IC_{11}	21			31
AT IC_{12} + DM IC_{11}	23			50
AT IC_{10} + DM IC_{18}	28			39
AT IC_{12} + DM IC_{18}	30			63
AT IC_{41} + DM IC_{33}	74			86

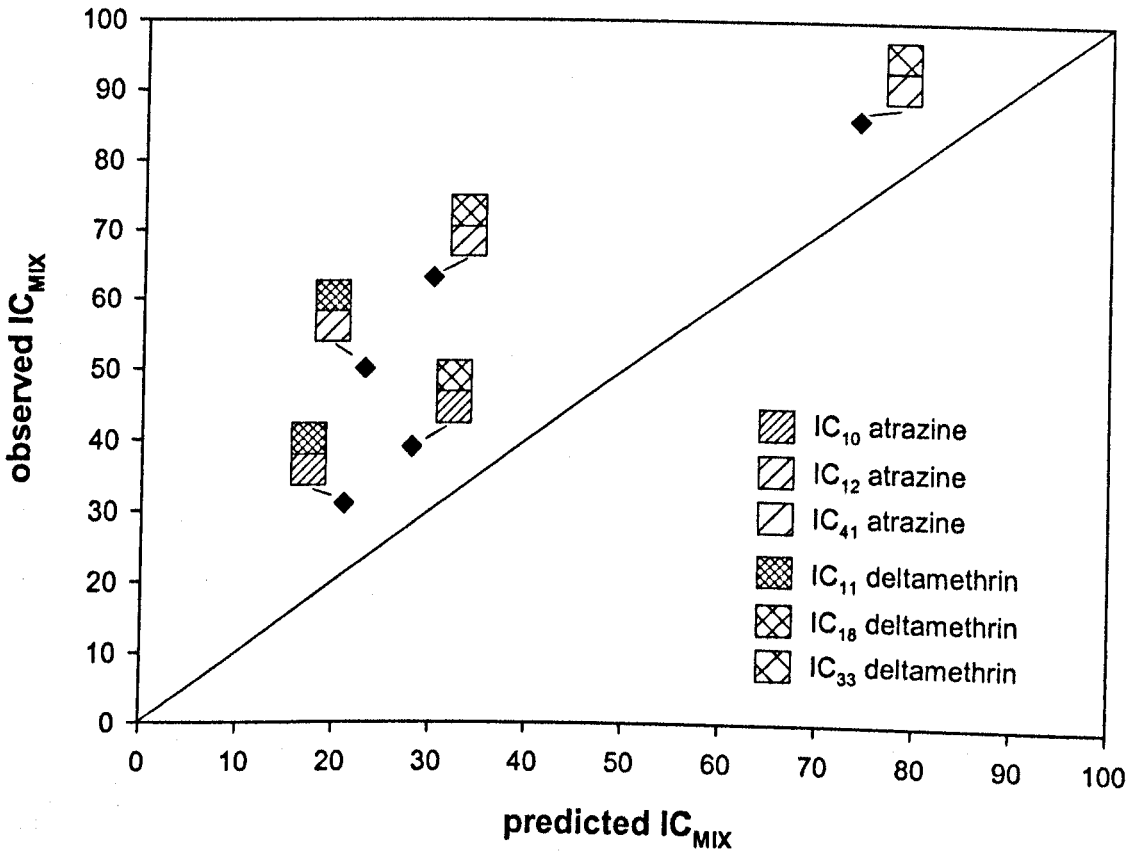


Figure 5.15 Observed versus predicted feeding inhibition expressed as IC_x values in *S. vetulus* exposed to mixtures of atrazine and deltamethrin, Experiment 15.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.34 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 15, as plotted in Figure 5.15

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
AT IC_{10}+ DM IC_{11}	21	31
AT IC_{12} + DM IC_{11}	23	50
AT IC_{10} + DM IC_{18}	28	39
AT IC_{12} + DM IC_{18}	30	63
AT IC_{41} + DM IC_{33}	74	86

Table 5.35 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the binary mixtures.
Experiment 16: *S. vetulus* exposed to binary mixtures of deltamethrin and atrazine

Compound	Feeding inhibition = %	Concentration	½ of concentration	predicted IC-value
Atrazine	40	6.35 mg L^{-1}	3.18	18
Atrazine	46	7.38 mg L^{-1}	3.69	21.5
Atrazine	56	9.46 mg L^{-1}	4.73	29.5
Deltamethrin	27	0.25 μg L^{-1}	0.125	15
Deltamethrin	37	0.39 μg L^{-1}	0.195	22
Deltamethrin	47	0.57 μg L^{-1}	0.285	30
AT IC_{18}+ DM IC_{15}	33			39
AT $IC_{21.5}$ + DM IC_{15}	36.5			73
AT IC_{18} + DM IC_{22}	40			64
AT $IC_{21.5}$ + DM IC_{18}	43.5			83
AT $IC_{29.5}$ + DM IC_{30}	59.5			91

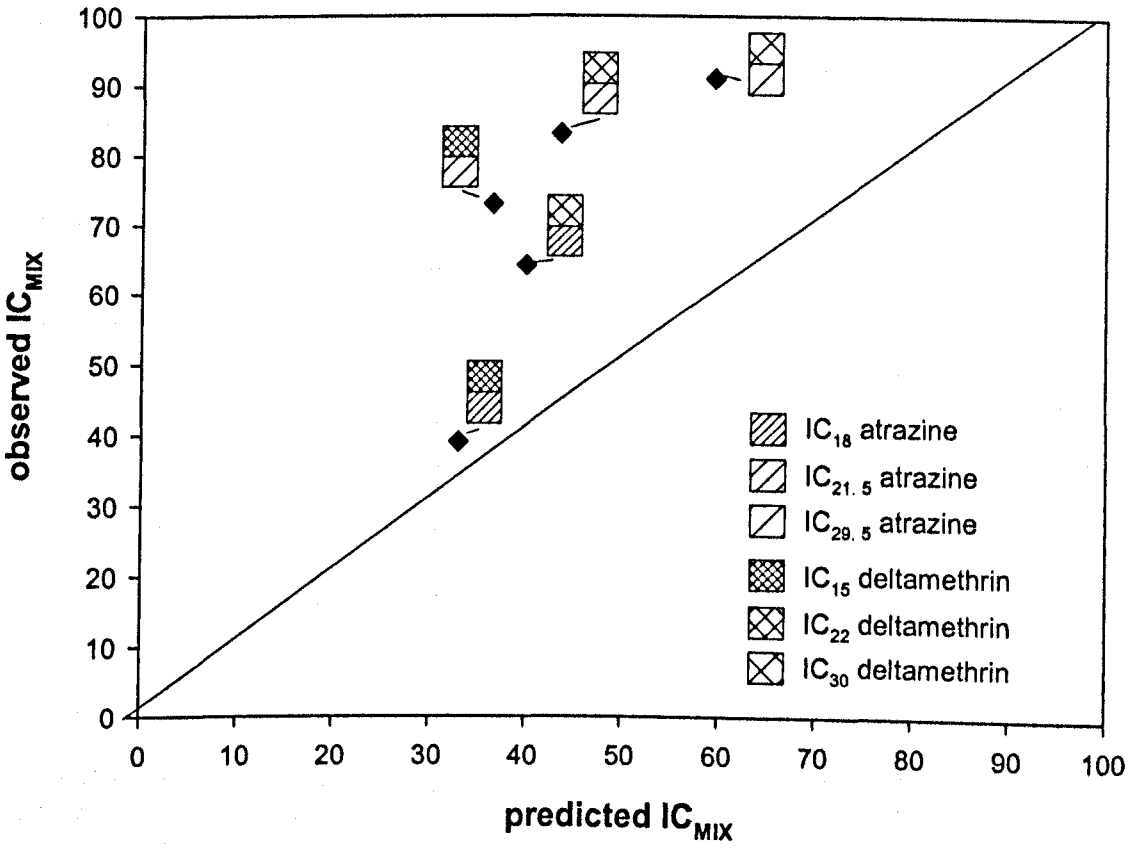


Figure 5.16 Observed versus predicted feeding inhibition expressed as IC_x values in *S. vetulus* exposed to mixtures of atrazine and deltamethrin, Experiment 16.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.36 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 16, as plotted in Figure 5.16

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
AT IC_{18}+ DM IC_{15}	33	39
AT $IC_{21.5}$ + DM IC_{15}	36.5	73
AT IC_{18} + DM IC_{22}	40	64
AT $IC_{21.5}$ + DM IC_{18}	43.5	83
AT $IC_{29.5}$ + DM IC_{30}	59.5	91

Table 5.37 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effecte values for the tertiary mixtures.
Experiment 17: *C. dubia* exposed to tertiary mixtures of deltamethrin, atrazine and cadmium

Compound	Feeding inhibition = %	Concentration	1/3 of concen-tration	predicted IC-value
Deltamethrin	8	0.059 $\mu g\ L^{-1}$	0.02	0.75
Deltamethrin	9	0.063 $\mu g\ L^{-1}$	0.021	1
Atrazine	26	10.25 $mg\ L^{-1}$	3.42	0.75
Atrazine	27	10.37 $mg\ L^{-1}$	3.46	0.75
Cadmium	14	0.3 $\mu g\ L^{-1}$	0.1	8
Cadmium	17	0.43 $\mu g\ L^{-1}$	0.143	9
Cadmium	36	2.13 $\mu g\ L^{-1}$	0.71	22
DM $IC_{0.75}$+ AT $IC_{0.75}$ + Cd IC_8	9.5			35
D1 IC_1+ AT $IC_{0.75}$ + Cd IC $_8$	9.75			40
DM $IC_{0.75}$+ AT $IC_{0.75}$ + Cd IC $_8$	9.5			33
DM $IC_{0.75}$+ AT $IC_{0.75}$ + Cd IC $_9$	10.5			38
DM IC_1+ AT $IC_{0.75}$ + Cd IC $_9$	10.75			47
DM IC_1+ AT $IC_{0.75}$ + Cd IC $_{22}$	23.75			54

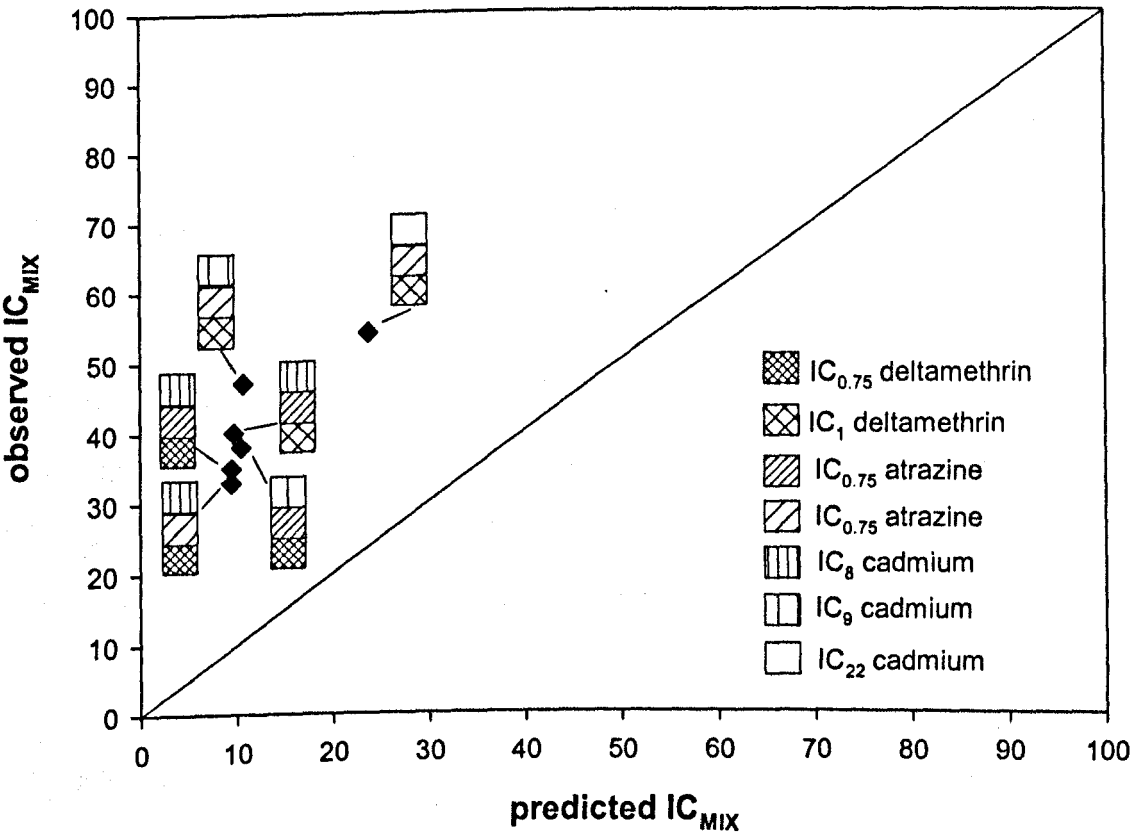


Figure 5.17 Observed versus predicted feeding inhibition expressed as IC_x values in *C. dubia* exposed to tertiary mixtures of deltamethrin, atrazine and cadmium, Experiment 17.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.38 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 17, as plotted in Figure 5.17

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
DM $IC_{0.75}$+ AT $IC_{0.75}$ + Cd IC_8	9.5	35
D1 IC_1+ AT $IC_{0.75}$ + Cd IC_8	9.75	40
DM $IC_{0.75}$+ AT $IC_{0.75}$ + Cd IC_8	9.5	33
DM $IC_{0.75}$+ AT $IC_{0.75}$ + Cd IC_9	10.5	38
DM IC_1+ AT $IC_{0.75}$ + Cd IC_9	10.75	47
DM IC_1+ AT $IC_{0.75}$ + Cd IC_{22}	23.75	54

Table 5.39 Feeding inhibition effect values for the individual compounds, converted into IC_x values, and feeding inhibition effect values for the tertiary mixtures.
Experiment 18: *C. dubia* exposed to tertiary mixtures of deltamethrin, atrazine and cadmium

Compound	Feeding inhibition = %	Concentration	½ of concentration	predicted IC-value
Deltamethrin	0	0 $\mu g\ L^{-1}$	0	0
Deltamethrin	4	0.042 $\mu g\ L^{-1}$	0.014	0.5
Atrazine	15	8.66 mg L^{-1}	2.89	0.5
Atrazine	16	8.83 mg L^{-1}	2.94	0.5
Cadmium	0	0 $\mu g\ L^{-1}$	0	0
Cadmium	2	0.011 $\mu g\ L^{-1}$	0.0037	1
Cadmium	41	2.98 $\mu g\ L^{-1}$	0.993	26
DM IC_0+ AT $IC_{0.5}$ + Cd IC_0	0.5			20
DM $IC_{0.5}$+ AT $IC_{0.5}$ + Cd IC_0	1			26
DM IC_0+ AT $IC_{0.5}$ + Cd IC_0	0.5			28
DM IC_0+ AT $IC_{.5}$ + Cd IC_1	1.5			39
DM $IC_{0.5}$+ AT $IC_{0.5}$ + Cd IC_1	2			44
DM $IC_{0.5}$+ AT $IC_{0.5}$ + Cd IC_{26}	27			65

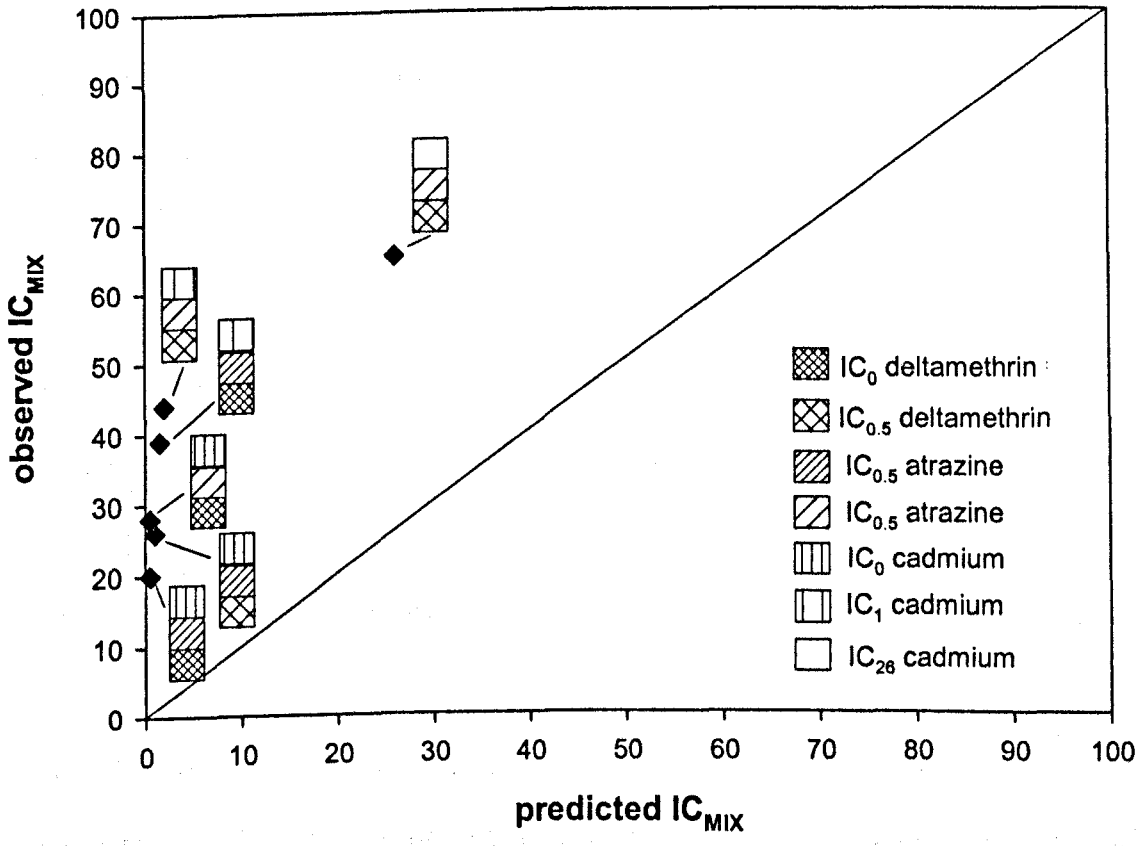


Figure 5.18 Observed versus predicted feeding inhibition expressed as IC_x values in *C. dubia* exposed to tertiary mixtures of deltamethrin, atrazine and cadmium, Experiment 18.
♦ indicates data point. Boxes refer to mixture components (see legend)

Table 5.40 Predicted IC_{MIX} values compared to observed IC_{MIX} values for Experiment 18, as plotted in Figure 5.18

Mixture	Predicted IC_{MIX}	Observed IC_{MIX}
DM IC_0+ AT $IC_{0.5}$ + Cd IC_0	0.5	20
DM $IC_{0.5}$+ AT $IC_{0.5}$ + Cd IC_0	1	26
DM IC_0+ AT $IC_{0.5}$ + Cd IC_0	0.5	28
DM IC_0+ AT IC_5 + Cd IC_1	1.5	39
DM $IC_{0.5}$+ AT $IC_{0.5}$ + Cd IC_1	2	44
DM $IC_{0.5}$+ AT $IC_{0.5}$ + Cd IC_{26}	27	65

Results from the feeding inhibition experiments showed that the combined toxic effects of the 3 different chemicals on feeding rates in both species of cladocerans, *C. dubia* and *S. vetulus*, appeared indeed to be additive or more-than-additive with very few exceptions.

Experiments 1 to 4 studied effects of binary mixtures of cadmium and atrazine on feeding rates in *C. dubia*. In experiments 1 to 3 (Figures 5.1 – 5.3), toxicity of each binary mixture was slightly more-than-additive, but corresponded well with the predicted effects in a linear relationship. Experiment 4 however (Figure 5.4) shows a notably more-than-additive effect, with the observed IC increasing exponentially way, with a steep increase at the lower concentrations and a levelling off at the higher concentrations. Experiments 5 and 6, presenting combination effects of cadmium and deltamethrin (Figures 5.5 and 5.6), again depict the toxic effects of the mixtures to act in an additive (Figure 5.5) or slightly more-than-additive (Figure 5.6) manner, corresponding linearly to the predicted toxicity values.

Experiments 7 to 10 (Figures 5.7 to 5.10) studied the toxic effects of binary mixtures of atrazine and deltamethrin on *C. dubia*. The first 3 experiments (Figures 5.7 to 5.9) show toxicity of the 2 pesticides in combinations to be more-than-additive, and the relationship between predicted and observed inhibition values (ICs) is exponential. The effects on feeding of the combination of the 2 pesticides are much higher than predicted at low and intermediate concentrations, and level off at the high concentrations, resulting in almost total inhibition of feeding. Experiment 10 (Figure 5.10) deviated from the other 3 experiments in that toxicity of the binary mixtures appeared only slightly more-than-additive and linear.

The experiments studying the mixture effects of the 3 chemicals on feeding depression in *S. vetulus* showed similar results as those with *C. dubia*. Mixture effects

on feeding in this species were also additive or more-than-additive. Experiment 11 (Figure 5.11) shows toxic effects of binary mixtures of cadmium and atrazine. At the lowest and highest concentration, there appeared to be less than additivity, but intermediate values are slightly more-than-additive or exactly additive. Experiment 12 (Figure 5.12) shows combination effects of cadmium and deltamethrin, again slightly more-than-additive, corresponding in a linear way to the predicted values. Experiments 13 to 16 (Figures 5.13 to 5.16) represent the binary mixtures of atrazine and deltamethrin. Here, toxicity of the combinations responds well to additivity at the lower concentrations, whereas at the higher concentrations, there appears to be a more-than-additive effect of the binary mixtures (Figures 5.14, 5.15 and 5.16). Generally the mixture experiments with this species showed a less pronounced synergistic effect among the 3 chemicals, especially less synergy between atrazine and deltamethrin than was found for *C. dubia*.

The tertiary mixtures of atrazine, deltamethrin and cadmium, experiments 17 and 18, again have a much more-than-additive effect on reducing feeding rates of *C. dubia*. In both tertiary mixture feeding experiments, the observed additive effects of the mixture increase exponentially, with additivity effects markedly more-than-additive at the lower concentrations and levelling off approaching total inhibition of feeding.

Interestingly, in some experiments, concentrations of individual toxicant solutions that did not cause any inhibition of feeding contributed to overall toxicity of the mixture. In experiment 1 (Figure 5.1, Table 5.6) the concentration of the cadmium solution 1 did not cause any feeding inhibition when tested singly. However, when that solution was combined with a concentration of atrazine predicted to cause an inhibition of feeding of 1.5 or 2%, respectively (Table 5.6), it led to an observed inhibition effect of 20%, much higher than that expected. This phenomenon was again observed in

experiment 7, with binary combinations of atrazine and deltamethrin. In this experiment, a deltamethrin solution that did not cause any inhibitory effect on its own caused an increase in the toxicity of atrazine (Figure 5.7, Tables 5.17 and 5.18): observed toxicity of an atrazine and deltamethrin mixture was 3% instead of 1% and 16% instead of 1%. In experiment 8, a non-effective concentration of deltamethrin caused an even higher increase in atrazine toxicity: 28% instead of a predicted 0.5% and 46% instead of a predicted 1% (Figure 5.8, Tables 5.19 and 5.20). In the tertiary mixture experiment, experiment 18, two concentrations of single solutions of deltamethrin and cadmium had a more-than-additive effect in the mixture compared to the effect predicted from the single compounds (Figure 5.18, Tables 5.39 and 5.40).

5.5 DISCUSSION

The general consensus among researchers reporting results of empirical testing of chemical mixtures has been that greater than additive toxicity is rather the exception, with most mixture toxicity corresponding to either concentration addition or response addition (Könemann, 1981; Broderius & Kahl, 1985; Deneer *et al.*,1987; Deneer *et al.*, 1988; Broderius *et al.*, 1995). However, most of these studies concentrated on mixtures of large numbers of chemicals, whereas examinations of binary and tertiary combinations of reactive chemicals, the focus of this study, have repeatedly found synergistic effects in a range of species and test conditions. The effect of a mixture of chemicals depends on the individual components, as well as on their relative concentrations, the test conditions, the test organisms, and the toxicity parameters investigated. Therefore, the joint toxicity of chemicals has been found to vary greatly. Though the model of concentration addition appears to be a valid approach for generally estimating the risk of chemical mixtures, many researchers have found

interactive joint toxicity, which deviates from the non-interactive joint action of concentration addition and response addition. Such joint interactive toxicity is not directly predictable from the toxicity of the individual components, as has been stated by Broderius (1990) and by Kraak *et al.* (1994).

The results found in this study do not conform to either of these non-interactive models, but exhibit an interactive joint toxicity, or synergy, between the 3 chemicals investigated. Each of the 3 chemicals acts through a different mode of action. The pyrethroid deltamethrin is a sodium-channel blocker (Mayes & Barron, 1991), whereas cadmium acts by inhibiting calcium transport at the membrane Ca^{2+} ATPase level (IPCS INCHEM, 1992). The toxicity of atrazine to non-photosynthetic organisms is not fully understood, but it has been shown to induce cytochrome P450 and general esterase activity in insects (Kao *et al.*, 1995). Although the different toxic mechanisms of the 3 chemicals would suggest non-interactive, less-than-additive toxicity, they all affect feeding rates in cladocerans at sublethal concentrations. In that way, the combined toxicity of these chemicals to feeding could be expected to act additively. Additionally, reactive chemicals have been shown to interact in different ways, and such biochemical interactions can lead to synergistic effects (Pape-Lindstrom & Lydy, 1997, Forget *et al.*, 1999).

Mixtures of different toxic concentrations of metals have been found to react in a very unpredictable way, with additive, more-than-additive and less-than additive effects reported. Naddy *et al.* (1995), studying effects of binary mixtures of selenium, arsenic and molybdenum on acute survival and reproduction of *C. dubia*, reported additive effects from binary mixtures of selenium and molybdenum or selenium and arsenic, however a binary mixture of molybdenum and arsenic produced less-than-additive effects. They suggested that the toxic effect produced by the metal mixture was

mainly due to the action of the most toxic compound, selenium, and that arsenic and molybdenum acted antagonistically. The dominant effect of the most toxic compound in a metal mixture has also been reported by Eaton (1973), who studied chronic toxicity of a mixture of cadmium, copper and zinc on the fathead minnow *P. promelas*. He found interactive joint toxicity effects between the metals, with zinc dominating in the tertiary mixture as the most effective compound. He also concluded that the effect of the mixture was not predictable in a simply additive manner from that of the individual compounds (Eaton, 1973). In our study, toxicity in the mixtures did not appear to be due to one specific compound, but increased with the addition of increasing amounts of the individual components.

Toxicity of binary mixtures of copper, cadmium and zinc was found to be more-than-additive when using a luminescent bacterial bioassay (Preston *et al.*, 2000), and cadmium and zinc were found to act synergistically on acute survival of white shrimp (Vanegas *et al.*, 1997). A binary mixture of copper and cadmium reportedly exerted toxicity in a simply additive way on acute lethality in the amphipod *Gammarus italicus* (Pantani *et al.*, 1990). When studying metal mixture toxicity on a sensitive sublethal endpoint, filtration rate in mussels, Kraak *et al.* (1994) determined different combination effects, which could not be predicted from the effects of the single compounds. Mixtures of copper and zinc were found to be less-than-additive, zinc and cadmium was additive, and copper and cadmium was more-than-additive, a tertiary mixture was additive again. These partly consistent and partly contradictory results suggest that knowledge of specific targets is necessary to explain the effects observed. Results from studies on daphnids suggest that when a mixture is comprised of a large number of metals, the effect is additive, but for mixtures of 2 or 3 metals, no general trend can be seen (Spehar & Fiandt, 1986; Enserink *et al.*, 1991).

Only few studies have investigated combination effects of metals and organic pesticides, and results vary again depending on the chemicals and different parameters. Pantani *et al.* (1990) found that in acute lethality tests with the amphipod *G. italicus*, binary mixtures of the organophosphorous insecticides methyl parathion and methyl azinphos with copper produced less-than-additive toxicity, whereas binary mixtures with cadmium varied: less-than-additive for cadmium and methyl azinphos, and additive for cadmium and methyl parathion. In a different study using acute lethality tests with a different amphipod, *H. azteca*, Steevens & Benson (2001) reported binary mixtures of methyl mercury and the organochlorine insecticide dieldrin to interact independently, while methyl mercury interacted additively with the organophosphorous insecticide chlorpyrifos. The biochemical modes of action of both pesticides are different from that of the metal, suggesting independent interaction; however, they both show a possibility of joint interaction due to their biochemical mechanisms. The fact that only one combination did indeed show an interactive effect led the authors to the conclusion that, again, the chemical species and animal model as well as exposure conditions are of great importance for the assessment of chemical mixtures, and a more detailed knowledge of toxicological interaction mechanisms is required for the understanding and predicting of the toxicological effects of such mixtures.

A third study, investigating the effects of binary mixtures of the metals arsenic, copper and cadmium with organophosphorous and carbamate insecticides found much greater than additive effects (Forget *et al.*, 1999). Acute lethality tests with the copepod *T. brevicornis* as well as measurements of AChE-inhibition reportedly indicated more-than-additive toxicity of all the binary mixtures, with the most strongly synergistic effects found for combinations of cadmium + dichlorvos and

cadmium + carbofuran. The authors suggested that all 3 insecticides as well as arsenic and copper have been found to inhibit AChE-activity, which could explain an additive toxicity. However, reported toxicity was much higher than additive, and cadmium combinations were especially potent, indicating that some other interaction mechanisms took place.

The results of the cadmium + atrazine and cadmium + deltamethrin experiments correspond with the additive or slightly more-than-additive toxicity effects reported from these other studies. Toxicity of the combinations in our study was found to be additive or slightly more-than-additive in all cases with the exception of experiment 4 (Figure 5.4, Tables 5.11 and 5.12), which shows a much more-than-additive effect of the cadmium + atrazine combination. All other experiments consistently revealed to be more-than-additive. This could be explained by noting that although the compounds have different toxicological modes of action, each is capable of producing a reduction in feeding in both cladoceran species. This feeding inhibition can be due to different underlying toxicological mechanisms: a general reduction in fitness due to the low levels of toxicants present could lead to a reduction in filtration rate, or direct poisoning of the gut function could lead to reduced absorption of food and cells passing through the gut without being digested. This last mechanism has been suggested to explain the potential of different chemicals to inhibit feeding in daphnids at sublethal concentrations (Allen *et al.*, 1995; Taylor *et al.*, 1998). Different studies showed that animals that had been exposed to certain toxic substances showed a reduced feeding rate even after transferral to clean medium, and cadmium as well as pyrethroid insecticides were found to produce this delayed toxic effect on feeding rates of daphnids (McWilliam, 2001). A combination of both physiological effects

induced by the toxicant mixture as well as interactions between the chemicals could explain the slightly more-than-additive effects observed in some of the experiments.

The binary mixtures of deltamethrin and atrazine appear to have a more pronounced joint interactive effect, with most the feeding experiments using *C. dubia* (experiments 7, 8 and 9) as well as, less pronounced, those with *S. vetulus* (14, 15 and 16) and also the binary and tertiary mixture experiments (17 and 18) showing an exponentially rising observed effect in feeding reduction. This suggests that, in addition to the general reduction in fitness, there might be an interaction between the 2 chemicals, leading to increased toxicity when they are present in combination.

There are different theories on the possible mechanisms of chemical synergy of mixtures. These include increase in rate of uptake, formation of toxic metabolites, reduction of excretion, alteration of distribution, and inhibition of detoxification, which appears to be the most popular theory (Marking, 1977).

Toxicity of atrazine, as mentioned earlier, is not well understood for non-photosynthetic organisms, however, several toxic effects of sublethal atrazine concentrations have been observed in invertebrates. Kao *et al.* (1995) found that atrazine induced cytochrome P450 and general non-specific esterase activity in larvae of southern armyworm (*Spodoptera eridania*). This mechanism has been offered to explain synergy of mixtures of atrazine with several organophosphorus insecticides observed in acute toxicity tests with larvae of the chironomid *C. tentans* (Pape-Lindstrom & Lydy, 1997; Forget *et al.*, 1999; Belden & Lydy, 2000; Anderson & Lydy, 2002; Jin-Clark *et al.*, 2002). Thus, Pape-Lindstrom & Lydy theorised that the increased activation of these oxidases and esterases in the presence of atrazine led to increased transformation of phosphorothionates such as methyl-parathion and chlorpyrifos, and phosphorodithionates, such as malathion, to their more toxic oxo-

analogs. In contrast, mevinphos, a member of the phosphate class of organophosphorous insecticides, is deactivated by oxidation, and indeed less-than-additive toxicity was reported from binary mixtures of this compound and atrazine (Pape-Lindstrom & Lydy, 1997). Correspondingly, Kao *et al.* (1995) reported that toxicity of the pyrethroid permethrin, which is detoxified by esterase-catalysed hydrolysis as well as by cytochrome P450, was decreased in the presence of atrazine. This study, however, focused on the analysis of enzyme and detoxification products from the animal's midgut tissue. It also determined a reduced toxicity of parathion and carbaryl in combinations with atrazine. In this study, the presence of atrazine in a solution containing the pyrethroid deltamethrin led to an increase in toxicity, not a decrease. This could be due the fact that the specific cytochrome P450 isozymes induced by atrazine did not detoxify deltamethrin in the same way as was found for permethrin, or that they acted differently in the cladoceran than in the insect. Another possibility is that the specific effect of the chemicals on gut poisoning, which caused the reduction in feeding, was produced so rapidly that an increased internal detoxification did not reduce the toxic overall effect. Atrazine has also been found to increase respiration rates in chironomid larvae (Lydy *et al.*, 2000). Sublethal stress can cause increased activity and increased respiration, resulting in increased uptake and toxicity. The uptake of hydrophobic chemicals has been shown to be limited by the flow of water over breathing surfaces in fish (Erickson & McKim, 1990). Therefore, increased water movement and respiration could lead to an increased uptake of a hydrophobic compound such as deltamethrin.

In any case, mixture toxicity with atrazine and pyrethroids has been found, just like with metals or metals and insecticides, to produce wildly differing effects depending on concentrations, test species and parameters. Stratton (1983) reports toxicity of

combinations of atrazine and permethrin to a range of microorganisms to vary with species. Effects of the mixture on the photosynthesis and nitrogenase activity of the cyanobacterium *A. inequalis* were found to be additive, whereas the same mixture acted synergistically on growth of the fungus *Pythium ultimum*, and antagonistically on growth of the fungus *Trichoderma viride*. Tripathi & Agarwal (1997) reported that mixtures of deltamethrin and carbaryl or dichlorvos led to a much greater than additive toxicity in acute lethality studies with the snail *Lymnaea acuminata*. They suggested that both the carbamate and the organophosphate reduced rates of detoxification of the pyrethroid by inhibiting esterase activity.

The exact mechanism by which atrazine and deltamethrin interacted to produce the more-than-additive toxic effect on feeding is not known. More research on biochemical interactions of these chemicals would be required to better understand those complex interactions. However, from a risk assessment point of view exact biochemical modes of action are less interesting than effects on ecologically important parameters such as feeding at environmentally realistic concentrations. The fact that such chemically diverse compounds as were investigated in this study show at least additive or more-than-additive toxic effects on feeding rates in cladocerans has implications for risk assessment, predictions and control of toxic substances in aquatic systems, as agrochemicals and metals commonly appear in more or less complex mixtures.

Results from this study show that the additivity potential of combinations of chemicals does not necessarily decrease when using a more sensitive parameter, such as feeding, compared to survival, opposite to results reported from studies by Hermens *et al.* (1984) and Deneer *et al.* (1988), who investigated effects of complex mixtures. Studies on binary and tertiary mixtures by Kraak *et al.* (1994) and Forget *et*

al. (1999) corresponded with this study in showing additivity or more-than-additivity to sublethal parameters like filtration rate or AChE-activity.

Our results also support the important findings that chemicals can add to the overall increased toxicity of a mixture at concentrations at or below their individual NOEC levels. In some of our experiments, individual solutions that did not have an effect on feeding when tested singly produced an effect in combination with another chemical. These results are supported by Spehar & Fiandt (1986) and by Kraak *et al.* (1994) working with metals, and by Könemann (1981), Hermens & Leeuwangh (1982) and Deneer *et al.* (1988) working with organic chemicals. This is a strong argument in favour of mixture toxicity experiments, as it indicates that NOEC levels can be underprotective when predicting the toxicity of chemical mixtures.

Generally laboratory-based studies such as those reported here should be complemented with in situ studies, to guarantee predictive value and environmental applicability of results. In natural systems, complex interactions between chemical and biological factors often lead to very different effects. For example, a study on effects of mixtures of atrazine and the pyrethroid insecticide bifenthrin on a pond mesocosm found certain interactive effects of the 2 chemicals on zooplankton numbers, but mostly effects of atrazine were masked by the much more pronounced effects of bifenthrin (Hoagland & Drenner, 1993). More studies on interactive effects of different environmental pollutants are needed to improve our understanding of the environmental risks of these increasingly common mixtures, and field, mesocosm, and in situ studies must be a part of a critical and realistic assessment and comprehension.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

The findings of this study are divided into different areas of discussion which are summarised below:

- Different cladoceran species within the same genus occupying similar habitats can exhibit differences in acute and chronic sensitivity to chemicals irrespective of their size
- Acute toxicity (LC_{50} values) of chemicals is often affected by the presence of food (algae)
- Feeding inhibition is a suitable endpoint for assessing effects of low, sublethal, environmentally relevant concentrations of some chemicals, but not all
- Contaminant-induced feeding inhibition in daphnids follows a non-linear relationship which can be described by an allosteric decay curve, the shape of which varies depending on the compound and the test organisms
- Binary and tertiary mixtures of the three chemicals investigated in this study produced greater-than-additive effects, the extent of which varied depending on the compounds in the mixture and the different test species
- The sublethal endpoint of contaminant-induced feeding inhibition was sensitive to those interactive mixture effects
- Chemicals below their individual NOECs contributed to the overall effects of the mixtures

Ecotoxicological tests and bioassays have mainly been developed for temperate systems, using species typical of temperate conditions. Standard toxicity test organisms have been established and are employed for toxicity testing world-wide, irrespective of whether they are ecologically representative of the systems investigated. The Holarctic cladoceran species *Daphnia magna* is ubiquitously used for assessing the effects of aqueous toxicants. However, *D. magna* is a species typical of temperate conditions and small ephemeral ponds. As part of the European Community TROCA project investigating effects of pesticides on tropical wetlands, acute and sublethal sensitivity of three different cladoceran species to three pesticides and one metal were investigated. *Ceriodaphnia dubia*, a standard toxicity species typical of subtropical conditions, and *Ceriodaphnia cornuta* and *Simocephalus vetulus*, typical of tropical systems, all of them occurring in permanent lakes and streams, were found to be more sensitive to a range of chemicals than values reported for *D. magna*. Differences in sensitivity were also found between the 3 species, with the largest species, *S. vetulus*, exhibiting significantly higher sensitivity to the herbicide atrazine than the other 2 species, whereas sensitivity to the pyrethroid deltamethrin was highest for *C. cornuta*. These findings indicate that although for general risk assessment purposes, the selection of a representative species to test effects of toxic substances might be acceptable and certainly more practical, differences in sensitivities between species, even those closely related, have to be considered when determining potential impact effects, and locally representative species are preferable for assessing effects of pollutants on sensitive ecosystems.

The role of food in modifying toxicity to aquatic organisms especially important for laboratory-based toxicity testing. Results from this study found acute toxicity of the chemicals investigated to be reduced when food was present as opposed to when food

was absent. This supports the argument that for comparability reasons, absence of food in acute toxicity tests is essential. However, food-poor environments are not fully representative of natural systems (Rand, 1995). The reasons for this effect have been discussed and investigated, and although some uptake and binding of toxic molecules to the organic material occurs, the most important reasons seem to be an increase in general health and fitness of test animals when food is present, leading to higher resistance to toxicants (Taylor, 2001). This leads back to the classic dilemma facing those using ecotoxicological test data: to understand complex natural relationships, simplified tests and models have to be developed, in which all parameters can be controlled. This simplification of test conditions however can result in oversimplified systems that are no longer representative of natural conditions. Research into all areas of ecology, physiology, chemistry, toxicology and others must continue to improve our understanding of these relationships, and laboratory tests should be increasingly complemented by mesocosm and *in situ* studies, an ideal situation, which of course is usually limited by availability of funds.

The use of bioassays involving contaminant-induced feeding inhibition as a parameter for assessing toxicity offers a sensitive and ecologically relevant tool for assessing effects of low concentrations of toxic substances, singly and in combinations. Results from this study found feeding inhibition to occur at much lower exposure levels than those that produced lethality. Several different compounds were found to affect feeding at sublethal concentrations, including cadmium, a pyrethroid, and a herbicide. However, the organophosphate did not affect feeding at sublethal levels. These findings correspond with results reported from other studies (McWilliam & Baird, 2002), suggesting that for *in situ* tests feeding inhibition and lethality should be assessed in combination.

Determination of contaminant-induced feeding inhibition also proved to be a consistent, repeatable, fast and practical tool, and the bioassays with *C. dubia* and *S. vetulus* are useful for assessing impacts of toxic substances on subtropical and tropical systems. *S. vetulus* is a potential candidate for carrying out *in situ* tests, and an *in situ* chamber has already been developed and tested in the laboratory, however, it could not be tested in a tropical test site due to time constraints. As basic life-cycle parameters and acute and sublethal sensitivities to a range of chemicals of this organism are known, it would be relatively easy to employ this species in an *in situ* bioassay similar to the *D. magna in situ* bioassay (McWilliam, 2001), which would be directly applicable to tropical systems.

The basic life-cycle parameters such as age at first reproduction, brood size, and interval between broods were determined for *C. dubia* and the 2 tropical cladocerans in this study. This data in combination with the data on relative inhibition of feeding rates at different chemical concentrations can also be used for assessing population dynamics of these organisms in a Dynamic Mass Budget Model (Kooijman, 1993; Nogueira, 1996). These models calculate rates of growth and fecundity for a given organism, considering reduced feeding due to exposure to toxic substances. Using these models, one can make predictions about how certain sublethal chemical concentrations might affect population dynamics of the cladocerans by inhibiting feeding and thus affecting population growth. It is possible to estimate the concentration of a specific chemical at which feeding would be reduced to a level which is insufficient to allow the animal to survive and reproduce, even if this concentration does not produce a marked acute or chronic lethality in those organisms.

The contaminant-induced feeding inhibition curves for *C. dubia* and *S.vetulus* varied depending on the species and the compound investigated. Feeding inhibition was fitted to a non-linear allosteric decay model, which best described the relationship between exposure concentration and feeding rate. The shape of this curve varied greatly, and the steepness of the curve had implications for the predicted effects. Since the binary and tertiary mixtures were prepared with a fraction of the solutions, which were individually assessed for their effects (50% for binary mixtures, 33% for tertiary mixtures), the predicted effect was calculated to correspond to the one expected from the curve. Therefore, the predicted effects were much less than half of the effects of individual chemical solutions if the curve was steep, as was the case for deltamethrin and atrazine and *C. dubia*, and more than half if the curve was flatter, as was the case for cadmium. The determination of feeding inhibition curves is therefore of great importance when trying to assess the effects of mixtures as additive, more-than-additive, or less-than-additive. The individual amounts of the different chemicals present in the mixture are also of great importance for the effect of the overall mixture. It would be interesting to study feeding inhibition curves with a number of additional compounds from different chemical groups to see whether similarities between feeding inhibition curves could be used as a predictive tool for toxicity of binary and tertiary mixtures and even mixtures containing more than 3 compounds. Ideas for further work could include the generation of such a database with a range of compounds from different groups. The *C. dubia* feeding bioasssay is a quick assessment tool, and the current refinement of the most time-consuming step, the counting of the algae, with the help of a fluorescent spectrophotometer, will further decrease the time necessary for completing one experiment. At the same time, it is a relatively complex miniature ecosystem, and the parameter of inhibition of feeding is

of real ecological importance. The sensitivity of this bioassay to mixtures of reactive chemicals, which has been demonstrated in this study, makes it a valuable tool for increasing our understanding of how such sublethal concentrations of chemicals could affect the function and dynamics of individuals, populations and communities.

The binary and tertiary mixture investigated in this study all produced more-than-additive effects, especially the mixtures containing the organic pesticides. The additive effect between the different chemicals could be explained by the fact that, although all 3 compounds have different biochemical modes of action, they all produce an inhibitory effect on feeding rates. This effect could be due to either a reduction in general fitness of the organisms, or a direct poisoning of the gut, leading to a reduced uptake of algal cells. Most probably, it is a combination of both effects. The pronounced greater-than-additive toxic effect of deltamethrin and atrazine suggests a chemical interaction between the 2 compounds, increasing either uptake or decreasing detoxification. For further work, an investigation into binary mixtures of other, different compounds could lead to a better understanding of such mixture effects, and studies on internal biochemical effects with regard to detoxification products would help to understand the biochemical mechanisms leading to such synergistic or antagonistic interactions between chemicals.

However, for the assessment of interactive effects of mixtures of environmental pollutants, more studies with sensitive species and parameters need to be performed. Models developed, such as the concentration addition and independent action model, appear to be of predictive value for some mixtures, especially large mixtures of non-reactive chemicals, but not for others, like reactive chemicals with specific modes of action. These models have often been developed using relatively simple bacterial or algal growth bioassays, which measure an all-or-nothing effect, but complex whole

organisms, such as crustaceans and other animals, react less predictably, and effects can be due to numerous interactions. The model of concentration addition might be a good general model, offering a close enough approximation for many situations of mixture toxicity, but enough exceptions to simple additivity have been reported to keep in mind the fact that CA is only a model, which does not hold true for every case. Much more work is needed by ecotoxicologists, as well as medical researchers, to be able to understand the complex interactions of chemicals on a molecular, biochemical, physiological, as well as ecological level.

It is also important to note that the *C. dubia* feeding inhibition bioassay also detected a contribution to overall toxicity from individual chemicals below their NOEC. The possibility of such low concentrations, which do not seem to have an effect, to contribute to the toxicity of a mixture, is an area of discussion amongst researchers studying and modelling chemical mixtures. Many models, such as the independent action model, do not include this possibility, although from a logical point of view, it is not surprising that such low concentrations might accumulate to produce an overall effect. The fact that those low concentrations do sometimes lead to accumulating mixture effects has been shown in this study as well as in a number of other studies using higher test organisms (e.g. Spehar & Fiandt, 1986; Deneer *et al.*, 1988; Kraak *et al.*, 1994). It supports the need for recognition of interaction between the multiple chemicals released into aquatic systems and a change in laws and regulations controlling discharges of chemicals. The precise ways in which the multitudes of anthropogenic chemicals interact in affecting aquatic organisms, ecosystems, and ourselves, will have to be the focus of much more research in years to come.

REFERENCES

Abdullah, A.R., Bajet, C.M., Matin, M.A., Nhan, D.D. and Sulaiman, A.H. (1997). Ecotoxicology of pesticides in the tropical paddy field ecosystem. *Environmental Toxicology and Chemistry* **16** (1), 59-70.

Alabaster, J.S. & Lloyd, R. (1980). Water Quality Criteria for Freshwater Fish, London: Butterworths for the Food and Agricultural Organisation.

Allen, Y., Calow, P. & Baird.D.J. (1995). A mechanistic model of contaminant-induced feeding inhibition in *Daphnia magna. Environmental Toxicology and Chemistry* **14** (9), 1625-1630.

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M. & Grimme, L.H. (2000). Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: Mixtures composed of similarly acting chemicals. *Environmental Toxicology and Chemistry* **19** (9), 2341-2347.

Altenburger, R., Boedeker, W., Faust, M. & Grimme, L.H. (1996). Regulations for combined effects of pollutants: consequences from risk assessment in aquatic toxicology. *Food and Chemical Toxicology* **34** 1155-1157.

American Society for Testing Materials (ASTM) (1980) *Standard practices for conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians E729 - 80*, pp. 272-296. Philadelphia, PA, USA: ASTM

Anderson, B.C. (1944). Toxicity thresholds of various substances found in industrial wastes as determined by the use of *Daphnia magna. Sewage Works Journal* **16** 1156-1165.

Anderson, B.C. (1980) Aquatic invertebrates in tolerance investigations from Aristotle to Naumann. Philadelphia: American Society for Testing and Materials, pp. 3-35.

Anderson, P.D. & Weber, L.J. (1975). The toxicity to aquatic populations of mixtures containing certain heavy metals. *Proceedings of the International Conference on Heavy Metals in the Environment, Toronto, 27-31 October 1975*, pp. 933-954.

Anderson, T.D. and Lydy, M.J. (2002). Increased toxicity to invertebrates associated with a mixture of atrazine and organophosphate insecticides. *Environmental Toxicology and Chemistry* **21** (7), 1507-1514.

APHA-AWWA-WEF (1995). Standard methods for the examination of water and wastewater (18th ed.). American Public Health Association/ Water Environment Federation. Washington D.C.

Armbrust, K.L. (1999). Photochemical processes influencing pesticide degradation in rice paddies. *Journal of Pesticide Science* **24** (1), 69-73.

Backhaus, T., Altenburger, R., Boedeker, W., Faust, M., Scholze, M. & Grimme, L.H. (2000). Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to *Vibrio fischeri. Environmental Toxicology and Chemistry* **19** (9), 2348-2356.

Bailey, H.C., DiGiorgio, C., Kroll, K., Miller, J.L., Hinton, D.E. and Starretts, G. (1996). Development of procedures for identifying pesticide toxicity in ambient waters: carbofuran, diazinon, chlorpyrifos. *Environmental Toxicology and Chemistry* **15** (6), 837-845.

Bailey, H.C., Elphick, J.R., Krassoi, R. & Lovell, A. (2001). Joint acute toxicity of diazinon and ammonia to *Ceriodaphnia dubia. Environmental Toxicology and Chemistry* **20** (12), 2877-2882.

Bailey, H.C., Krassoi, R., Elphick, J.R., Mulhall, A.-M., Hunt, P., Tedmanson, L. & Lovell, A. (2000). Application of *Ceriodaphnia dubia* for whole effluent toxicity tests in the Hawkesbury- Nepean watershed, New South Wales, Australia: Method development and validation. *Environmental Toxicology and Chemistry* **19** (1), 88-93.

Bailey, H.C., Miller, J.L., Miller, M.J., Wiborg, L.C., Deanovic, L. and Shed, T. (1997). Joint acute toxicity of diazinon and chlorpyrifos to *Ceriodaphnia dubia. Environmental Toxicology and Chemistry* **16** (11), 2304-2308.

Baird, D.J. (1992). Predicting population response to pollutants: in praise of clones. A comment on Forbes and Depledge. *Functional Ecology* **6** 616-617.

Baird, D.J. (1993). Can toxicity testing contribute towards ecotoxicology? *Functional Ecology* **7** 510-511.

Baird, D.J., Barber, I., Bradley, M.C., Calow, P. & Soares, A.M.V.M. (1989b). The *Daphnia* bioassay: a critique. *Hydrobiologia* **188/189** 403-406.

Baird, D.J., Barber, I., Bradley, M.C., Soares, A.M.V.M. & Calow, P. (1991). A comparative study of genotype sensitivity to acute toxic stress using clones of *Daphnia magna. Ecotoxicology and Environmental Safety* **21** 257-265.

Baird, D.J., Barber, I. & Calow, P. (1990). Clonal variation in general responses of *Daphnia magna* Straus to toxic stress. I: chronic life history effects. *Functional Ecology* **4** (399-407).

Baird, D.J., Soares, A.M.V.M., Girling, A., Barber, I., Bradley, M.C. and Calow, P. (1989a) The long term maintenance of *Daphnia magna* Straus for use in ecotoxicity tests: problems and prospects. In: *Proceedings of the First European Conference of Ecotoxicology*, pp. 144-148. Lyngby, Denmark.

Barata, C. & Baird, D.J. (1998). Phenotypic plasticity and constancy of life-history traits in laboratory clones of *Daphnia magna* Straus: effects of neonatal length. *Functional Ecology* **12** 412-419.

Barata, C. & Baird, D.J. (2000). Determining the ecotoxicological mode of action of chemicals from measurements made on individuals: results from instar-based tests with *Daphnia magna* Straus. *Aquatic Toxicology* **48** 195-209.

Barata, C., Baird, D.J. & Markich, S.J. (1999). Comparing metal toxicity among *Daphnia magna* clones: an approach using concentration-time-response surfaces. *Archives of Environmental Contamination and Toxicology* **37** 326-331.

Barata, C., Baird, D.J., Minarro, A. & Soares, A.M.V.M. (2000). Do genotype responses always converge from lethal to non-lethal toxicant exposure levels? Hypothesis testing using clones of *Daphnia magna* Straus. *Environmental Toxicology and Chemistry* **9** 2314-2322.

Barber, I., Baird, D.J. & Calow, P. (1994). Effect of cadmium and ration level on oxygen-consumption, RNA concentration and RNA-DNA ratio in 2 clones of *Daphnia magna* Straus. *Aquatic Toxicology* **30** (3), 249-258.

Barker, D.M. & Hebert, P.D.N. (1986). Secondary sex ratios of the cyclic parthenogen *Daphnia magna* (Crustacea: Cladocera) in the Canadian arctic. *Canadian Journal of Zoology* **64** 1137-1143.

Barry, M.J. (1996). Effects of an organochlorine pesticide on different levels of biological organization in *Daphnia*. *Ecotoxicology and Environmental Safety* **34** (3), 239-251.

Barry, M.J., Logan, D.C., Ahokas, J.T. & Holdway, D.A. (1995). Effect of algal food concentration on toxicity of two agricultural pesticides to *Daphnia carinata*. *Ecotoxicology and Environmental Safety* **32** (3), 273-279.

Beisinger, K.E. & Christensen, G.M. (1972). Effects of various metals on survival, growth, reproduction and metabolism of *Daphnia magna. Journal of the Fisheries Research Board of Canada.* **29** 1691-1700.

Belanger, S.E., Farris, J.L. & Cherry, D.S. (1989). Effects of diet, water hardness, and population source on acute and chronic copper toxicity to *Ceriodaphnia dubia. Archives of Environmental Contamination and Toxicology* **18** 601-611.

Belden, J.B. & Lydy, M.J. (2000). Impact of atrazine on organophosphate insecticide toxicity. *Environmental Toxicology and Chemistry* **19** (9), 2266-2274.

Berenbaum, M.C. (1985). The expected effect of a combination of agents: the general solution. *Journal of Theoretical Biology* **114** 413-431.

Berner, D.B. (1986). Taxonomy of *Ceriodaphnia* (Crustacea: Cladocera) in U.S. Environmental Protection Agency cultures. EPA/640/4-86/032. Cincinnati: OH. Environmental Protection Agency.

Bitton, G., Rhodes, K. and Koopman, B. (1996). Ceriofast: an acute toxicity test based on *Ceriodaphnia dubia* feeding behavior. *Environmental Toxicology and Chemistry* **15** (2), 123-125.

Bitton, G., Rhodes, K., Koopman, B. & Cornejo, M. (1995). Short-term toxicity assay based on daphnid feeding behavior. *Water Environment Research* **67** (3), 290-293.

Bliss, C.I. (1939). The toxicity of poisons applied jointly. *Annual Journal of Applied Biology* **26** 585-615.

Bocquené, G., Bellanger, C., Cadiou, Y. & Galgani, F. (1995). Joint action of combinations of pollutants on the acetylcholinesterase activity of several marine species. *Ecotoxicology* **4** 266-279.

Bradley, M.C., Naylor, C., Calow, P., Baird, D.J., Barber, I. and Soares, A.M.V.M. (1993) Reducing variability in *Daphnia* toxicity tests- A case for further standardisation. In: *Progress in the Standardisation of Aquatic Bioassays.* Soares A.M.V.M & Calow P. (eds.) Michigan: Lewis Publishers, pp. 57-70.

Brecken-Folse, J.A., Mayer, F.L., Pedigo, L.E. & Marking, L.L. (1994). Acute toxicity of 4-nitrophenol, 2,4-dinitrophenol, terbufos and trichlorfen to grass shrimp (*Palaemonetes* spp.) and sheepshead minnows (*Cyprinodon variegatus*) as affected by salinity and temperature.

Environmental Science and Technology **13** 67-77.

Broderius, S.J. (1990). Modeling the joint toxicity of xenobiotics to aquatic organism: Basic concepts and approaches. In: *Aquatic Toxicology and Risk Assessment (14th Vol)*, ASTM STP 1124, Mayes M.A. and M.G. Barron (eds.), Philadelphia, PA: American Society for Testing and Materials.

Broderius, S.J. & Kahl, M. (1985). Acute toxicity of organic chemical mixtures to the fathead minnow. *Aquatic Toxicology* **6** 307-322.

Broderius, S.J., Kahl, M.D. & Hoglund, M.D. (1995). Use of joint toxic response to define the primary mode of toxic action for diverse industrial organic chemicals. *Environmental Toxicology and Chemistry* **9** 1591-1605.

Broderius, S.J. & Smith, L.L. (1979). Lethal and sublethal effects of binary mixtures of cyanide and hexavalent chromium, zinc, or ammonia to the fathead minnow (*Pimephales promelas*) and rainbow trout (*Salmo gairdneri*). *Journal of the Fisheries Research Board Canada* **36** 164-172.

Bucksteeg, W., Thiele, H. & Stoltzel, K. (1955). Die Beeinflussung von Fischen durch Giftstoffe aus Abwässern. *Vom Wasser* **22** 194-211.

Buikema, A.L.Jr. & Cairns, J.Jr. (1980) (eds.) *Aquatic Invertebrate Bioassays*. ASTM STP 715. Philadelphia: American Society for the Testing of Materials.

Buhl, K.J., Hamilton, S.J., Schmulbach, J.C. (1993). Acute toxicity of the herbicide bromoxynil to *Daphnia magna*. *Environmental Toxicology and Chemistry* **12** 1455-1468.

Cairns, J., Heath, A.G. & Parker, B.C. (1975). The effects of temperature upon the toxicity of chemicals to aquatic organisms. *Hydrobiologia* **47** 135-160.

Calabrese E.J. (1991). *Multiple Chemical Interactions*, MI: Lewis.

Calamari, D. and Marchetti, R. (1973). The toxicity of mixtures of metals and surfactants ot rainbow trout (*Salmo gairdneri* Rich.). *Water Research* **7** 1453-1464.

Calamari D. & Vighi M. (1992) A proposal to define quality objectives for aquatic life for mixtures of chemical substances. *Chemosphere* ***25*** (4), 531-542.

Calow, P. (1992). The 3 R's of ecotoxicology. *Functional Ecology* **6** 617-619.

Canton, J.H. & Sloof, W. (1982). Toxicity and accumulation studies of cadmium (Cd^{2+}) with freshwater organisms of different trophic levels. *Ecotoxicology and Environmental Safety* **6** 113-128.

Carpy, S.A., Kobel, W. and Doe, J. (2000). Health risk of low-dose pesticides mixtures: a review of the 1985-1998 literature on combination toxicology and health risk assessment. *Journal of Toxicology and Environmental Health* **B3** 1-25.

Castillo, L.E. (2000) Pesticide impact of intensive banana production on aquatic ecosystems in Costa Rica. PhD Thesis, University of Stockholm, Sweden .

Castillo, L.E., De La Cruz, E. and Ruepert, C. (1997). Ecotoxicology and pesticides in tropical aquatic ecosystems of Central America. *Environmental Toxicology and Chemistry* **16** (1), 41-51.

Chow-Fraser, P. & Sprules, W.G. (1992). Type-3 functional response in limnetic suspension feeders as demonstrated by *in situ* grazing rates. *Hydrobiologia* **232** 175-191.

Christensen, E.R., Chen, D., Nyholm, N. & Kusk, K.O. (2001). Joint action of chemicals in algal toxicity tests: influence of response level and dose-response regression model. *Environmental Toxicology and Chemistry* **20** (10), 2361-2369.

Cooney, J.D., DeGraeve, G.M., Moore, E.L., Lenoble, B.J., Pollock, T.L. & Smith, G.J. (1992a). Effects of environmental and experimental design factors on culturing and toxicity testing of *Ceridaphnia dubia. Environmental Toxicology and Chemistry* **11** 839-850.

Cooney, J.D., DeGraeve, G.M., Moore, E.L., Palmer, W.D. & Pollock, T.L. (1992b). Effects of food and water quality on culturing of *Ceriodaphnia dubia. Environmental Toxicology and Chemistry* **11** 823-837.

Cowgill, U.M. (1987). Critical analysis of factors affecting the sensitivity of zooplankton and the reproducibility of toxicity results. *Water Research* **21** (12), 1453-1462.

Cowgill, U.M., Keating, K.I. and Takahashi, I.T. (1985). Fecundity and longevity of *Ceriodaphnia dubia/affinis* in relation to diet at two different temperatures. *Journal of Crustacean Biology* **5** (3), 420-429.

Cowgill, U.M. and Milazzo, D.P. (1991). The sensitivity of *Ceriodaphnia dubia* and *Daphnia magna* to seven chemicals utilizing the three-brood test. *Archives of Environmental Contamination and Toxicology* **20** 211-217.

Crane, M. (1995). Effect of zinc on four populations and two generations of *Gammarus pulex* (L.). *Freshwater Biology* **33** 119-126.

Cox, E.J., Naylor, C. & Calow, P. (1992). Frozen algae as food for *Daphnia magna* Straus in toxicity testing. *Ecotoxicology and Environmental Safety* **24** 58-62.

Davies, P.E., Cook, L.S.J. and Goenarso, D. (1994). Sublethal responses to pesticides of several species of Australian freshwater fish and crustaceans and rainbow trout. *Environmental Toxicology and Chemistry* **13** (8), 1341-1354.

Dawson, D.A. & Wilke, T.S. (1991). Evaluation of the frog embryo teratogenesis assay: *Xenopus* (Fetax) as a model system for mixture toxicity hazard assessment. *Environmental Toxicology and Chemistry* **10** 941-948.

Day, K. & Kaushik, N.K. (1987). Short-term exposure effects of zooplankton to the synthetic pyrethroid, fenvalerate, and its effects on rates of filtration and assimilation of the alga, *Chlamydomonas reinhardii*. *Archives of Environmental Contamination and Toxicology* **16** 423-432.

Day, K.E., Kaushik, N.K. & Solomon, K.R. (1987). Impact of fenvalerate on enclosed fresh-water planktonic communities and on *in situ* rates of filtration of zooplankton. *Canadian Journal of Fisheries and Aquatic Sciences* **44** (10), 1714-1728.

Day, K.E. and Maguire, R.J. (1990). Acute toxicity of isomers of the pyrethroid insecticide deltamethrin and its major degradation products to *Daphnia magna*. *Environmental Toxicology and Chemistry* **9** 1297-1300.

De Infante, A. (1985). A note about *Daphnia* in Venezuelan waterbodies. *Hydrobiologia* **119** 81-82.

De March, B.G.E. (1987a). Mixture toxicity indices in acute lethal toxicity tests. *Archives of Environmental Contamination and Toxicology* **16** 33-37.

De March, B.G.E. (1987b). Simple similar action and independent joint action- two similar models for the joint effects of toxicants applied as mixtures. *Aquatic Toxicology* **9** 291-304.

DeGraeve, G.M. & Cooney, J.D. (1987). *Ceriodaphnia*: an update on effluent toxicity testing and research needs. *Environmental Toxicology and Chemistry* **6** 331-333.

DeGraeve, G.M., Cooney, J.D., Marsh, B.H., Pollock, T.L. and Reichenbach, N.G. (1992). Variability in the performance of the 7-d *Ceriodaphnia dubia* survival and reproduction test: an intra- and interlaboratory study. *Environmental Toxicology and Chemistry* **11** 851-866.

DeMott, W.R. (1982). Feeding selectivities and relative ingestion rate of *Daphnia* and *Bosmina*. *Limnology and Oceanography* **27** 518-527.

Deneer, J.W. (2000). Toxicity of mixtures of pesticides in aquatic systems. *Pest Management Science* **56** 516-520.

Deneer, J.W., Seinen, W. & Hermens, J.L.M. (1988a). Growth of *Daphnia magna* exposed to mixtures of chemicals with diverse modes of action. *Ecotoxicology and Environmental Safety* **15** 72-77.

Deneer, J.W., Sinnige, T.L., Seinen, W. & Hermens, J.L.M. (1987). Quantitative structure-activity relationships for the toxicity and bioconcentration factor of nitrobenzene derivatives towards the guppy, *Poecilia reticulata*. *Aquatic Toxicology* **10** 115-129.

Deneer, J.W., Sinnige, T.L., Seinen, W. & Hermens, J.L.M. (1988b). The joint acute toxicity to *Daphnia magna* of industrial organic chemicals at low concentrations. *Aquatic Toxicology* **12** 33-38.

Dodson, S.I., Merritt, C.M., Shannahan, J.-P. & Shults, C.M. (1999). Low exposure concentrations of atrazine increase male production in *Daphnia pulicaria*. *Environmental Toxicology and Chemistry* **18** (7), 1568-1573.

Donkin, P. (1997). Effect of neurotoxic pesticides on the feeding rate of marine mussels (*Mytilus edulis*). *Pesticide Science* **49** 196-209.

Douglas, W.S., McIntosh, A. & Clausen, J.C. (1993). Toxicity of sediments containing atrazine and carbofuran to larvae of the midge *Chironomus tentans*. *Environmental Toxicology and Chemistry* **12** 847-853.

Dumont, H.J. (1980). Zooplankton and the science of biogeography: the example of Africa. In: W.C. Kerfoot (ed.), *Evolution and Ecology of Zooplankton Communities*, Univ. Press of New England, Hanover, pp. 685-696.

Dumont, H.J. (1994). On the diversity of the Cladocera in the tropics. *Hydrobiologia* **272** 27-38.

Durairaj, S. & Selvarajan, V.R. (1995). Synergistic action of organophosphate pesticides on fish, *Oreochromis mossambicus. Journal of Environmental Biology* **16** 51-53.

Eaton, J.G. (1973). Chronic toxicity of a copper, cadmium and zinc mixture to the fathead minnow (*Pimephales promelas* Rafinesque). *Water Research* **7** 1723-1736.

EIFAC (1987). European Inland Fisheries Advisory Commission: Working party on Water Quality Criteria for European Freshwater Fish, Water quality criteria for European freshwater fish. Revised report on combined effects on freshwater fish and other aquatic life of mixtures of toxicants in water, 75 pp.: EIFAC Technical Paper (37) Rev.1.

Elnabarawy, M.T., Welter, A.N. & Robideau, R.R. (1986). Relative sensitivity of three daphnia species to selected organic and inorganic chemicals. *Environmental Toxicology and Chemistry* **5** 393-398.

Enserink, E.L., Maas-Diepeveen, J.L. & Van Leeuwen, C.J. (1991). Combined toxicity of metals; an ecotoxicological evaluation. *Water Research* **25** 679-687.

Enserink, L. (1995) Food mediated life-history strategies in *Daphnia magna* Straus: their relevance to ecotoxicological evaluations. PhD Thesis, Landbouw Universiteit, NL.

Environment Canada (1992). Biological test method: Test of reproduction and survival using the cladoceran *Ceriodaphnia dubia.* Environmental Protection Series. EPS 1/RM/21. Ottawa, ON.

Erickson, R.J. & McKim, J.M. (1990). A simple flow-limited model for exchange of organic chemicals at fish gills. *Environmental Toxicology and Chemistry* **9** 159-165.

Ernst, W.R., Jonah, P., Doe, K., Julien, G. and Hennigar, P. (1991). Toxicity to aquatic organisms of off-target deposition of endosulfan applied by aircraft. *Environmental Toxicology and Chemistry* **10** (103-114).

Fairchild, J.F., La Point, T.W. & Schwartz, T.R. (1994). Effects of an herbicide and insecticide mixture in aquatic mesoscosms. *Archives of Environmental Contamination and Toxicology* **27** 527-533.

Farnworth, E.G. & Golley, B.G. (1974). *Fragile ecosystems- Evaluation of research and applications in the neotropics*, New York: Springer.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vighi, M. and Grimme, L.H. (2001). Predicting the joint algal toxicity of multi-component *s* -triazine mixtures at low-effect concentrations of individual toxicants. *Aquatic Toxicology* **56** 13-32.

Fernandez-Casalderrey, A., Ferrando, M.D. & Andreu-Moliner, E. (1992). Filtration and ingestion rates of *Brachionus calyciflorus* after exposure to endosulfan and diazinon. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **103** (2), 357-361.

Fernandez-Casalderrey, A., Ferrando, M.D. & Andreu-Moliner, E. (1993). Effects of endosulfan on survival, growth and reproduction of *Daphnia magna. Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **106** (2), 437-441.

Fernandez-Casalderrey, A., Ferrando, M.D. & Andreu-Moliner, E. (1994). Effects of sublethal concentrations of pesticides on the feeding behavior of *Daphnia magna. Ecotoxicology and Environmental Safety* **27** (1), 82-89.

Fernando, C.H. (1980a). The freshwater zooplankton of Sri Lanka, with a discussion of tropical freshwater zooplankton composition. *International Revue des. Hydrobiol.* **65** (1), 85-125.

Fernando, C.H. (1980b). The species and size composition of tropical freshwater zooplankton with special reference to the Oriental Region (South East Asia). *Int. Rev. ges. Hydrobiol.* **65** 411-426.

Fernando, C.H. (1994). Zooplankton, fish and fisheries in tropical freshwaters. *Hydrobiologia* **272** 105-123.

Fernando, C.H., Paggi, J.C. & Rajapaksa, R. (1987). *Daphnia* in tropical lowlands. In: R.H.Peters & R. de Bernardi (eds.), *Daphnia. Mem. Ist. ital. Idrobiol.* **45** 107-141.

Ferrando, M.D. & Andreu, E. (1993). Feeding behavior as an index of copper stress in *Daphnia magna* and *Brachionus calyciflorus. Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **106** (2), 327-331.

Finney, D.J. (1971). *Probit Analysis: third edition. Cambridge University Press*

Fisken, Ø. (1997). Allocation patterns and diel vertical migration: modelling the optimal *Daphnia*. *Ecology* **78** 1446-1456.

Forbes, V.E. & Depledge, M.H. (1993). Testing vs. research in ecotoxicology: a response to Baird and Calow. *Functional Ecology* **7** 509-510.

Forbes, V.E. & Forbes, T.L. (1993). Ecotoxicology and the power of clones. *Functional Ecology* **7** (511-512).

Forget, J., Pavillon, J.F., Beliaeff, B. and Bocquene, G. (1999). Joint action of pollutant combinations (pesticides and metals) on survival (LC50 values) and acetylcholinesterase activity of *Tigriopus brevicornis* (Copepoda, Harpactoida). *Environmental Toxicology and Chemistry* **18** (5), 912-918.

Forget, J., Pavillon, J.F., Menasria, M.R. & Bocquene, G. (1998). Mortality and LC50 values for several stages of the marine copepod *Tigriopus brevicornis* (Mueller) exposed to the metals arsenic and cadmium and the pesticides atrazine, carbofuran, dichlorvos and malathion. *Ecotoxicology and Environmental Safety* **40** 239-244.

Franks, N.P., Lieb, W.R. (1997). Inhibitory synapses- Anaesthetics set their sites on ion channels. *Nature* **389** (6649), 334-335.

Frost, B.W. (1972). Effects of size and concentration of food particles on the feeding behaviour of the marine planktonic copepod *Calanus pacificus*. *Limnology and Oceanography* **17** 805-815.

Frost, B.W. (1975). A threshold feeding behaviour in *Calanus pacificus*. *Limnology and Oceanography* **20** 263-267.

Fulton, M.H. & Scott, G.I. (1991). The effect of certain intrinsic and extrinsic variables on the acute toxicity of selected organophosphorous insecticides to the mummichog, *Fundulus heteroclitus*. *Journal of Environmental Science and Health* **B26** (5&6), 459-478.

Gerritsen, J. & Porter, K.G. (1982). The role of surface-chemistry in filter feeding by zooplankton. *Science* **216** 1225-1227.

Gilbert, J.J. & Durand, M.W. (1990). Effect of *Anabaena flos-aquaea* on the ability of *Daphnia* and *Keratella* to feed and reproduce on unicellular algae. *Freshwater Biology* **24** 577-596.

Gillooly, J.F. and Dodson, S.I. (2000). Latitudinal patterns in the size distribution and seasonal dynamics of new world freshwater cladocerans. *Limnology and Oceanography* **45** (1), 22-30.

Gliwicz, M.Z. (1975). Effects of zooplankton grazing on photosynthetic activity and composition of phytoplankton. *Verhandlungen der internationalen Vereinigung für theoretische und angewandte Limnologie* **19** (966-973),

Gliwicz, M.Z. & Sieniawska, A. (1986). Filtering activity of *Daphnia* in low concentrations of a pesticide. *Limnology and Oceanography* **31** (5), 1132-1138.

Gophen, M. (1976). *Freshwater Biology* **6** 451-455.

Greco, W., Unkelbach, H.-D., Poech, G., Suehnel, J., Kundi, M. and Boedeker, W. (1992). Consensus on concepts and terminology for combined action assessment: the Saariselkae Agreement. *ACES* **4** (3), 65-69.

Grimme, L.H., Faust, M., Boedeker, W. & Altenburger, R. (1996). Aquatic toxicity of chemical substances in combination: still a matter of controversy. *Human Ecological Risk Assessment* **2** (3), 426-433.

Hagler, J.R., Waller, G.D. & Lewis, B.D. (1989). Mortality of honeybees (Hymenoptera: Apidae) exposed to permethrin and combinations of permethrin with piperonyl butoxide. *Journal of Apicultural Research* **28** (4), 208-212.

Haney, J.F. (1985). Regulation of cladoceran filtering rates in nature by body size, food concentration, and diel feeding patterns. *Limnology and Oceanography* **30** 397-411.

Haney, J.F., Sasner, J.J. & Ikawa, M. (1995). Effects of products released by *Aphinizomenon flos-aquaea* and purified saxitoxin on the movements of *Daphnia carinata* feeding appendages. *Limnology and Oceanography* **40** 263-272.

Hart, R.C. (1986). Aspects of the feeding ecology of turbid water zooplankton. *In situ* studies of community filtration rates in silt-laden Lake Le Roux, Orange River, South Africa. *Journal of Plankton Research* **8** 401-426.

Hartgers, E.M., Heugens, E.H.W. & Deneer, J.W. (1999). Effects of lindane on the clearance rate of *Daphnia magna*. *Archives of Environmental Contamination and Toxicology* **36** 399-404.

Hatch, A.C. & Burton, G.A.Jr. (1999). Phototoxicity of fluoranthene to two freshwater crustaceans, *Hyalella azteca* and *Daphnia magna*: measures of feeding inhibition as a toxicological endpoint. *Hydrobiologia* **400** 243-248.

Hayes, W.J.Jr. & Laws, E.R.Jr. (1991). *Handbook of Pesticide Toxicology (Vol 2)*, Academic Press Inc.

Hebert, P.D.N. (1978). The population biology of *Daphnia* (Crustacea: Daphniidae). *Biological Reviews* **55** 387-426.

Helgen, J.C. (1987). Feeding rate inhibition in crowded *Daphnia pulex*. *Hydrobiologia* **154** 113-119.

Henriques, W., Jeffers, R.D., Lacher, Jr.T.E. and Kendall, R.J. (1997). Agrochemical use on banana plantations in Latin America: Perspectives on ecological risk. *Environmental Toxicology and Chemistry* **16** (1), 91-99.

Hermens, J., Canton, H., Steyger, N. & Wegman, R. (1984). Joint effects of a mixture of 14 chemicals on mortality and inhibition of reproduction of *Daphnia magna*. *Aquatic Toxicology* **5** 315-322.

Hermens, J. & Leeuwangh, P. (1982). Joint toxicity of of mixtures of 8 and 24 chemicals to the guppy (*Poecilia reticulata*). *Ecotoxicology and Environmental Safety* **6** 302-310.

Hermens, J., Leeuwangh, P. & Musch, A. (1985). Joint toxicity of mixtures of groups of organic aquatic pollutants to the guppy (*Poecilia reticulata*). *Ecotoxicology and Environmental Safety* **9** 321-326.

Hewlett, P.S. & Plackett, R.L. (1959). A unified theory for quantal repsonses to mixtures of drugs: noninteractive action. *Biometrics* **15** 591-609.

Hickey, C.W. (1989). Sensitivity of four New Zealand cladoceran species and *Daphnia magna* to aquatic toxicants. *New Zealand Journal of Mrine and Freshwater Research* **23** 131-137.

Hill, I.R. (1989). Aquatic organisms and pyrethroids. *Pesticide Science* **27** (429-457),

HMSO (1983). *Acute toxicity testing with aquatic organisms*, Her Majesty's Stationary Office. 68pp.

Hoagland, K.D. and Drenner, R.W. (1993). Freshwater community responses to mixtures of agricultural pesticides: effects of atrazine and bifenthrin. *Environmental Toxicology and Chemistry* **12** (4), 627-637.

Horn, W. (1981). Phytoplankton losses due to zooplankton grazing in a drinking water reservoir. *Internationale Revue der Gesamten Hydrobiologie* **66** 787-810.

Horning, W.B.I. & Weber, C.I. (1985). Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/4-85/014. U.S.Environmental Protection Agency, Cincinnati, OH.

Howe, G.E., Gillis, R. & Mowbray, R.C. (1998). Effect of chemical synergy and larval stage on the toxicity of atrazine and alachlor to amphibian stages. *Environmental Toxicology and Chemistry* **17** (3), 519-525.

Howe, G.E., Marking, L.L., Bills, T.D., Rach, J.J. & Mayer Jr, F.L. (1994). Effects of water temperature and pH on toxicity of terbufos, trichlorfon, 4-nitrophenol and 2,4-dinitrophenol to the amphipod *Gammarus pseudolimnaeus* and rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry* **13** 51-66.

IPCS INCHEM (1992). International Program on Chemical Safety. Cadmium: Environmental Aspects, http://www.inchem.org/docments/ehc/ehc/ehc135.htm:

Jak, R.G., Maas, J.L. & Scholten, M.C.T. (1996). Evaluation of laboratory derived toxic effect concentrations of a mixture of metals by testing fresh water plankton communities in enclosures. *Water Research* **30** (5), 1215-1227.

Janssen, C.R., Ferrando, M.D. & Persoone, G. (1993). Ecotoxicological studies with the freshwater rotifer *Brachionus calyciflorus*. I: Conceptual ramework and applications. *Hydrobiologia* **255/256** 21-32.

Jin-Clark, Y., Lydy, M.J. & Zhu, K.Y. (2002). Effects of atrazine and cyanazine of chlorpyrifos toxicity in *Chironomus tentans* (Diptera: Chironomidae). *Environmental Toxicology and Chemistry* **21** (3), 598-603.

Johnston, G., Collett, G., Walker, C., Dawson, A., Boyd, I. & Osborn, D. (1989). Enhancement of malathion toxicity to the hybrid red-legged partridge following exposure to prochloraz. *Pesticide Biochemistry and Physiology* **35** 107-112.

Johnston, G., Walker, C.H., Dawson, A. & Furnell, A. (1990). Interactive effects of pesticides in the hybrid red-legged partridge. *Functional Ecology* **4** 309

Juchelka, C.M. & Snell, T.M. (1994). Rapid toxicity assessment using rotifer ingestion rate. *Archives of Environmental Contamination and Toxicology* **26** 35-44.

Jung, K. & Bitton, G. (1997). Use of Ceriofast™ for monitoring the toxicity of industrial effluents: comparison with the 48-h acute *Ceriodaphnia* toxicity test and Microtox®. *Environmental Toxicology and Chemistry* **16** (11), 2264-2267.

Kao, L.M., Wilkinson, C.F. & Brattsten, L.B. (1995). In-vivo effects of 2,4-D and atrazine on cytochrome P-450 and insecticide toxicity in Southern armyworm (*Spodoptera eridania*) larvae. *Pesticide Science* **45** 331-334.

Keating, K.I. (1985). A system of defined (*sensu stricto*) media for the daphnid (Cladocera) culture. *Water Research* **19** 73-78.

Keating, K.I. & Caffrey, P.B. (1989). Selenium deficiency induced by zinc deprivation in a crustacean. *Proceedings of the National Academy of Sciences* **86** 6436-6440.

Kersting, K. & Van der Leeuw, W. (1974). The use of the Coulter Counter for measuring the feeding rates of *Daphnia magna*. *Hydrobiologia* **49** 233-237.

Kersting, K. and Van Wijngaarden, R. (1992). Effects of chlorpyrifos on a microecosystem. *Environmental Toxicology and Chemistry* **11** 365-372.

Klein, W. (1989) Mobility of environmental chemicals, including abiotic degradation. In: *Ecotoxicology and Climate* P.Bourdeau, J.A.Haines, W.Klein, C.R.Krishna Murti (eds.) , pp. 65-78. Suffolk, UK: John Wiley & Sons.

Klüttgen, B., Kuntz, N. & Ratte, T. (1996). Combined effects of 3,4-dichloroaniline and food concentration on life-table data of two related cladocerans, *Daphnia magna* and *Ceriodaphnia quadrangula*. *Chemosphere* **32** (10), 2015-2028.

Koivisto, S. (1995). Is *Daphnia magna* an ecologically representative zooplankton species in toxicity tests? *Environment and Pollution* **90** 263-267.

Könemann, W.H. (1981). Quantitative structure-analysis relationships in fish toxicity studies. Part 1: Relationship for 50 industrial pollutants. *Toxicology* **19** 209-221.

Kooijman, S.A.L.M. (1993). *Dynamic Energy Budgets in Biological Systems: Theory an Application in Ecotoxicology*, Cambridge, UK: Cambridge University Press.

Kraak, M.H.S., Lavy, D., Schoon, H., Toussaint, M., Peeters, W.H.M. & Van Straalen, N.M. (1994). Ecotoxicity of mixtures of metals to the zebra mussel *Dreissena polymorpha*. *Environmental Toxicology and Chemistry* **13** 109-114.

Kuivila, K.M. & Foe, C.G. (1995). Concentrations, transport and biological effects of dormant spray pesticides in the San Francisco estuary, California. *Environmental Toxicology and Chemistry* **14** (7), 1141-1150.

Kuntz, D.J., Rao, N.G.S., Berg, I.E., Khattree, R. & Chaturvedi, A.K. (1990). Toxicity of mixtures of parathion, toxaphene and/or 2,4-D in mice. *Journal of Applied Toxicology* **10** (4), 257-266.

Lahr, J. (2000) Effects of insecticides on invertebrates in temporary ponds in the Sahel. PhD Thesis, Universiteit Den Helder, NL.

Lampert, W. (1977). Studies on the carbon balance of *Daphnia pulex* DE GEER as related to environmental conditions; IV. Determination of the "threshold" concentration as a factor controlling the abundance of zooplanktonic species. *Archives of Hydrobiology* **3/4** 361-368.

Lampert, W. (1982). Further studies on the inhibitory effect of the toxic blue-green algae *Microcystis aeruginosa* on the filtering rate of zooplankton. *Archives of Hydrobiology* **95** 207-220.

Lampert, W. (1987). Feeding and nutrition in *Daphnia*. *Memorie Instituto Italianos Idrobiologica* **45** 143-192.

Lampert, W. (1993). Ultimate causes of diel vertical migration of zooplankton: new evidence for the predator-avoidance hypothesis. *Archives of Hydrobiol.Beih. (Ergebn. Limnol)* **39** 79-88.

Lampert, W., Fleckner, W., Pott, E., Schrober, U. and Storkel, K. (1989). Herbicide effects on planktonic systems of different complexity. *Hydrobiologia* **188/189** 415-424.

Lampert, W., Fleckner, W., Rai, H. & Taylor, B.E. (1986). Phytoplankton control by grazing zooplankton- a study on the spring clear water phase. *Limnology and Oceanography* **31** (3), 478-490.

Landrum, P.F., Nihart, S.R., Eadie, B.J.& Herche, L.S. (1987). Reduction in bioavailability of organic contaminants to the amphipod *Pontoporeia hoyi* by dissociated organic matter of the sediment interstitial water. *Environmental Toxicology and Chemistry* **6** 11-20.

LaRocca, C.A., Francisco, D.E. & DiGiano, F.A. (1994). Effects of diet on survival, reproduction and sensitivity of *Ceriodaphnia dubia. Water Environment Research* **66** (7), 905-911.

Lee, S.I., Na, E.J., Cho, Y.O., Koopman, B. & Bitton, G. (1997). Short term toxicity test based on agal uptake by *Ceriodaphnia dubia. Water Environment Research* **69** (7), 1207-1210.

Lewis, W.M., Hamilton, S.K. & Saunders, J.F. (1995). Rivers of northern South America. In: *Ecosystems of the world.* 22. *River and stream ecosystems,* C.E.Cushing, K.W.Cummins & G.W.Minshall (eds.), pp. 219-256. Amsterdam: Elsevier.

Lichtenstein, E.P., Liang, T.T. & Anderegg, B.N. (1973). Synergism of insecticides by herbicides. *Science* **181** 847-849.

Lilius, H., HaestbackaT. and Isomaa, B. (1995). A comparison of the toxicity of 30 reference chemicals to *Daphnia magna* and *Daphnia pulex. Environmental Toxicology and Chemistry* **14** (12), 2085-2088.

Lynch, M. (1978). Complex interactions between natural coexploiters- *Daphnia* and *Ceriodaphnia. Ecology* **59** (3), 552-564.

Lynch, M. & Shapiro, J. (1981). Predation, enrichment, and phytoplankton community structure. *Limnology and Oceanography* **26** 86-102.

Macek, K.J. (1975). Acute toxicity of pesticide mixtures to bluegills. *Bulletin of Environmental Contamination and Toxicology* **14** (6), 648-652.

Makino, W., Haruna, H. & Ban, S. (1996). Diel vertical migration and feeding rhythm of *Daphnia longispina* and *Bosmina coregoni* in Lake Toya, Hokkaido, Japan. *Hydrobiologia* **337** 133-143.

Maltby, L. (1994). Stress, shredders and streams: using *Gammarus* energetics to assess water quality. In: *Water Quality and Stress Indicators in Marine and Freshwater Ecosystems. Linking Levels of Organisation, Individuals, Populations and Communities.* Sutcliffe, D.W. (ed.), Freshwater Biological Association, pp. 98-110,

Marking, L.L. (1977). Methods for assessing additive toxicity of chemical mixtures. STP 634. American Society for Testing and Materials, Philadelphia, PA.

Marking, L.L. and Dawson, V.K. (1975). Method for assessment of toxicity or efficacy of mixtures of chemicals. *U.|S.Fisheries and Wildlife Service Invest. Fisheries Control* **67** 1-8.

Matsumura, F. (1989) Biotic degradation of pollutants. In: *Ecotoxicology and Climate* P.Bourdeau, J.A.Haines, W.Klein, C.R.Krishna Murti (eds.) pp. 79-89. Suffolk,UK: John Wiley & Sons.

Matsumura-Tundisi, T., Tundisi, J.G. & Tavares, L.S. (1984). Diel migration and vertical distribution of cladocera in Lake D. Helvecio (Minas Gerais, Brazil). *Hydrobiologia* **113** 299-306.

Matthiessen, P., Whale, G.F., Rycroft, R.J. & Sheahan, D.A. (1988). The joint toxicity of pesticide tank mixtures to rainbow trout. *Aquatic Toxicology* **13** (1), 61-75.

Matveev, M. (1993). An investigation of alleopathic effects of *Daphnia*. *Freshwater Biology* **29** 99-103.

Mayes M. A. & Barron M.G. (editors) (1991). *Aquatic Toxicology and Risk Assessment: Fourteenth Volume*, American Society of Testing and Materials (ASTM).

McCarty, L.S. & Mackay, D. (1993) enhancing ecotoxicological modeling and assessment: body residues and modes of toxic action. *Environmental Science and Technology* **27** 1719-1728.

McCarty, L.S., Ozburn, G.W., Smith, A.D. and Dixon, D.G. (1992). *Hazard Assessment*: toxikinetic modeling of mixtures of organic chemicals. *Environmental Toxicology and Chemistry* **11** 1037-1047.

McLoughlin, N., Yin, D.Q., Maltby, L., Wood, R.M. & Yu, H.X. (2000). Evaluation of sensitivity and specificity of two crustacean biochemical biomarkers. *Environmental Toxicology and Chemistry* **19** (8),

McMahon, J.W. (1965). Some physical factors influencing the feeding behaviour of *Daphnia magna* Straus. *Canadian Journal of Zoology* **43** 603-610.

McMahon, J.W. & Rigler, F.H. (1965). Feeding rate of *Daphnia magna* Straus in different foods labeled with radioactive phosphorus. *Limnology and Oceanography* **10** 105-113.

McWilliam, R.A. (2001) Toxic Anorexia in *Daphnia magna* Straus as an *in situ* indicator of the ecological impact of pollutants. PhD Thesis, University of Stirling, UK.

McWilliam, R.A. & Baird, D.J. (2002). Post-exposure feeding depression: a new toxicity endpoint for use in laboratory studies with *Daphnia magna*. *Environmental Toxicology and Chemistry* **21** (6), 1198-1205.

Mian, L.S. and Mulla, M.S. (1992). Effects of pyrethroid insecticides on nontarget invertebrates in aquatic ecosystems. *Journal of Agricultural Entomology* **9** (2), 73-98.

Mokry, L.E. and Hoagland, K.D. (1990). Acute toxicities of five synthetic pyrethroid insecticides to *Daphnia magna* and *Ceriodaphnia dubia*. *Environmental Toxicology and Chemistry* **9** 1045-1051.

Monserrat, J. & Montserrat, B.A. (1995). Effects of temperature and salinity on the toxicity of a commercial formulation of methyl parathion to *Chasmagnathus granulata* (Decapoda, Grapsidae). *Brazilian Journal of Medical and Biological Research* **28** (1), 74-78.

Moore, D.R.J. & Caux, P.-Y. (1997). Estimating low toxic effects. *Environmental Toxicology and Chemistry* **16** (4), 794-801.

Moore, M. and Folt, C. (1993). Zooplankton body size and community structure: effects of thermal and toxicant stress. *Tree* **8** (5), 178-183.

Moore, M.V., Folt, C.F. & Stemberger, R.S. (1996). Consequences of elevated temperatures for zooplankton assemblages in temperate lakes. *Archives of Hydrobiology* **135** 289-319.

Mount, D.I. & Norberg, T.J. (1984). A seven-day life-cycle cladoceran toxicity test. *Environmental Toxicology and Chemistry* **3** 425-434.

Mount, D.I. & Stephan, C.E. (1967). A method for establishing acceptable limits for fish-malathion and the butoxyethanol ester of 2,4-D. *Transitions of the American Fisheries Society* **96** 185-193.

Murray, D. (1994). *Cultivating Crisis, The Human Cost of Pesticides in Latin America.* Austin, TX, USA: University of Texas Press.

Naddy, R.B., LaPoint, T.W. and Klaine, S.J. (1995). Toxicity of arsenic, molybdenum and selenium combinations to *Ceriodaphnia dubia*. *Environmental Toxicology and Chemistry* **14** (2), 329-336.

Newsted, J.L. (1995). Development of toxic equivalency factors for PCB congeners and the assessment of TCDD and PCB mixtures for rainbow trout. *Environmental Toxicology and Chemistry* **14** 861-871.

Niederlehner, B.R., Cairns, J. and Smith, E.P. (1998). Modeling acute and chronic toxicity of nonpolar narcotic chemicals and mixtures to *Ceriodaphnia dubia. Ecotoxicology and Environmental Safety* **39** 136-146.

Nogueira, A.J.A. (1996) Modelling the physiological performance of *Daphnia* (Crustacea: Cladocera) under stress. PhD Thesis. Stirling University, UK.

Norberg-King, T.J., Durhan, E.J., Ankley, G.T. & Robert, G. (1991). Application of toxicity identification evaluation procedures to the ambient waters of the Colusa Basin Drain, California. *Environmental Toxicology and Chemistry* **10** 891-900.

Nriagu, J.O. (1990). Food contamination with cadmium in the environment. In: *Food Contamination from Environmental Sources.* Advances in Science and Technology 23, J.O. Nriagu (ed.). John Wiley and Sons, New York, pp.59-84.

O.E.C.D. (1981). *Daphnia* spp. Acute immobilisation test and reproduction test. Guidelines for testing of chemicals. Organisation of Economic Cooperation and Development Publications Office, Paris. *O.E.C.D. Guidelines for the Testing of Chemicals.* **202**

Pantani, C., Ghetti, P.F., Cavacini, A. & Muccioni, P. (1990). Acute toxicity of equitoxic binary mixtures of some metals, surfactants and pesticides to the freshwater amphipod *Gammarus italicus* Goedm. *Environmental Technology* **11** 1143-1146.

Pape-Lindstrom, P.A. & Lydy, M.J. (1997). Synergistic toxicity of atrazine and organophosphate insecticides contravenes the response addition mixture model. *Environmental Toxicology and Chemistry* **16** (11), 2415-2420.

Parrish, P.P. (1985) Aquatic Toxicity Tests. In: Anonymouspp. 31-67. USA: Hemisphere Publishing Corporation.

Parrott, J.L. & Sprague, J.B. (1993). Patterns in toxicity of sublethal mixtures of metals and organic chemicals determined by Microtox and by DNA, RNA and protein content of fathead minnows (*Pimephales promelas*). *Canadian Journal of Fisheries and Aquatic Science* **50** 2245-2253.

Patra, R.W.R. (1999). Effects of temperature on the toxicity of chemicals to Australian fish and invertebrates. PhD Thesis, Sydney University, AUS.

Patterson, P.W., Dickson, K.L., Waller, W.T. & Rodgers, J.H. (1992). The effects of nine diet and water combinations on the culture health of *Ceriodaphnia dubia. Environmental Toxicology and Chemistry* **11** 1023-1035.

Payne, A.I. (1986). *The ecology of tropical lakes and rivers.* Great Britain: John Wiley & Sons Ltd.

Pennak, R.W. (1978). *Freshwater Invertebrates of the United States (2nd ed.)*, New York, USA: Wiley.

Persoone, G., Van de Vel, A., Van Steertegem, M. and De Nayer, B. (1989). Predictive value of laboratory tests with aquatic invertenbrates: influence of experimental conditions. *Aquatic Toxicology* **14** 149-166.

Peters, E.C., Gassman, N.J., Firman, J.C., Richmond, R.H. and Power, E.A. (1997). Ecotoxicology of tropical marine ecosystems. *Environmental Toxicology and Chemistry* **16** (1), 12-40.

Peters, R.H. (1984). Empirical analysis of zooplankton filtering and feeding rates. *Limnology and Oceanography* **29** (4), 763-784.

Pilling, E.D., Bromley-Challenor, K.A.C., Walker, C.H. & Jepson, P.C. (1995). Mechanisms of synergism between the pyrethroid insecticide λ-cyhalothrin and the imidazole fungicide prochloraz, in the honeybee (*Apis mellifera* L.). *Pesticide Biochemistry and Physiology* **51** 1-11.

Plackett, R.L. (1952). Quantal responses to mixtures of poisons. *Journal of the Royal Statistical Society* **14** (2), 141-163.

Plackett, R.L. & Hewlett, P.S. (1963). Quantal responses to mixtures of poisons. *Journal of the Royal Statistical Society* **B14** 141-163.

Plackett, R.L. and Hewlett, P.S. (1967). A comparison of two approaches to the construction of models for quantal responses to mixtures of drugs. *Biometrics* **23** 27-44.

Pöch, G. (1993). *Combined Effects of Drugs and Toxic Agents. Modern Evaluation in Theory and Practice*, New York, NY, USA: Springer Verlag.

Porter, K.G. (1976). Enhancement of algal growth and productivity by grazing zooplankton. *Science* **192** 1332-1334.

Porter, K.G., Feig, Y.S. & Vetter, E.F. (1983). Morphology, flow regimes, and filtering rates of *Daphnia, Ceriodaphnia,* and *Bosmina* fed natural bacteria. *Oecologia (Berlin)* **58** 156-163.

Porter, K.G., Gerritsen, J. & Orcutt Jr, J.D. (1982). The effect of food concentration on swimming patterns, feeding behaviour, ingestion, assimilation, and respiration by *Daphnia. Limnology and Oceanography* **27** 935-949.

Preston, B.L., Snell, T.W., Robertson, T.L. and Dingmann, B.J. (2000a). Use of freshwater rotifer *Brachionus calyciflorus* in screening assay for potential endocrine disruptors. *Environmental Toxicology and Chemistry* **19** (12), 2923-2928.

Preston, S., Coad, N., Townend, J., Killham, K. & Paton, G.I. (2000b). Biosensing the acute toxicity of metal interactions: are they additive, synergistic, or antagonistic? *Environmental Toxicology and Chemistry* **19** (3), 775-780.

Ramcharan, C.W., Dodson, S.I. & Lee, J. (1992). Predation risk, prey behaviour, and feeding rate in *Daphnia pulex. Canadian Journal of Fisheries and Aquatic Science* **49** 159-165.

Rand, G.M. (1995). Fundamentals of aquatic toxicology: effects, environmental fate, and risk assessment (2nd ed.), USA: Taylor & Francis.

Reeve, M.R. (1963). The filter feeding of *Artemia* IL in pure cultures of plant cells. *Journal of Experimental Biology* **40** 195-205.

Robinson, K.A. (1998) The influence of prey-surface contamination on aquatic invertebrate predators with contrasting modes of feeding. PhD Thesis, University of Stirling, UK.

Rose, R.M., Warne, M.St.J. & Lim, R.P. (2001). The presence of chemicals exuded by fish affects the life-history response of *Ceriodaphnia* cf. *dubia* to chemicals with different mechanisms of action. *Environmental Toxicology and Chemistry* **20** (12), 2892-2898.

Saenger, P. and Holmes, N. (1992) Physiological, temperature tolerance, and behavioral differences between tropical and temperate organisms. In: *Pollution in Tropical Aquatic Systems.*Hawker, D.V. & Connell, D.W. (eds.) CRC Press, Inc. Boca Raton, FL. pp. 69-95.

Sambasiva Rao, K.R.S., Prasada Rao, K.S., Ahammad Sahib, I.K. & Ramana Rao, K.V. (1985). Combined action of carbaryl and phenthoate on a freshwater fish (*Channa punctatus* Bloch). *Ecotoxicology and Environmental Safety* **10** 209-217.

Schubauer-Berigan, M.K. & Dierkes, J.R. (1993). pH-dependent toxicity of Cd, Cu, Ni, Pb and Zn to *Ceriodaphnia dubia, Pimphales promelas, Hyalella azteca* and *Lumbriculus variegatus. Environmental Toxicology and Chemistry* **12** 1261-1266.

SCOPE (1987). Scientific Committee on Problems of the Environment: Methods for Assessing the Effects of Mixtures of Chemicals, Chichester: John Wiley.

Servizi, J.A., Gordon, R.W. & Martens, D.W. (1987). Acute toxicity of Garlon 4 and Roundup herbicides to salmon, *Daphnia* and trout. *Bulletin of Environmental Contamination and Toxicology* **39** 15-22.

Sethunathan, N. (1989). Biodegradation of pesticides in tropical rice ecosystems. In: *Ecotoxicology and Climate* P.Bourdeau, J.A.Haines, W.Klein, C.R.Krishna Murti, (eds.) **SCOPE 38** (John Wiley & Sons, Suffolk, UK), pp. 247-264.

Shehan, P.J., Miller, D.R., Butler, G.C. & Bourdeau, P. (1984). *Effects of Pollutants at the Ecosystem Level.* SCOPE 22, Chichester: Wiley.

Sloof, W., Canton, J.H. & Hermes, J.L.M. (1983). Comparison of the susceptibility of 22 freshwater species to 15 chemical compounds; 1. Sub-acute toxicity tests. *Aquatic Toxicology* **4** 113-128.

Solon, J.M. & Nair III, J.H. (1970). The effect of a sublethal concentration of LAS on the acute toxcity of various phosphate pesticides to the fathead minnow (*Pimephales promelas* Rafinesque). *Bulletin of Environmental Contamination and Toxicology* **5** (5), 408-413.

Song, M.Y., Stark, J.D. and Brown, J.J. (1997). Comparative toxicity of four insecticides, including imidacloprid and tebufenozide, to four aquatic arthropods. *Environmental Toxicology and Chemistry* **16** (12), 2494-2500.

Spehar, R.L. & Fiandt, J.T. (1986). Acute and chronic effects of water quality criteria-based metal mixtures on three aquatic species. *Environmental Toxicology and Chemistry* **5** 917-931.

Sprague, J.B. (1985) Factors that modify toxicity. In: *Fundamentals of Aquatic Toxicology.* Rand, G.M. & Petrocelli, S.R. (eds.), Bristol, PA, USA: Taylor & Francis, pp. 124-163.

Starkweather, P.L. (1978). Diel variation in feeding behaviour of *Daphnia pulex.* Influences of food density and nutritional history on mandibular activity. *Limnology and Oceanography* **23** 307-317.

Steen, R.J.C.A., Leonards, P.E.G., Brinkmann, U.A.T. & Barcelo, D. (1999). Ecological risk assessment of agrochemicals in European estuaries. *Environmental Toxicology and Chemistry* **18** (7), 1574-1581.

Steevens, J.A. and Benson, W.H. (2001). Toxicokinetic interactions and survival of *Hyalella azteca* exposed to binary mixtures of chlorpyrifos, dieldrin, and methyl mercury. *Aquatic Toxicology* **51** 377-388.

Stevenson, R.R. & Watts, S.A. (1984). Chronic toxicity tests with *Daphnia magna*: the effects of different food and temperature regimes on survival, reproduction and growth. *Environmental Pollution* **36** 95-107.

Stratton, G.W. (1983). Interaction effects of permethrin and atrazine combinations towards several nontarget microorganisms. *Bulletin of Environmental Contamination and Toxicology* **31** 297-303.

Stross, R.G. & Hill, J.C. (1965). Diapause induction in *Daphnia* requires two stimuli. *Science* **150** 1462-1464.

Sunderam, R.I.M., Cheng, D.M.H. & Thompson, G.B. (1992). Toxicity of endosulfan to native and introduced fish in Australia. *Environmental Toxicology and Chemistry* **11** 1469-1476.

Sunderam, R.I.M., Thompson, G.B., Chapman, J.C. and Cheng, D.M.H. (1994). Acute and chronic toxicity of endosulfan to two Australian cladocerans and their applicability in deriving water quality criteria. *Archives of Environmental Contamination and Toxicology* **27** 541-545.

Tait, R.D., Shiel, R.J. & Koste, W. (1984). Structure and dynamics of zooplankton communities, Alligator Rivers Region, N.T., Australia. *Hydrobiologia* **113** 1-13.

Talling, J.F. & Lemoalle, J. (1998). *Ecological dynamics of tropical inland waters*, Great Britain: Cambridge Unversity Press.

Taylor, B.E. (1985). Effects of food limitation on growth and reproduction of *Daphnia*. *Archiv für Hydrobiologie (Beih. Ergebn. Limnol.)* **21** 285-296.

Taylor, G.J. (2001) The influence of particle sorption on the bioavailability of contaminants to *Daphnia magna*. PhD Thesis, Stirling University, UK.

Taylor, G.J., Baird, D.J. and Soares, A.M.V.M. (1998). Surface binding of contaminants by algae: consequences for lethal toxicity and feeding to *Daphnia magna* Straus. *Environmental Toxicology and Chemistry* **17** (3), 412-419.

Tessier, A.J. and Bizina, E.V. (2001). Grazer-resource interaction in the plankton: Are all daphniids alike? *Limnology and Oceanography* **46** (7), 1585-1595.

Tidou, A.S., Moreteau, J.-C. and Ramade, F. (1992). Effects of lindane and deltamethrin on zooplankton communities of experimental ponds. *Hydrobiologia* **232** 157-168.

Tripathi, A.M. & Agarwal, R.A. (1997). Synergism in tertiary mixtures of pesticides. *Chemosphere* **35** (10), 2365-2374.

Tripathi, A.M. and Agarwal, R.A. (1998). Molluscicidal and anti-AChE activity of tertiary mixtures of pesticides. *Archives of Environmental Contamination and Toxicology* **34** 271-274.

U.S. Environmental Protection Agency (1982). *Daphnia* chronic toxicity test EG-2, August 1982. *In*: Environmental effects test guidelines. EPA 560/6-82/002. U.S.Environmental Protection Agency, Office of Toxic Substances, Washington DC.

U.S.Environmental Protection Agency (1986). Guidelines for the health risk assessment of chemical mixtures. *Federal Regulations* **51** 34014-34025.

U.S. Environmental Protection Agency (1991). Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 4th ed., edited by C.I.Weber. EPA-600/4-90/027.

U.S.Environmental Protection Agency (1993). Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 4th ed. EPA 600/4-90/027F. Cincinnati, OH.

U.S. Environmental Protection Agency (1994). Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms, 3rd ed. EPA 600/4-91/002. Cincinnati OH.

Van Leeuwen, C.J., Luttmer, W.J. & Griffioen, P.S. (1985). The use of cohorts and populations in chronic toxicity studies with *Daphnia magna*: a cadmium example. *Ecotoxicology and Environmental Safety* **9** 26-39.

Vanegas, C., Espina, S., Botello, A.V. & Villanueva, S. (1997). Acute toxicity and synergism of cadmium and zinc in white shrimp, *Penaeus setiferus*, juveniles. *Bulletin of Environmental Contamination and Toxicology* **58** 87-92.

Veith, G.D., DeFoe D.L. & Bergstedt, B.V. (1979) Measuring and estimating the bioconcentration factor of chemicals in fish. *Journal of the Fisheries Research Board of Canada* **36** 1040-1048.

Vidal, J. (1980). *Marine Biology* **56** 111-134.

Villaroel, M.J., Ferrando, M.D. & Andreu, E. (1998). Toxic anorexia as a sensitive endpoint in *Daphnia magna*. *Journal of Environmental Science and Health* **B33** 151-160.

Villarroel, M.J., Ferrando, M.D., Sancho, E. and Andreu, A. (1999). *Daphnia magna* feeding bahavior after exposure to tetradifon and recovery from intoxication. *Ecotoxicology and Environmental Safety* **44** 40-46.

Viswanathan, P.N. & Krishna Murti, C.R. (1989). Effects of temperature and humidity of ecotoxicology of chemicals. In: P.Bourdeau, J.A.Haines, W.Klein and C.R.Krishna Murti, eds. *Ecotoxicology and Climate* **SCOPE 38** (John Wiley & Sons, Suffolk, UK), 139-154.

Vouk, V.V., Butler, G.C., Upton, A.C., Parke, D.V. & Asher, S.C. (1987). Methods for Assessing the Effects of Mixtures of Chemicals., New York, NY, USA: John Wiley & Sons.

Waddell, Y.T. (1993) Feeding responses of *Daphnia magna* under exposure to toxic chemicals. PhD Thesis, University of Sheffield,UK.

Winner, R.W. & Farrell, M.P. (1976). Acute and chronic toxicity of copper to 4 species of *Daphnia*. *Journal of the Fisheries Research Board Canada* **33** 1685-1691.

Winner, R.W. (1989). Multigeneration life-span tests of the nutritional adequacy of several diets and culture waters for *Ceriodaphnia dubia*. *Environmental Toxicology and Chemistry* **8** 513-520.

Winner, R.W. & Whitford, T.C. (1987). The interactive effects of a cadmium stress, a selenium deficiency and water temperature on the survival and reproduction of *Daphnia magna* Straus. *Aquatic Toxicology* **10** 217-224.

Wolanski, E. (1992). Hydrodynamics of tropical coastal marine systems. In: *Pollution in Tropical Aquatic Systems*. Hawker D.W. and Connell D.W. (eds.). CRC, Boca Raton, FL, USA, pp. 169-188.

Wong, C.K. (1989). Effects of cadmium on the feeding behavior of the fresh water cladoceran *Moina macrocopa. Chemosphere* **18** (7-8), 1681-1687.

Woods, M., Kumar, A. & Correll, R. (2002). Acute toxicity of mixtures of chlorpyrifos, profenofos, and endosulfan to *Ceriodaphnia dubia. Bulletin of Environmental Contamination and Toxicology* **68** 801-808.

Xiu, R., Xu, Y. and Gao, S. (1989). Toxicity of the new pyrethroid insecticide, deltamethrin, to *Daphnia magna. Hydrobiologia* **188/189** 411-413.

Zaret, T.M. & Suffern, J.S. (1976). Vertical migration in zooplankton as a predator avoidance mechanism. *Limnology and Oceanography* **21** 804-813.

APPENDIX 1

UNIVERSITY OF STIRLING, *ECOTOXICOLOGY GROUP*
STANDARD OPERATING PROCEDURE

Installation Date:
Status:

Title: Laboratory Feeding Inhibition Bioassay for the Cladoceran *Ceriodaphnia dubia.*

(i) PURPOSE:

Standardisation of procedure for assessment and processing of *Ceriodaphnia dubia* feeding inhibition test to assess sublethal effects of chemicals, effluents and ambient waters.

(ii) SCOPE:

To be applied in all laboratory feeding inhibition tests utilising *C.dubia.*

(iii) RESPONSIBILITIES:

None.

(iv) DEFINITIONS:

None.

(v) FORMS:

None.

(vi) REFERENCES:

Allen Y. *et al.*(1995), A mechanistic model of contaminant-induced feeding inhibition in *Daphnia magna.* Environmental Toxicology and Chemistry (14) 1625-1630.
ASTM (1980), Standard practise for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. E-729-80, ASTM Philadelphia, PA.

(vii) PROCEDURE:

Materials (for acclimation of experimental animals)
150mL narrow rim glass bottles with screw cap
ASTM moderately hard water

Seaweed extract (*Ascophyllum nodosum)* (Marinure, Glenside Organics Ltd.)- concentrate made up to a solution of 1:100, adding 9mL to 1L nanopure water (18MΩ), with optical density of 0.6-0.7 at 400nm in a 1 in 10 dilution
Chlorella vulgaris at a concentration of $2x10^6$ cells mL^{-1}

Materials (for exposure in test)- 8 treatments + control, 4 replicates
144, 5-6 day old *C.dubia* (after release of first brood)
36 x 7ml glass bottles with screw cap
50mL of each exposure solution, in ASTM moderately hard water, containing *C.vulgaris* at a concentration of $2x10^6$ cells mL^{-1}
C.vulgaris at concentration of $1x10^8$ cells mL^{-1}: 800µL per 40mL
3mL plastic pipettes
1mL glass pipettes

Materials for measuring of feeding rates
Can be carried out using either a Coulter Counter or a UV spectrophotometer.
FOR THE COULTER COUNTER:
Coulter Multisizer Model II (Coulter Electronics Ltd.) fitted with a 70µm orifice tube
Accuvette disposable sample containers (Coulter Electronics Ltd.)
Isoton II electrolyte (Coulter Electronics Ltd.)
200-1000µL micropipette

FOR THE UV SPECTROPHOTOMETER:
UV spectrophotometer equipped to hold a cuvette with a 4cm pathlength
Cuvettes, with 4cm pathlength
ASTM hard or moderately hard water

Procedure

Acclimation of *C.dubia:*

Volume of ASTM required depends on number of animals necessary for experiments. For a test with 8 treatments + control, with 4 replicates, 144 animals are needed. 30 animals require one 150mL glass bottle with 120mL volume. 150 animals have to be kept in 5 individual glass bottles, so about 600mL ASTM is needed.

Make up 600mL of moderately hard ASTM in a conical flask and add seaweed extract at a concentration of 5mL L^{-1}. Add *C.vulgaris* at a concentration of $2x10^6$ cells mL^{-1}. Put ~30 *C.dubia* neonates (<24h old) into the glass jars, using a clean plastic pipette, making sure animals are kept under water and not exposed to air. Keep the animals at 25°C in a light cycle of 16:8 hours light:dark.

Add *C.vulgaris* at a concentration of $2x10^6$ cells mL^{-1} each day, and put animals in fresh medium every two days, until animals release their first brood (5-6 days).

Feeding Inhibition Test

Prepare about 50mL of each exposure solution and the control solution and add relative amount of *C.vulgaris* to make up to a concentration of $2x10^6$ cells mL^{-1}.

Add 7mL of relative solutions to each 7mL glass bottle with a 5mL micropipette.

Put 4 animals into each exposure bottle, using a fine glass pipette. Make sure to only add minimal, and equal, amounts of water when transferring animals from acclimation vessel to test bottles.

Close all bottles with aluminium foil and/or a lid to avoid evaporation.

Put all bottles in a box or tray and transfer to an incubator or constant temperature room at 25°C and leave animals to feed for 24 hours in the dark.

Count samples of exposure solutions in the Coulter Counter to make sure the concentrations at t=0h were $2x10^6$ cells mL^{-1}, or make notes of concentrations. Keep some samples of exposure solution to be counted at t=24h, to make sure no changes occur over 24 hours.

After 24 hours, remove animals from bottles. First make notes of dead and alive animals and any neonates, if there should be any. Then make sure the algal solution in the exposure bottles is evenly mixed, as algae tend to settle on the bottom, by shaking the bottle vigorously and stirring up algae with a glass pipette. Remove animals from the bottles using a fine glass pipette.

Algae samples may be stored in a fridge at 4°C overnight, or frozen if necessary, before measuring feeding rates. For best results however algae samples should be processed on the same day, as a certain degree of cell damage and degradation will occur.

Measurement of feeding rates.

COULTER COUNTER:

Make sure algal sample solution is well mixed by vigorously shaking container. Withdraw a 0.5mL sample using a micropipette and add to 19.5mL Isoton II in an accuvette disposable sample container.

Gently mix sample in accuvette by turning upside town, making sure not to create any bubbles that can interfere with the counting process.

Place accuvette on sample platform in Coulter Counter with the orifice tube and foil submerged in the sample.

Set Coulter Counter to automatically sample 500µL of sample. Make 3 counts per sample.

Multiply the average of the 3 counts by 80 to obtain the final number of cells per millilitre. Blank jars give the initial number of cells per millilitre. Make sure the blank cell concentration did not change significantly between t=0h and t=24h.

UV SPECTROPHOTOMETER

Mix jar containing algal sample well and fill a cuvette with a 4cm pathlength with the sample. Measure 2 samples for each jar.

Measure optical density of sample in a UV spectrophotometer at 440nm using ASTM as a blank.

CALCULATION OF FEEDING RATES:

Feeding rates are calculated using the equation given in Allen (1995), which is as follows:

$$F = \frac{V.(C_0 - C_t)}{t.N}$$

where:
F= feeding rate of single animal (cells ind^{-1} hr^{-1})
V= volume of suspension (mL)
C_0= initial cell concentration (cells mL^{-1})
C_t= final cell concentration (cells mL^{-1})
t= time animals were allowed to feed (hours)
N= number of animals per replicate

UNIVERSITY OF STIRLING, *ECOTOXICOLOGY GROUP*
STANDARD OPERATING PROCEDURE

Installation Date:
Status:

Title: Laboratory Feeding Inhibition Bioassay for the Cladoceran *Simocephalus vetulus.*

(i) PURPOSE:

Standardisation of procedure for assessment and processing of *Simocephalus vetulus* feeding inhibition test to assess sublethal effects of chemicals, effluents and ambient waters.

(ii) SCOPE:

To be applied in all laboratory feeding inhibition tests utilising *S.vetulus.*

(iii) RESPONSIBILITIES:

None.

(iv) DEFINITIONS:

None.

(v) FORMS:

None.

(vi) REFERENCES:

Allen Y. *et al.*(1995), A mechanistic model of contaminant-induced feeding inhibition in *Daphnia magna.* Environmental Toxicology and Chemistry (14) 1625-1630.
ASTM (1980), Standard practise for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. E-729-80, ASTM Philadelphia, PA.

(vii) PROCEDURE:

Materials (for acclimation of experimental animals)
180mL wide rim glass bottles with screw cap
ASTM hard water
Seaweed extract (*Ascophyllum nodosum)* (Marinure, Glenside Organics Ltd.)-concentrate made up to a solution of 1:100, adding 9mL to 1L nanopure water (18MΩ), with optical density of 0.6-0.7 at 400nm in a 1 in 10 dilution
Chlorella vulgaris at a concentration of $2x10^6$ cells mL^{-1}

Materials (for exposure in test)- 8 treatments + control, 4 replicates
144, 7-8 day old *S.vetulus* (after release of first brood)
36 x 60ml wide rimmed glass bottles with screw cap
200mL of each exposure solution, in ASTM hard water, containing *C.vulgaris* at a concentration of $2x10^6$ cells mL^{-1}
C.vulgaris at concentration of $1x10^8$ cells mL^{-1}: 800µL per 40mL
3mL plastic pipettes

Materials for measuring of feeding rates
Can be carried out using either a Coulter Counter of a UV spectrophotometer.
FOR THE COULTER COUNTER:
Coulter Multisizer Model II (Coulter Electronics Ltd.) fitted with a 70µm orifice tube
Accuvette disposable sample containers (Coulter Electronics Ltd.)
Isoton II electrolyte (Coulter Electronics Ltd.)
200-1000µL micropipette

FOR THE UV SPECTROPHOTOMETER:
UV spectrophotometer equipped to hold a cuvette with a 4cm pathlength
Cuvettes, with 4cm pathlength
ASTM hard or moderately hard water

Procedure

Acclimation of *S.vetulus:*

Volume of ASTM required depends on number of animals necessary for experiments. For a test with 8 treatments + control, with 4 replicates, 144 animals are needed. 20 animals require one 180mL glass bottle with 150mL volume. 150 animals have to be kept in 8 individual glass bottles, so about 1200mL ASTM is needed.

Make up 1200mL of hard ASTM in a conical flask and add seaweed extract at a concentration of 5mL L^{-1}. Add *C.vulgaris* at a concentration of $2x10^6$ cells mL^{-1}. Put ~20 *S.vetulus* neonates (<24h old) into the glass jars, using a clean plastic pipette, making sure animals are not exposed to air. Keep the animals at 25°C in a light cycle of 16:8 hours light:dark.

Add *C.vulgaris* at a concentration of $2x10^6$ cells mL^{-1} each day, and put animals in fresh medium every two days, until animals release their first brood (7-8 days).

Feeding Inhibition Test

Prepare about 200mL of each exposure solution and the control solution and add relative amount of *C.vulgaris* to make up to a concentration of $2x10^6$ cells mL^{-1}.

Add 40mL of relative solutions to each 60mL glass bottle.

Put 4 animals into each exposure bottle, using a clean plastic pipette. Make sure to only add minimal, and equal, amounts of water when transferring animals from acclimation vessel to test bottles.

Close all bottles with aluminium foil and/or a lid to avoid evaporation.

Put all bottles in a box or tray and transfer to an incubator or constant temperature room at 25°C and leave animals to feed for 24 hours in the dark.

Count samples of exposure solutions in the Coulter Counter to make sure the concentrations at t=0h were $2x10^6$ cells mL^{-1}, or make notes of concentrations. Keep some samples of exposure solution to be counted at t=24h, to make sure no changes occur over 24 hours.

After 24 hours, remove animals from bottles. First make notes of dead and alive animals and any neonates, if there should be any. Then make sure the algal solution in the exposure bottles is evenly mixed, as algae tend to settle on the bottom, by shaking the bottle vigorously and stirring up algae with a glass pipette. Remove animals from the bottles using a pipette.

Algae samples may be stored in a fridge at 4°C overnight, or frozen if necessary, before measuring feeding rates. For best results however algae samples should be processed on the same day, as a certain degree of cell damage and degradation will occur.

Measurement of feeding rates.

COULTER COUNTER:

Make sure algal sample solution is well mixed by vigorously shaking container. Withdraw a 0.5mL sample using a micropipette and add to 19.5mL Isoton II in an accuvette disposable sample container.

Gently mix sample in accuvette by turning upside town, making sure not to create any bubbles that can interfere with the counting process.

Place accuvette on sample platform in Coulter Counter with the orifice tube and foil submerged in the sample.

Set Coulter Counter to automatically sample 500µL of sample. Make 3 counts per sample.

Multiply the average of the 3 counts by 80 to obtain the final number of cells per millilitre. Blank jars give the initial number of cells per millilitre. Make sure the blank cell concentration did not change significantly between t=0h and t=24h.

UV SPECTROPHOTOMETER

Mix jar containing algal sample well and fill a cuvette with a 4cm pathlength with the sample. Measure 2 samples for each jar.

Measure optical density of sample in a UV spectrophotometer at 440nm using ASTM as a blank.

CALCULATION OF FEEDING RATES:

Feeding rates are calculated using the equation given in Allen (1995), which is as follows:

$$F = \frac{V.(C_0 - C_t)}{t.N}$$

where:
F= feeding rate of single animal (cells ind^{-1} hr^{-1})
V= volume of suspension (mL)
C_0= initial cell concentration (cells mL^{-1})
C_t= final cell concentration (cells mL^{-1})
t= time animals were allowed to feed (hours)
N= number of animals per replicate

APPENDIX 2

Table containing feeding inhibition data for mixture experiments Chapter 5. Data for 4 replicates, feeding inhibition= % inhibition of control.

Experiment 1 Cd+AT	Observed effect (% control feeding inhibition, 4 replicates	Experiment 8 AT+DM	Observed effect (% control feeding inhibition, 4 replicates
1	89; 91;78;64	1	86;52;70;70
2	48; 72;71;72	2	20;28;22;18
3	82;81;74;84	3	54;54;54;53
4	58;44;60;52	4	22;25;11;13
5	27;16;27;54	5	17;16;8;13
Experiment 2 Cd+ AT		Experiment 9 AT+DM	
1	75;100;92;92	1	81;68;60;70
2	58;73;71;62	2	22;23;28;27
3	95;82;79;87	3	41;32;53;59
4	66;61;75;84	4	15;15;11;18
5	68;65;64;44	5	0;0;0;0;
Experiment 3 Cd+AT		Experiment 10 AT+DM	
1	84;98;88;82	1	80;68;84;67
2	66;57;63;66	2	78;62;76;82
3	95;93;83;82	3	88;74;68;64
4	64;58;61;60	4	60;73;64;52
5	58;36;52;40	5	61;55;49;48
Experiment 4 Cd+AT		Experiment 17 Cd+DM+AT	
1	92;92;96;90	1	49;61;80;70
2	43;35;36;42	2	61;58;55;65
3	55;57;44;55	3	78;54;60;79
4	25;21;34;34	4	59;78;54;58
Experiment 5 Cd+DM		5	60;58;46;47
1	94;92;98;93	6	54;46;44;40
2	87;100;96;-	Experiment 18 Cd+DM+AT	
3	89;97;97;95	1	77;70;82;92
4	95;95;92;91	2	69;76;65;85
5	80;87;82;78	3	70;69;76;73
Experiment 7 DM+AT		4	59;54;71;59
1	95;85;77;79	5	61;57;54;51
2	46;66;58;61	6	31;33;36;41
3	98;96;97;97		
4	85;78;78;-		