FEEDING BEHAVIOUR OF THE PRAWN *Macrobrachium rosenbergii* AS AN INDICATOR OF PESTICIDE CONTAMINATION IN TROPICAL FRESHWATER

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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.

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ABSTRACT

The purpose of this research was to develop and standardize a novel feeding bioassay with *Macrobrachium rosenbergii* for use in the laboratory and allowing it to be easily deployed under field conditions. Standardization of the test aimed to minimize feeding rate variations and to ensure that subsequent statistical analyses have sufficient power to consistently detect changes in feeding rates. These were accomplished through the development of a post-exposure feeding toxicity test under laboratory, microcosm and *in situ*/field conditions. This procedure was proven to be repeatable and economical. *M. rosenbergii* as test animals were available in terms of quantity and uniformity in sizes.

The standard guidelines and procedures for *M. rosenbergii* bioassay developed from this study include the size of test animals (9-10 mm), density in exposure containers (10 animals in 500 mL of medium in the laboratory, 10 animals in field chambers with 98.6 mL volume), exposure time (24 hours), feeding period for post-exposure feeding (4 hours) and number of replicates for the feeding test (10 replicates for individual measurements).

The tiered approach used in the preliminary risk assessment of pesticide using TOXSWA was capable of screening the risk level of pesticide in the study area, identifying profenofos and dimethoate as test chemicals for the lethal and sub-lethal experiments. This model was beneficial in the preliminary risk assessment of pesticides in the tropics, since it was not necessary to set up laboratory work. This method could also provide preliminary data to support the environmental planner and decision/policy maker. This is an alternative way to develop a cost efficient model to inform and warn the risk of pesticide use.

The effects of pH, temperature and hardness on control post-exposure feeding rates of *M. rosenbergii* were assessed and indicated that *M. rosenbergii* was very sensitive to acidic and basic conditions.

The use of post-exposure feeding inhibition as the endpoint under laboratory conditions revealed that prawns were sensitive to pesticides (chlorpyrifos, dimethoate and profenofos) and a heavy metal (zinc). Post-exposure feeding rate inhibition could be used as a sublethal endpoint as the EC_{50} values obtained for chlorpyrifos and zinc were lower than their lethal levels.

Mortality of prawn was also another endpoint used to define the toxicity of pesticides such as carbendazim, in which mortality occurred during exposure, but post-exposure feeding rate of the surviving animals did not decrease.

The microcosm experiments were able to link the laboratory toxicity tests and the effects observed in the field. Microcosm studies provided another dimension to studies looking at pesticide effects on aquatic systems. In this research, carbendazim affected feeding and survival rates in the microcosm set-up but in the laboratory only mortality showed a significant difference ($P < 0.05$).

In situ bioassays were able to show the effects of pesticides on post-exposure feeding rates using the methods developed. Post-exposure feeding rates were significantly lower than control in farms using pesticides while in uncontaminated sites (pesticide-free), the post-exposure feeding rates did not decrease. However, mortality was observed even in the uncontaminated sites which could be attributed to other factors such as low dissolved oxygen and presence of some other unidentified chemical substances. The degree of mortality and the effect on feeding rates depends not only on the type and concentration of the known pesticide but also on water quality parameters.

The basic methods developed for *in situ* bioassay from this research is a simple, easy and fast way to determine the effect of pesticides because the results can be seen in the field. The procedures developed and results obtained from this study can be used as a basis for further toxicity studies on *M. rosenbergii* and other potential tropical species.

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Glossary

Acute effects – results occurring rapidly from short-term exposure to a chemical, such as within a few hours, days, or weeks for fish and other aquatic organisms. Acute effects are generally considered critical, such as lethality or mortality.

Acute exposure – exposing organisms to a chemical once or several times within a short period of time, such as hours or days.

Aquatic toxicity tests – conducted to identify and assess the possible adverse effects of a chemical on aquatic organisms. These tests supply basic information for assessment of risk of chemicals on the organism and its environment.

Aquatic toxicology - the study of the adverse toxic effects of chemicals and other foreign materials on aquatic organisms, using both qualitative and quantitative processes.

Bioassay – a test to assess the strength or potency of a chemical by evaluating its effect on an organism against that of a standard preparation; also conducted to determine the extent of response the test organism has on exposure to a chemical.

Biocoenosis – describes all organisms living and interrelating together in a certain habitat; common synonyms are biotic community and ecological community

Chronic or subchronic toxic effects – results occurring when a chemical produces harmful effects from a single or repeated exposure over a long term period. These effects may be lethal or sublethal. If the exposure concentration is low, there may be a relatively long latency (time to occurrence) period for the expression of these effects.

Chronic exposure – exposing organisms to concentrations of a chemical continuously or at frequent periods over a long period of time, such as weeks, months, or years.

EC_{50} - median effective concentration; the concentration estimated to cause a specific effect (i.e. behavioural or physiological) on 50 % of the test organisms after a certain period of exposure (such as 24, 48 or 96 hours).

Exposure – the contact between an organism and a chemical to obtain a reaction. The most significant factors related to exposure in toxicity assessments are the duration, frequency and kind of exposure and the chemical concentration.

Gravid – carrying developing eggs or young

Half-life – time required to reduce the initial concentration of a chemical by 50%.

Irreversible toxic effects – result from serious damage or injury to an organism from exposure, and may lead to death.

LC_{50} – medial lethal concentration; the concentration estimated to cause a 50% mortality among test organisms over a certain time period (such as 24, 48 or 96 hours).

Lethal effect – death or mortality of organisms, quantifiably expressed by the number or percentage of organisms killed. May be an indirect result of sublethal effects, for e.g. behavioral changes (e.g., swimming or olfactory) may reduce the ability of aquatic organisms to find food or to escape from predators which will eventually lead to their death.

Local effects – occur at the primary site of contact, either on an organism (e.g. skin discoloration or inflammation in fish) or a location or space (e.g. farm canal or river) exposed to various organic and inorganic compounds.

MRL - maximum residue limit; the maximum allowable amount of pesticide residue in food

Non-selective chemical – a chemical that has undesirable effects on numerous cells and tissues of organisms, and may be effective in small concentrations.

Reversible toxic effects – occur through normal repair mechanisms such as regeneration of damaged tissues, and possible only if the organism is able to escape from the toxic medium and exposed to an uncontaminated environment.

Risk presents a probability that certain conditions will adversely affect an organism or environment.

Selective chemical – a chemical that has an adverse effect on only one type of cell or tissue without harming others that are nearby. The cells or tissues affected and unaffected may be in the same or different organisms. If they are in different organisms, the chemical is *species-specific* in its selective activity.

Setae – stiff hair-like projections covering bodies of crustaceans, containing nerves used for sensory functions

Species-specific – pertains only to a certain species

Subchronic exposure – exposing organisms to chemicals in intermediate duration, such as a month to several months, or less than a complete reproductive life cycle, or during the sensitive early stages of development.

Sub-lethal effect – behavioural changes (growth, development and reproduction), biochemical changes (blood enzyme and ion levels), and histological changes. These effects are expressed by quantifiable criteria such as percent egg hatchability, changes in length and weight, percent enzyme inhibition, number of skeletal abnormalities, decrease in feeding rate and tumor incidence.

Systemic effects – occur when the chemical is absorbed or distributed to a site distant from its primary site of entry, e.g. effects on nervous system or other internal organs.

Toxicant – chemical that produces an adverse response (effect) in a system resulting in serious damage to its structure or function, including death.

Toxicity – the potential of a chemical to harm a living organism, usually a function of the chemical concentration and length of exposure. Chemical substances are usually compared using toxicity data.

Toxicological interaction – a biological response resulting from exposure to two or more chemicals. This response is different from the response each of the chemicals may effect from an organism when exposed to each of the chemicals alone.

Toxicity tests – conducted to identify and assess a chemical's adverse effects on organisms using standardized, repeatable conditions to allow comparison with other chemicals tested. These tests are also used to evaluate the concentrations of the chemical and the duration of exposure required to produce a certain effect.

Xenobiotic – a compound, such as a chemical or pesticide, which is foreign to a body or living organism, and introduced into the environment by artificial means

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CHAPTER 1

INTRODUCTION

1.1 General introduction

The rapid agricultural development in Thailand over the last few decades has essentially aimed at increasing productivity. An expanding agricultural sector has responded to an increasing domestic demand, supplied agro-industry with agricultural products as raw materials and exported food products to neighbouring and developed countries. Adoption of modern technology and the use of agricultural inputs, namely fertilizers and pesticides have been a key part of this development. Pesticides, in particular, have long been associated with hazardous impacts on human beings, animals, vegetation and the environment (Murty, 1986; Jeyaratnam, 1990; DeLorenzo *et al.*, 2001; Siriruttanapruk and Anantagulnathi, 2004). The Department of Agriculture is responsible for controlling pesticides through the Hazardous Substances Act, B.E. 2535 (1992). In Thailand, it has been recognized that legislative measures alone are not enough to solve problems caused by pesticides since the law on hazardous substances mainly focuses on the control of imports and trade of hazardous substances.

This growth in the use of pesticides in Thailand reflects global trends. Worldwide pesticide use has risen steadily, increasing to 20.6 % from 1990 to 1998 (Dinham, 1993; Yudelman *et al.*, 1998). According to the Foundation for Advancements in Science and Education (FASE), exports of hazardous pesticides from U.S. ports grew by 26 % between 1992 and 1994, from 45.4 to 57.3 thousand tonnes, respectively, totaling more than 156.4 thousand tonnes during this period, the majority of which were exported to the developing world (PANNA InfoPubs, 1996). But the developed countries especially those located in North America and Western Europe still remained as the main users of pesticides in the world.

By 1998, world pesticide consumption reached US$ 34 billion, with North America and Western Europe being the major consumers (26.4 and 26.3 % of the total world pesticide consumption, respectively). The Asia/Oceania region used 47.3 % of the total consumption (Yudelman *et al.*, 1998).

Marketing of pesticides worldwide is strongly influenced by a small group of transnational corporations (TNCs) based in Western Europe and the United States. U.S.-based firms dominate the Latin American market, whereas European TNCs dominate the African and Asian markets. TNCs consider Latin America and Southeast Asia as important markets, including the richer less-developed countries (LDCs), which already use significant amounts of pesticides, such as Brazil, China, Malaysia, Mexico and Thailand (Frey, 1995).

Thailand began using pesticides such as chemical and synthetic substances during World War II with the importation of DDT [1,1,1-trichloro-2,2-bis-(p-chlorophenyl) ethane] for an anti-malaria campaign (Sirisingh, 2000). In addition to being used to kill mosquitoes, DDT was used as an insecticide for health reasons amongst the allied troops. After World War II ended, DDT continued to be widely used to kill mosquitoes and flies. Since 1960, following the "Green Revolution", a greater variety of chemicals and synthetics were introduced into the country mainly for crop protection, particularly those classified as organochlorines (OC), organophosphates (OP) and carbamates (Staring, 1984).

Thailand has an agricultural land area of about 21.6 million ha out of the 51 million ha available, or about half of the country's total land area. Agriculture is practiced by 60 % of the total population and rice is the dominant crop produced on 40 % of the cropped land area (Falvey, 2000). Cassava, flowers, fruit, rubber, sugar cane and vegetables are also major crops for local consumption and export. The Thai government substantially supports research on crop production, the results of which are later transferred to farmers. Advanced technologies and the expansion of agricultural area have led to changes in crop cultivation and pesticide use has become part of an accepted and standard culture method. Diversification of Thai agriculture from rice culture to horticulture including value-added export production has resulted

in increased use of pesticides (Falvey, 2000; Jungbluth, 2000). Whereas the frequency of pesticide application is up to four times per one crop cycle of 4-5 months for rice, weekly application is often used in horticulture.

Thailand has not remained as an agricultural society although the agricultural sector continues to be important. Bank of Thailand (1995) reported that the industrial sector grew from 25 % of Gross Domestic Product (GDP) in 1970 to 38 % in 1993 and manufacturing from 16 % of GDP to 26 %, while the agriculture sector decreased from 27 % to 12 % for the same period. By 1996 the GDP share of the agricultural sector had declined to 10.7% (Bank of Thailand, 1998) indicating that Thailand's economy has become increasingly based on the industrial and manufacturing sectors. The growth of these sectors together with zero tax on pesticide importation (Jungbluth, 2000) had encouraged the development of a pesticide agribusiness sector, including pesticide formulating companies and retailers. Many pesticide banned in developed countries are imported, sold and mixed with other pesticides in Thailand. These practices tend to remain undetected because of the limited range of analytical procedures available locally. Jungbluth (2000) reported that a large number of companies import and sell pesticides in Thailand, which include around 69 formulating and repackaging plants, 438 distributors and 5,000 retailers. The business is driven by diversification in agricultural practices especially towards fruit and vegetables production. The growth in the industrial and manufacturing sectors have made conditions favourable for pesticides to be imported, formulated and reformulated locally and used in large amounts each year, creating potential problems for human health and the environment in Thailand (Tabucanon and Boonyatumanond, 1998).

Pesticide use in Thailand has increased considerably through the years. Figure 1.1 shows the increasing trend of pesticide imports over a 20-year period (1980 to 1999). The most important class of pesticides in terms of quantity are the herbicides, followed by insecticides, fungicides and others such as acaricide, rodenticide and molluscicide (Sirisingh, 2000). Ten compounds that were mostly imported for agricultural uses based on the amount imported in 1996 were the herbicides glyphosate, 2,4-D, atazine, ametryn; insecticides methamidophos, monocrotophos,

methyl parathion, endosulfan and fungicides copper oxychloride, mancozeb (fungicide).

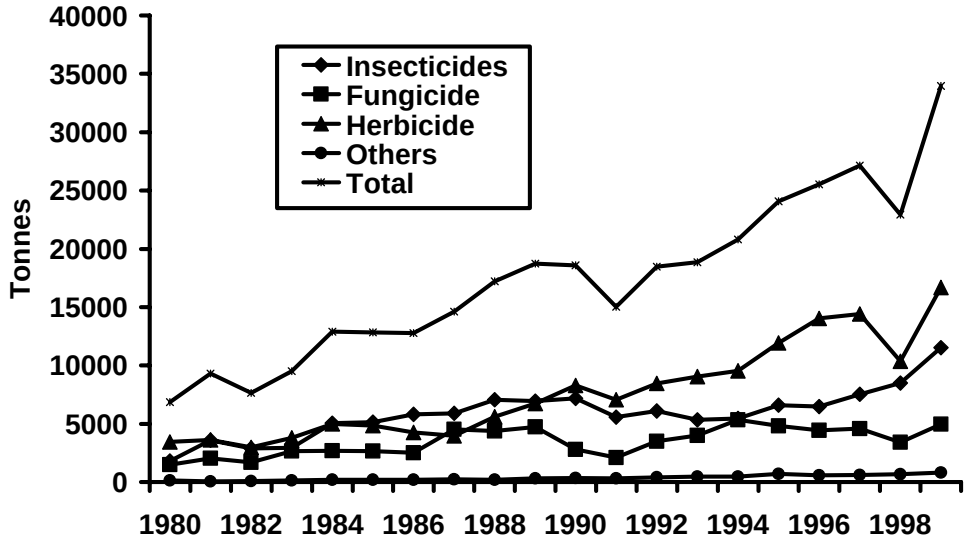


Figure 1.1 Quantity of pesticides imported into Thailand, 1980-1999. Source: Agricultural Regulatory Division, Thai Department of Agriculture

1.2 The aquatic environment

The aquatic environment is composed of many complex and diverse ecosystems with living and non-living components interacting with each other. The physical and chemical processes within these ecosystems can affect biological activities and impacts of external substances such as chemicals on organisms. These substances may be introduced into the system through effluent discharges, run-offs and spills. The aquatic environment is susceptible to contamination from these chemicals depending on factors such as the type of chemical and its properties, the proximity of the ecosystem to site of entry, concentration entering the ecosystem, period and method of introduction (once or continuous), and condition of the ecosystem (e.g. dissolved oxygen and pH levels) to enable it to resist chemical onslaught or to return to its original conditions when contamination is removed (e.g.

neutralizing with another substance or flushing by tidal action) (Rand and Petrocelli, 1985).

Water, the major component in these systems, is one of the most important inputs in agriculture. Thus it is not surprising to find rice and horticulture cultivation areas to be located near aquaculture areas, sharing the same water resources. Farm water supply canals may receive water drained from farms using pesticides for their crops resulting in contamination of the water resource. The presence of pesticides in paddy fields has been found to affect the paddy field ecosystem including fish and other biota (Abdullah *et al.*, 1997). In Thailand, where 70% of the common agricultural chemicals could be found in the soil and water, there is a rising public concern over the use of chemicals in agriculture (Falvey, 2000).

1.3 Impacts of pesticides

Pesticides are used to control or eliminate pests, weeds, fungi and other unwanted species in the agricultural system. However, when they are applied, even non-target organisms, the environment they are in and the users are also affected (Rand and Petrocelli, 1985; Abdullah *et al.*, 1997; Kennedy, 1998). The over-use and misuse of pesticides are detrimental to the health of users, consumers and the environment (Sirisingh, 2000; Tabucanon and Boonyatumanond, 1998). The extent and varied impacts on human health from pesticide exposure through food, occupation and the environment have been widely covered by FAO (2002; 2003).

Figure 1.2 illustrates the direct and indirect impacts of pesticides on humans and the environment.

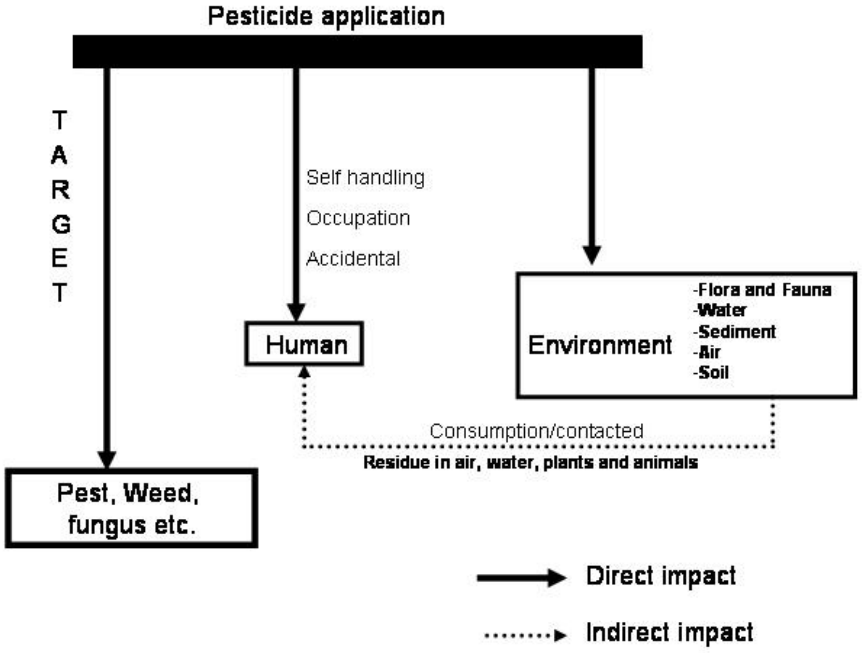


Figure 1.2 Impact of pesticide application on humans and the environment (D.Little, personal communication)

1.3.1 Human impacts

Risk to human health is always the primary issue whether contact with pesticides is direct or indirect. There are many ways that man may be directly exposed to pesticides, at different doses and exposure times. Wong and Ng (1984) as cited by Jeyaratnam (1990) described the levels of risk of human exposure to pesticides, where all population groups are at risk on a long-term low-level exposure, pesticide manufacturers, formulators, mixers, applicators and pickers have long-term high level exposure and those with single and short-term very high-level exposure include suicides and mass poisoning, pesticide formulators, mixers, applicators and pickers. Exposure to pesticides has been documented to cause health problems and defects such as attention deficit and hyperactivity disorders in children, birth defects, brain damage, cancer, chronic neurotoxic effects, infertility, miscarriages, Parkinson's

disease and weakening of immune system (Purdey, 1994; GAO, 2002; Flower *et al*., 2004).

In Thailand, 3,035 cases of occupational disease cases have been reported in 2001, of which 87.4% were pesticide poisoning (Siriruttanapruk and Anantagulnath, 2004). In Zimbabwe, small-scale cotton growers in the districts of Sanyati and Chipinge experienced health effects from direct and indirect exposure to pesticides resulting in economic losses of Z$180 and Z$316 (1 US$ = Z$57 in January 2003; Z$99,416 in April 2006, approximately), equivalent to 45 % and 83 % of the annual household pesticide expenditures, respectively (Maumbe and Swinton, 2003). In addition, those who suffered from illnesses caused by pesticides had to spend between 2 to 4 days to recuperate.

Indirect impacts of pesticides on humans include consumption of food contaminated with pesticides as well as contact with pesticide residues in the air, water, soil, sediment, food materials, plants and animals (Seth *et al*., 1998). The use of pesticides in shrimp culture may expose consumers to their residues (Johnston *et al*., 1998) but an emphasis on antibiotics has focused attention away from pesticide residues in shrimps (Graslund and Bengtsson, 2001). Organochlorines such as dioxins and polychlorinated biphenyls (PCBs) have the potential to contaminate farmed fish and shrimps at high concentrations, through their presence in fishmeal used in their feed (Stokstad, 2004).

The pesticides hexachloro-cyclohexane (HCH) and DDT have been found in varying levels from trace amounts to more than the acceptable limits in vegetables, edible oil and oil seeds, cereals, pulses, coffee, tea, milk and milk products, meats, eggs and fish in India (Seth *et al*., 1998). In addition, residue levels in humans were also found, with the occupationally exposed population having three times higher residue levels than the general population.

Although oganochlorine pesticide residues at levels below the maximum residue limit (MRL) have been found in green mussels growing along the Gulf of

Thailand, concerns have been raised regarding the presence of pesticide residues in food in Thailand (Tabucanon and Boonyatumanond, 1998).

Similarly, in China, pesticide contamination of vegetables has reached critical levels, where pesticide levels up to 8.4 times above standard were found (Dai *et al*., 1998). Furthermore concerns on pesticide residue levels in tea leaves and fruit also exist.

1.3.2 Environmental impacts

The use of large quantities of pesticides in LDCs has affected the atmosphere, water body, soil, ecosystem (Dai *et al*., 1998) resulting in the destruction of fauna and flora and pollution of the environment. A large body of literature has documented the effects of pesticides on animals (Carson, 1962; Kennedy *et al*., 1998; Minh *et al.*, 2002; Sakamoto *et al*., 2002; Kenntner *et al*., 2003).

Sources of pesticide contamination may include terrestrial runoff into rivers and streams from urban areas, agricultural areas and pesticide waste dumps. According to a report from FAO (2001), many countries in Africa stocked pesticide in certain areas and these became waste dump sites. All these sites contained some of the most dangerous insecticides such as aldrin, chlordane, DDT, dieldrin, endrin and heptachlor which have all been banned in most countries, along with organophosphates. This FAO report also emphasized that as pesticides deteriorate, they form by-products which may be more toxic than the original substance. In addition to pesticides, waste sites also contain contaminated pesticide sprayers, empty pesticide containers and huge quantities of heavily polluted soil.

The rice field ecosystem in tropical countries has been specifically at risk of negative impacts from pesticide use (Abdullah *et al*., 1997) as rice production has intensified in recent decades. In a study done by Varca and Tejada (1998), most of the pesticides used in rice fields (azinphos ethyl, endosulfan, cyfluthrin, chlorpyrifos, fenvalerate, cypermethrin, triazophos, etofenprox) were extremely toxic (48 h LC_{50} <

0.5 mg/L) to fish. Furthermore, carbofuran residue was detected in Nile tilapia, where residues were highest in the gut tissue.

In Japan, waters from rivers used for irrigation of rice paddies eventually flow into lakes, bringing with them the pesticides from the fields and potentially polluting the lake waters, aquatic plants and organisms (Nohara and Iwakuma, 1996; Shigehisa and Shiraishi, 1998). Insecticides detected in sediments, suspended particles and in water from a stream in Argentina posed an acute risk to aquatic life (Jergentz *et al*., 2005).

In Vietnam, concentrations of persistent organochlorides (OC) such as polychlorinated biphenyls (PCBs), 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) and its metabolites (DDTs), hexachlorocyclohexane isomers (HCHs), hexachlorobenzene (HCB) and chlordane compounds (CHLs) were analysed in bodies of resident and migratory birds from the Red River estuary in 1997 by Minh *et al.* (2002) who found that resident birds had greater concentrations of DDTs compared to migrants while HCH was greater in migrant birds. Moreover, when OC residues in avian species in Asia-Pacific were compared, the DDT residue levels in resident birds in North Vietnam were among the highest reported, implying that DDT has been used liberally in Vietnam during the period of analysis. The main food items of these birds were insects and other lower trophic organisms such as invertebrates, mollusks and crustaceans. Even though DDT has been banned since the early 1970s in the United States, some Asian countries still continue to use it.

In Thailand, more than 60% of pesticide is used on rice, cotton, fruit and vegetables, with fruit and vegetables increasing in importance due to their high value in both domestic and export markets (Tayaputch, 1998). Problems brought about by heavy pesticide use and mismanagement have emerged which called for more controls and better education regarding the hazardous effects of pesticide use (Tabucanon and Boonyatumanond, 1998). Despite the popularity gained by organic or pesticide-safe vegetables, pesticide use has continued to increase, contaminating the soil, water and the environment (Sirisingh, 2000).

1.4 Understanding pesticides in the aquatic environment and their impacts

Methods for measuring pesticides and their impacts are most developed in temperate countries. Greater wealth, public concern and government regulation has stimulated such development.

There are many methods to study the impact of pesticide on the environment, and some of them are listed in Table 1.1, including information on the results these methods can provide. The choice of method to use would depend on the objectives of the study being done, the methods to assess the risk, complexity of the research set up and relevance to real ecosystem. Each method has its own advantages and disadvantages.

Table 1.1 Methods to assess the impact of pesticide

Method	Results that can be obtained
1. Monitoring water, soil and sediment	Amount of pesticide residue occurring at a particular time and date
2. Acute toxicity tests	Lethal concentration effect for 50 % of the organisms
3. Chronic toxicity tests	Sublethal effect (behavioural, physiological and biochemical)
4. Microcosm and mesocosm	Multi-function effects : fate and effect data (based on made-up ecosystem)
5. Biodiversity changes	Total number and type of organisms change over time
6. Computer modeling	Predicted environmental concentration (PEC) in surface water
7. *In situ* bioassay	Multi-function effects: fate and effect data (based on actual field exposures)

1.4.1 Monitoring of water and sediment

Water or sediment samples are commonly analysed for specific toxic substances using High Performance Liquid Chromatography (HPLC) or Gas Chromatography (GC). The results obtained are compared with existing toxicity data or the maximum limited concentration data such as FAO environmental quality standards and the Environmental Projection Agency (EPA) environmental quality standards. The result could exceed the norm depending on the standard used for comparison. Most of the monitoring data are not up to date, with some updated every five years only. Thus this does not represent the current situation but could be used for the water quality and pesticide contamination database. This kind of data is useful for trend and projection of potential for pollution. According to CRC (1998), it is expensive to use HPLC and GC for analysis. Furthermore, there is a need to consider the logistics due to the nature of the chemicals. Analysis needs relevant standards to ensure the data generated are relevant and usable.

Environmental Quality Standards (EQS) are maximum concentration limits of substances in the water to protect the aquatic environment (Graham, 2004), expressed as maximum admissible concentration (MAC) for short-term exposure, and annual average (AA) for continuous and long-term exposure. In addition, the EQS of many substances also relies on other water quality parameters, such as hardness which affects the toxicity of metals, and temperature and pH which affects the conversion of the ammonium ion to its more toxic form, unionized ammonia.

1.4.2 Acute and chronic toxicity tests

Toxicity tests are laboratory-based tests conducted to assess the detrimental effects of chemicals on organisms. These tests can either be acute (short-term exposure of the organism at various concentrations of the test chemical) or chronic (long-term exposure at sub-lethal concentrations). The most common criterion in acute toxicity tests is mortality of the test organism. Data are expressed as the median lethal concentration (LC_{50}) and median effective concentration (EC_{50}). On the other hand, chronic toxicity tests are useful with chemicals that do not show adverse effects

on aquatic organisms in acute toxicity tests which might be mistaken as not toxic to these organisms (Van Leeuwen, 1995).

Acute toxicity tests measure effects of a certain substance on an organism over a period of time, usually between 24 and 96 hours (Adam, 1995). Acute or lethal toxicity tests are conducted to determine the concentration of chemical or pesticide which can cause a 50, 10 or 5 % mortality of the test organism. The median lethal concentration (LC50) to cause 50% mortality over a particular time period is calculated using the probit analysis or log transfer.

In chronic toxicity tests organisms are exposed to a toxicant over a longer period of time, usually a significant portion of an organism's lifetime, such as a tenth or more (Adam, 1995). The end result is to determine the long term effect of the toxicant on an organism through a change in its biological function such as reproduction.

However, the relevance of acute and chronic toxicity tests is limited as the results often do not reflect the real situation in the field. They do not take into consideration the complexity of the ecosystem, such as the presence of other species, water and soil dynamics and environmental factors such as dissolved oxygen, temperature, pH and hardness, which could affect the response of the target organism to the chemicals.

A major benefit though of these tests is that they allow a ranking of chemicals in terms of their toxicity.

1.4.3 Microcosm and mesocosm

Microcosm and mesocosm are defined by Leeuwen and Hermens (1996) as those artificial multi-species test systems that simulate the major characteristics of the natural environment to assess ecotoxicological effects and risk. Such systems are either terrestrial or aquatic and may contain plants, vertebrates and invertebrates. Mesocosms refer to larger and more complex systems than microcosms although

there is often no clear distinction. Data obtained from this set-up on pesticide effect are considered mid-way between the laboratory and field derived data. Van der Hoeven and Gerritsen (1997), Van den Brink *et al.* (1996 and 2000) and Cuppen *et al.* (2000) have done work using these systems, integrating ecology and toxicology thus giving a greater understanding of the impact of pesticides on the environment than from toxicity tests alone.

Microcosm and mesocosm test systems are achieved by introducing basic components of natural ecosystems and giving access to physiochemical, biological and toxicological parameters that can be controlled to some extent (Rand and Petrocelli, 1985). Results from these test systems could be analysed through classical parametric statistical methods, nonparametric approaches and/or multi-variate analysis. Microcosms and mesocosms are higher tier risk assessment methods used to validate the first tier risk assessment (Cuppen *et al.*, 2000). However, a disadvantage of using a multi-species test system such as these is the high cost of setting up and maintenance, leading to the question of whether they are cost-effective in the long run.

1.4.4 Biodiversity changes

Monitoring changes in biodiversity is an approach widely used in environmental and ecological assessments and pollution studies. Biodiversity refers to the range of organisms existing in a given ecological community or system. The number and types of organisms present in this ecosystem are compared with those of the previous years or a reference site (Kovacs, 1992).

Species and abundance relationships are expressed as diversity indices which are functions of the number of species and the number of individuals per species. These indices are widely used in ecological studies, but there are some conflicting views on their use (Kovacs, 1992). Diversity measures may give misleading results regarding the situation of environmental change, since other factors aside from environmental conditions affect biodiversity, such as presence of chemicals, seasonal variation, food availability and natural disturbances.

In terms of monitoring stress on the aquatic system, change in populations and communities may give an indication of the effects of stress, but not explain the way these effects are caused (Maltby, 1999). Furthermore, even if differences in the community structure were found, it would be impossible to determine how much was caused by a particular known stressor. Thus community-level measures such as biodiversity indices and scores are often insensitive to sub-lethal levels of stress. The use of such indices are, therefore, mainly for monitoring and ongoing environmental regulation but not for predictive ecotoxicological assessment.

1.4.5 Computer modeling

There has been great interest in using models to determine the fate and effect of toxic substances since the 1970s and currently computer simulations are one of the methods used to estimate the fate of toxic contaminants in aquatic environments (Halfon, 1989). Moreover, mathematical models have been useful in predicting the fate of contaminants yet these predictions are uncertain because of knowledge gaps between chemical structure and environmental behaviour.

Computer modeling can be used to predict pesticide concentrations but it needs considerable data and then monitoring effort to validate the results. Simple evaluative models have been introduced since the late 1970s, the main one of which is the fugacity model, where the relative quantity of substances is calculated and their distribution in the environment predicted, although there is no simulation of actual environmental conditions (Calamari and Vighi, 1992). Fugacity is based on the concept that a chemical substance has the ability to escape from a certain aquatic environmental compartment, such as sediment, water and fish. The relationship between fugacity and concentration of substance is linear (Mackay and Paterson, 1982) depending on temperature, pressure, nature of the substance and the environment being examined. The fugacity concept has been used to predict environmental distribution of substances, compare different substances and rank them based on their resemblance to a component in the environment. For example, it has been used to choose a number of herbicides that have the least likeness to water, which provided a basis to minimize the possibility of contaminating the water

(Calamari and Vighi, 1992). Despite the practical and concrete applications of the fugacity approach, there are problems and constraints in its use, such as other units of the biota (terrestrial plants for example) are not included, as it includes only aquatic animals. This could probably be due to the fact that there is not enough information on the bioconcentration factors (BCF) for terrestrial plants, although attempts have been made to include them in later fugacity models (Calamari *et al*., 1987). Other major problems cited by Calamari and Vighi (1992) in the use of fugacity models include the lack of quality basic data on the physico-chemical properties of substances, such as for water solubility and vapour pressure, and the lack of reference to the time it would take to attain equilibrium..

Another model developed to estimate and interpret the bioconcentration and bioavailability of xenobiotics in fish and other aquatic animals is the pharmacokinetic model, where bioconcentration refers to the accumulation of xenobiotics by aquatic organisms, and bioavailability refers to the rate and extent of a chemical entering an organism, mainly from water and sediment (Stehly *et al*., 1999a). Additionally, food web models are used to describe the activity of xenobiotics within the environment based on the quantity of contaminants and the biotic and abiotic factors affecting their movement. Thus, these models are useful in predicting the fate of xenobiotics in the environment and in measuring how much is accumulated in organisms, with work mainly done with Chinook salmon (Law, 1999), channel catfish and rainbow trout (Stehly *et al*., 1999b).

In 1996, the **TOX**ic substances in **S**urface **Wa**ters (TOXSWA) programme, one of the many software models which can help to assess the concentration of pesticide in water and sediment of small surface waters, was released by researchers from the Netherlands (Beltman and Adriaanse, 1999). Many versions have been released since then to improve its usage. TOXSWA is a computer model in which risk assessment starts from a simple risk screening (Tier 1) to more complex site specific risk assessment (Tier 2), describing pesticide behaviour in a small surface water system, including the sediment or in a water body at the edge-of-field scale such as a ditch, pond or stream near a single field (Beltman and Adriaanse, 1999). Additionally, the programme can calculate pesticide concentrations in the water layer in a

horizontal direction only and in the sediment layer in both horizontal and vertical directions.

Using computer models such as TOXSWA reduces the cost of laboratory and chemical analysis. Models also assist researchers and policy makers to focus on pesticides that pose high risks to a particular area. It is important to note that estimation models should be used within their scope and this limitation must be well defined (Van den Brink *et al*., 2003a).

Furthermore, the challenge for models such as TOXSWA is the "pesticide cocktail" i.e. a combination of pesticides used in one application which is in common use in LDCs. This type of pesticide use results in complexity and difficulty in studying the residue in the water body and sediments, and makes effects difficult to predict.

Computer models are useful in preliminary assessment of pesticide levels but may not be able to predict impacts on specific species or the environment as a whole.

1.4.6 *In situ* bioassay

In situ is a Latin phrase meaning *in its place*. A bioassay or biological assay measures the effect of a substance on a living organism (Rand and Petrocelli, 1985). *In situ* bioassays expose organisms directly to the conditions in the field where the actual responses of the organisms are affected by the various kinds of dynamics and exchanges among physical and chemical factors in the site (Kater *et al*., 2001).

In situ approaches have a considerable advantage in evaluating pesticide impacts under natural field conditions (Figure 1.3). They have been used to examine real pollution problems by exposing organisms directly where effluent is released or at a site where pollution is predicted to happen (Pascoe and Edwards, 1989). Biological, chemical and physical processes can be integrated into the test (Chappie and Burton, 1997).

Various endpoints in *in situ* bioassays are used to measure the organism's response to toxicity or stress. These endpoints can be lethal (mortality) or sublethal such as feeding depression or inhibition, growth, infertility, respiration, reproduction, enzyme activity, avoidance, moulting frequency, movement and deformity (Van Leeuwen, 1995; McLoughlin *et al*., 2000; Schulz, 2004). Since lethal effect is considered a crude measure, the use of sublethal physiological endpoints is preferred (McLoughlin *et al*., 2000). Specifically, feeding inhibition has been found as a sensitive endpoint to gauge the response of the organism to chemical exposure. Food is the basic requirement for growth and other physiological functions of the body and feeding is a daily and most frequent natural activity (Bond, 1996; McWilliam and Baird, 2002a). Furthermore, feeding declines in intensity or eventually stops in response to exposure to toxicants.

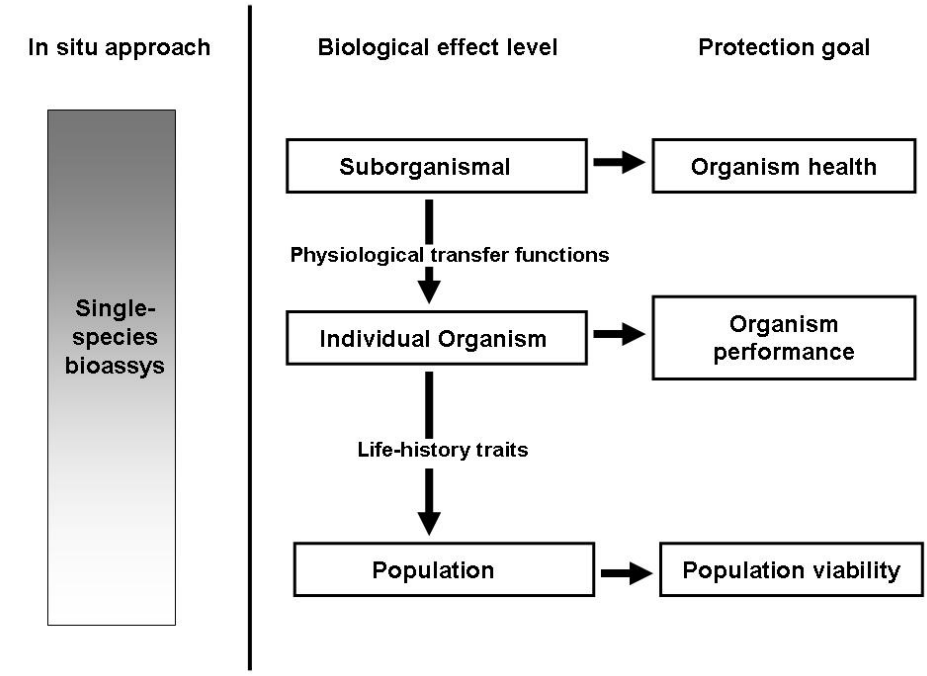


Figure 1.3 Flowchart of *in situ* approach (D Baird., personal communication).

In situ or field-based bioassays have several advantages over purely laboratory-based techniques (Pereira *et al*., 1999; McWilliam and Baird, 2002b; Moreira *et al*., 2005). The major advantage when studying contaminants in the water is that there is no delay between sample collection and testing, preventing the

volatilization or rapid degradation of chemicals such as pesticide present in the water (Crane *et al.*, 1995).

Successful *in situ* testings had been achieved with a number of species and among them studies by Chappie and Burton (1997), Maltby *et al.* (2002a) and McWilliam and Baird (2002b) are described below:

Chappie and Burton (1997) conducted *in situ* testing with *Chironomus tentans* (midge) and *Hyalella azteca* (amphipod) for 2 and 4 weeks exposure, respectively, to evaluate the effectiveness of the method. Two sites were used, one in a creek surrounded by agricultural and forest areas and the other a pond receiving runoff from parking lots, roads and commercial areas. In conducting the tests it was concluded that many uncertainties and unnatural features associated with laboratory bioassays were reduced, such as effects of collection and storage of samples, as well as taking into consideration irregular changes and pollution occurrences. In situ testing then was effective in the assessment of short- to long-term conditions of water and sediment quality.

Maltby *et al.*, (2002a) assessed the usefulness of a single species *in situ* testing on *Gammarus pulex* (amphipod) to observe its feeding behaviour as a biomonitor of water quality. Most organisms show signs of feeding inhibition as a response to stress, although it can also be brought about by many other factors in the environment. Thus for it to be effective as an indicator of water quality, the causes of feeding inhibition need to be known. Deployments were in both contaminated (near point-source discharges) and uncontaminated sites in rivers in England and Scotland for 6 days during winter and summer/autumn. It was concluded that this method was a good example of a short-term sublethal biomonitoring of water quality as it showed community- and ecosystem-level responses over time.

McWilliam and Baird (2002b) conducted *in situ* bioassays with *Daphnia magna* (daphnids) to assess toxic effluents in rivers, with post-exposure feeding as the endpoint. *In situ* deployments were implemented in central and southern Scotland at known contaminated sites with corresponding reference sites. Animals were exposed

for 24 hours and post-exposure feeding tests were conducted for another 4 hours. Results of the study showed that the *in situ* bioassay was reliable and was able to provide enough organisms for the post-exposure feeding tests. The bioassay was able to detect post-exposure feeding depression in *D. magna* exposed at contaminated sites which could be used as a reliable and sensitive tool to detect toxic conditions in those sites.

In situ tests are suitable to evaluate pesticide effects under natural field conditions and can be applied in the tropics. These tests combined with sublethal responses and lethality are more sensitive, ecologically relevant and robust as revealed in the studies described above. Costs in the culture of test animals, including the space and time needed to maintain such cultures can be reduced. Less developed countries (LDCs) can adapt *in situ* bioassays as tools for assessing pesticide effects. This will allow researchers and regulators to make decisions regarding pesticide use and regulations.

1.5 Developing methods to assess the effects of pesticides in the tropics

Less developed countries (LDCs) in the tropics import large amounts of pesticides for agriculture. Since the green revolution, agricultural areas have used pesticides in large quantities which have the potential to affect people and environment. Even though pesticides have been found to be effective and economical, problems came up when they were used inappropriately, such as the practice of using broad-spectrum pesticides which disrupt the natural balance of the ecosystem (Sirisingh, 2000) resulting in reduced species biodiversity (Pimentel, 1992). Pesticides can circulate through the ecosystem, causing damage in unexpected ways; they are seldom species-specific (Carson, 1962; Nimmo, 1985; Hellawell, 1986; Pimentel, 1992; Halwart, 2001). This further means that in controlling undesirable organisms (target) in the ecosystem, other organisms (non-target) might also be affected even though there is no intention to destroy them. In addition, most of these non-target organisms may be beneficial to the whole ecosystem.

In the absence of standard aquatic toxicity procedures for tropical species and conditions, researchers have used techniques derived from temperate regions despite differences in species composition and environmental conditions (Lacher and Goldstein, 1997). However, it is uncertain whether the fate and transport processes of pesticides in a tropical environment occur in the same way or at the same rates as in the temperate regions (Peters *et al.*, 1997). Moreover, there is a need to know whether closely related tropical species react in the same way as temperate species when given equivalent exposures to pesticides. Biodiversity, temperature and other environmental factors could interact and positively or negatively influence the intensity of pesticide effects (Lacher and Goldstein, 1997). In southern Vietnam, water quality in a river with pen culture of freshwater prawns deteriorated when water from rice fields flowed into it, resulting in mortality of prawns (Son et al., 2005). Prawn farmers there who are rice farmers themselves have no way of knowing of the possible impacts of the pesticides they use in their rice fields on prawns they culture in pens, or on the river system as a whole.

Participants (mostly from Asia-Pacific) in a workshop organized by the Australian Centre for International Agricultural Research (ACIAR) to review options for monitoring and minimizing pesticide contamination of agricultural products and the environment suggested developing simple, affordable test methods for monitoring and research of pesticide effects and residues as one of the action priorities (Kennedy et al., 1998). Furthermore, procedures for registration and regulation in different countries should be synchronized taking into account differences in climate and other factors affecting fate of pesticides.

As the tropics has a complex ecosystem with terrestrial and freshwater ecosystems containing more than two-thirds of the world's fauna and flora (Lacher and Goldstein, 1997), this may be an advantage in the area of developing techniques to assess pesticide contamination or bioassays, as researchers have more variety in choosing which species to work on, following acceptable selection criteria.

1.6 Aquatic species in toxicity tests

Many aquatic species have been used in pesticide toxicity tests to register new pesticides and to assess their impacts. However, these studies have been conducted mostly using temperate species and the information from these studies is being used by tropical countries for their own registration purposes or for impact assessments (Castillo *et al*., 1997).

Suitable organisms should be used along with suitable tests to obtain applicable, considerable and useful results from toxicity tests (Rand *et al*., 1995). The selection criteria for species that can be used as suggested by Rand *et al*. (1995) and Van Leeuwen (1995) are as follows:

1. Sensitive to a wide range of factors such as chemicals, water quality parameters
2. Easy to maintain in the laboratory or can be reproduced and cultured under laboratory conditions
3. Has adequate background information on its biology as reference database
4. Widely available and abundant
5. Native to , or representative of, the ecosystem being studied
6. Valuable in terms of economics, ecology and recreation

In freshwater environments in the temperate region, most of the acute and chronic tests have been conducted using vertebrates (brook trout, Coho salmon, Chinook salmon, rainbow trout, goldfish, common carp, fathead minnow, white sucker, channel catfish, bluegill, green sunfish, northern pike, threespine stickleback, zebra fish, guppy) and invertebrates (daphnids, amphipods, crayfish, stoneflies, mayflies, midges, snails, planaria, rotifers), chosen either for their sensitivity, ecological and economic importance, availability, history of some success in their use, handling in laboratory, and having enough information on their responses to toxicity (Cooney, 1995; Rand *et al*., 1995). Plants such as green algae and *Lemna* have also been used for acute and chronic tests (Couderchet and Vernet, 2003; Geoffroy *et al*., 2004).

There is scarce information on toxicological effects of pesticides on tropical aquatic organisms. Castillo *et al.* (1997) mentioned that corals (*Pocillopora damicornis*) and freshwater prawn (*Macrobrachium rosenbergii*) were used for herbicide toxicity studies in Central America. *M. rosenbergii* was further described as suitable for future assessment as a tropical test species for toxicity studies.

M. rosenbergii was also used for toxicity tests in Thailand (Utayopas, 1983; Siripatrachai, 1984), in India (Natarajan *et al*., 1992), in Mexico together with blue shrimp, *Peneaus stylirostris* (Lorenzo and Sanchez, 1989) and in Brazil (Lombardi *et al*., 2001).

In Japan, the freshwater shrimp, *Paratya compresa improvisa*, was used in bioassays to monitor toxicity of pesticides in rivers (Hatakeyama and Sugaya, 1989; Hatakeyama *et al*., 1991).

Recently, the cladoceran, *Ceriodaphnia cornuta*, was chosen to represent the invertebrates living in Saigon River and similar ecosystems and used for toxicity tests with pesticides and metals in Vietnam (Hong *et al.*, 2004). The chironomid, *Chironomus xanthus*, found all over South America, was also evaluated for its suitability to toxicity tests (Moreira-Santos et al., 2005).

Responses to environmental factors and especially to pesticides are site- and species-specific, as well as age-specific (Rand *et al*., 1995), and the lack of country-specific information and exchanges among countries needs to be addressed by research.

In Thailand, as in most tropical countries, this information gap especially regarding standard procedures to determine effects of pesticides on aquatic systems makes research results irrelevant, incomparable and unrepeatable. Even though several studies have been conducted in many countries and with the same species, the ages and sizes of test species as well as procedures used are not standardized. In addition, most of these studies dealt with lethal toxicity tests of pesticides in the

laboratory, and not on *in situ* or field bioassays to determine the lethal and sublethal effects of pesticides on aquatic organisms and the environment.

With Thailand's diverse flora and fauna, there is a wide range of species available for developing procedures to determine effects of pesticides on aquatic systems. One of the species that meets the selection criteria provided by Rand et al., (1995) and Van Leeuwen (1995) is the freshwater prawn, *Macrobrachium rosenbergii*.

M. rosenbergii is a native prawn species of Thailand and other Southeast Asian countries (New and Valenti, 2000) and has also been introduced into Africa, the Caribbean, Central and South America, Israel, Japan, Mauritius, Tahiti, Taiwan, and the United Kingdom for aquaculture studies (Sandifer and Theodore, 1985). Moreover, *M. rosenbergii* is an important species for culture, and its breeding and culture technologies have evolved since 1960. Commercial hatchery production in Thailand has been successful in producing juveniles for grow-out culture since 1981 (New and Valenti, 2000). Average prawns production between 1989 and 1998 was 8,300 tonnes/year with highest production in 1992 (10,306 tonnes) and 1994 (10,124 tonnes) (New, 2000). *M. rosenbergii* then has an advantage as its breeding and culture systems and technology are already well-established and the quality of post-larvae is likely to be consistent and stable.

1.7 Macrobrachium rosenbergii biology

1.7.1 Habitat and life cycle

M. rosenbergii is a tropical freshwater species but requires a brackishwater environment during its larval stage (New and Valenti, 2000) as the larvae die within a few days in either freshwater or high salinities (Sandifer and Theodore, 1985).

Wild adult *M. rosenbergii* live in freshwater. After ovaries mature, females experience a moult (pre-spawning or pre-mating moult) usually at night; then courting

and mating start (Ismael and New, 2000). The mating behaviour of the male involves four major steps, namely contact, seizure, mounting and copulation, with fertilization occuring externally (Karplus *et al.*, 2000). Furthermore, the male guards the female for 2-3 days more until her exoskeleton has hardened to fight against attacks by other prawns.

The eggs are spawned within hours following mating and are then fertilized by the sperm which the males deposited onto the thoracic region of the females, where the fertilized eggs are attached to special setae on the female's abdominal appendages (pleopods) (Sandifer and Theodore, 1985). The eggs change colour from orange to grey-brown in about three weeks. The female migrate to brackishwater and eggs hatch into free-swimming larvae. There are 11 substages in larval development over 20-50 days depending on temperature and availability of feed. Then, they become post-larvae, assume an adult position (dorsal side up) and follow a benthic lifestyle. In the wild, they migrate upstream to seek freshwater environments and develop into adults.

In Thailand, the majority of prawn hatcheries buys in or obtains gravid females, from culture ponds. Some hatcheries also obtain mature females from rivers believing that they can obtain better quality larvae from wild females than pond-reared ones but availability is affected by season (Daniels *et al.*, 2000) and scarcity as natural populations have been adversely affected by overfishing and destruction of spawning grounds (Tonguthai, 1992). Once the females in the hatcheries spawn, larval development takes place in round cement tanks until they reach the post-larval stage. Five days after becoming post-larva (PL_5) the PLs are now ready to be stocked in nursery or grow-out ponds.

1.7.2 Moulting

Crustaceans shed their old exoskeleton as they grow and increase in size in a process called moulting or ecdysis. This process results from a cyclic and continuous morpho-physiological occurrences in the life of crustaceans (Ismael and New, 2000). Sandifer and Theodore (1985) stated that the frequency of moulting is related to age, such that the early life stages moult more frequently as they are growing rapidly.

Moulting frequency is also affected by the health condition and sex of the prawn, quantity and quality of food and water quality.

1.7.3 Cannibalism

Prawns are cannibalistic and display aggressive and territorial behaviour (New and Valenti, 2000; Barros and Valenti, 2003) especially when stocking densities are high, such as in nursery systems (Alston and Sampaio, 2000) and food is insufficient. Cannibalistic behaviour causes stress and mortality especially on moulting individuals which are vulnerable to these attacks. Refuges for vulnerable animals can reduce cannibalism. Substrates in the form of aquatic plants, palm leaves, pebbles or shells can be provided in tanks and ponds to improve survival.

Aggressive interactions are particularly important among male prawns, and in conflict situations the aggressors are the bigger sized individuals (Karplus, 2005). This agonistic behaviour is further described as a growth-controlling mechanism, with the claws playing a major role in these encounters. Juvenile prawns were observed to exhibit aggressive behaviour during feeding activity, wherein the more aggressive individuals would drive the less aggressive individuals away from the food or even take away the food from their mouths. Detailed descriptions of dominance and hierarchy among adult prawns are found in Karplus (2005) where observations were conducted in the laboratory and in the field.

1.7.4 Feeding

The natural food preference of prawns depends on their age. Larvae are carnivorous (Lavens et al., 2000), feeding primarily on zooplankton (especially small crustaceans), while the postlarvae and adults are omnivorous, feeding on algae, aquatic plants, mollusks, aquatic insects, worms, and other crustaceans (Ismael and New, 2000). During the larval stages, prawns seize food by their thoracic appendages and since at this stage they are non-active hunters, they seize food items as they encounter them, thus the importance of live prey that remains suspended in the water

column (Lavens et al., 2000). Moreover, the size of the food is also important, such that the brine shrimp *Artemia* nauplii have been found to be more suitable than cladoceran *Moina* at the early prawn larval stages.

In hatcheries, larvae still rely on live feed because they have low digestive capacity and cannot digest artificial diets (New and Valenti, 2000). They also cannot swim long distances to search for food, thus a live and moving feed will give them more chance to catch food. After they metamorphose into post-larvae (PLs), they are able to swim and search for food. *Artemia* is an important feed in the prawn diet in hatcheries (Lavens et al., 2000) and continue to be used in nurseries (Alston and Sampiao, 2000). The free-swimming *Artemia* nauplius (first instar) is brownish-orange in colour due to the presence of the yolk (Sorgeloos, 1980), and has been a popular food for a range of organisms for culture and research (Persoone *et al*., 1980). One deterrent with using *Artemia* at a later postlarval stage is its cost. Thus nurseries use a combination of other diets as the prawns grow into juveniles. There have been many studies conducted to find ways to reduce the cost of feed in hatchery operations (Mohanta, 1997; Han, 2000; Mohanta and Rao, 2000; Barros and Valenti, 2003; Indulkar and Belsare, 2004).

Feeding activity may be reduced in the presence of pesticide or other stressors due to its effect on the senses of the organism. Prawns catch food based on sight, smell and taste (Bond, 1996) and when these senses are affected by chemicals, they will not be able to feed.

1.7.5 Environmental factors affecting feeding

There are many factors that relate to the growth and feeding activity of prawns. Since New and Valenti (2000) mentioned that the optimum ranges for most water quality variables in the environmental study of freshwater prawn ponds are not available, they suggested the data compiled by Zimmermann (1998) to be used to create the ideal range for water quality parameters for *M. rosenbergii* (Table 1.2)

Table 1.2 Ideal ranges for water quality variables of *Macrobrachium rosenbergii* (after Zimmermann, 1998)

Parameters	**Ideal range**
Temperature	25-32 ˚ C
Transparency	25-40 cm
Alkalinity ($CaCO_3$)	20-60 mg/L
Hardness ($CaCO_3$)	30-150 mg/L
Un-ionized ammonia	0.1-0.3 mg/L
Dissolved oxygen	3-7 mg/L
pH	7-8.5 mg/L

Prawns become stressed at dissolved oxygen levels below 2 ppm (Rogers and Fast, 1988). pH should not be more than 9.5 (Strauss et al.,1991). However, the most critical parameter is temperature (New and Valenti, 2000) which means that in conducting tests with freshwater prawns, temperature has to be within the acceptable ranges. Moreover, post-larvae (PLs) are more tolerant of lower temperatures when compared to juveniles or adults but in general, at temperatures below 12˚C or above 42˚C, prawns rapidly die. Most often temperature in the field is within the acceptable range and in the tropics temperature does not fluctuate much between day and night. However, acclimation is necessary when transferring test animals from transport water to the test medium to avoid temperature shock.

There have been previous studies conducted to determine the effects of some environmental parameters on *Macrobrachium sp.* Sarver *et al.* (1982) studied the low temperature tolerance of 0+ to 4-day-old postlarvae and reported a survival of 76.2 % over a 24-hour period at 19˚C, but only 3.8 % at 17˚C. Nelson *et al.* (1977) studied metabolic rates of *M. rosenbergii* juveniles and found that at zero salinity, the metabolic rate was higher at 20 to 27˚C than at 27 to 34˚C. According to Zimmermann (1998) freshwater prawns cease to grow and may not survive for long periods when water temperatures are below 19 or above 34˚C.

Increase in temperature from 17 to 32˚C caused an increase in oxygen consumption and nitrogenous excretion such as ammonia in juvenile prawns (Chen

and Kou, 1996). Thus it is necessary to maintain the temperature at a constant level during the tests since a combination of low oxygen, ammonia and presence of other chemicals such as pesticide could have sublethal or even lethal effects on the prawn.

In terms of pH, Hummel (1986) found that mass mortalities may occur at pH levels of 9.5 or greater. In addition, studying the effect of fluctuating pH values in green water rich in natural food and clear water systems on post-larval mortality in outdoor pools revealed higher mortalities for green than clear water; maximum pH values rose to 9.9 to 10.5 in green water compared to 8.9 to 9.0 in clear water. Díaz-Barbosa (1995) exposed postlarvae to abrupt chemical adjustments of pH. Mortality over time in these experiments varied with respect to pH. Postlarval mortality was 100 % after being subjected to pH 9.3 for 4 h. For pH 9.1 the same mortality was reached after 32 h. He concluded that reduced mortality of postlarvae might be obtained by avoiding pH levels above 8.9.

1.7.6 Response of prawns to toxicity tests

Table 1.3 summarizes the results of previous toxicity tests carried out with *M. rosenbergii* juveniles using different pesticides. The results reveal that *M. rosenbergii* is sensitive to a range of chemicals.

Table 1.3 Previous toxicity tests conducted with *Macrobrachium rosenbergii* using different pesticides and media

Contaminant	Effect	Type of water	Duration (days)	Reported Effect Concentration (µg/L)	Reference
Insecticide					
Endosulfan	LC_{50}	FW	1	1.6	Lombardi *et al.* (2001)
Endosulfan	LC_{50}	BW	1	21	Natarajan *et al.* (1992)
Malathion	LC_{50}	BW	1	220	Natarajan *et al.* (1992)
Carbofuran	LC_{50}	BW	1	105.8	Siripatrachai (1984)
Endrin	LC_{50}	BW	1	3.25	Siripatrachai (1984)
Herbicide					
Ametryne	LC_{50}	FW	1	27810	Lombardi *et al.* (2001)
Paraquat	LC_{50}	BW	3	45	Utayopas (1983)
Glyphosate	LC_{50}	BW	2	57740	Utayopas (1983)
Propanil	LC_{50}	BW	1	5040	Utayopas (1983)
2,4 – D	LC_{50}	BW	1	632120	Utayopas (1983)
Fungicide					
Copper oxychloride	LC_{50}	FW	1	240	Lombardi *et al.* (2001)
Copper sulphate	LC_{50}	BW	1	1220	Natarajan *et al.* (1992)

Note : FW = Freshwater ; BW = Brackishwater

1.8 Research framework and aim of thesis

The research for this thesis was conducted within the wider scope of the EU-funded Managing Agrochemicals in Multi-Use Aquatic Systems (MAMAS) project sponsored by the European Union. It is a collaborative research project implemented in two Asian countries (Sri Lanka and Thailand) with partnership support from aquaculture, development and ecotoxicology experts in the Netherlands (Alterra-Wageningen University), Portugal (University of Aveiro) and the United Kingdom (University of Stirling). One of the major difficulties facing the setting up of sustainable management plans in complex environmental systems in the tropics is the lack of sufficient and relevant information on important ecological, hydrological and land use processes that strengthen the value of natural resources. The MAMAS project was thus implemented to obtain baseline information on issues related to pesticide use and handling, development of cost-effective tests and diagnostic tools for risk assessment which would lead to the development of policy guidelines for the management of agrochemicals in Asian countries.

The lack of information on effects of pesticides on the tropical aquatic environment using tropical species has been recognized. The increased use of pesticides in Asia and its potential effects on aquatic systems needs to be studied so decisions can be made for pesticide use regulation and people educated on proper use of pesticides.

The objective of this thesis was to develop procedures to assess the lethal and sub-lethal effects of pesticide contamination with *M. rosenbergii* as the test animal. Figure 1.4 presents the research framework of this thesis.

Chapter 2 describes the materials and methods developed and used in this research, including the study sites in Central Thailand where the *in situ* bioassays were conducted.

Chapter 3 explains the tiered approach used in preliminary risk assessment of pesticides in the study areas. This chapter includes the methodologies used to assess

the pesticides and crops which have the highest impacts on the environment. The results from this chapter were used to determine the types of pesticides with the highest risk to the study areas. Specific pesticides from the actual worst case scenarios assisted the researcher in establishing a research programme which has more impact and relevance to the local situation rather than using the un-updated pesticide contamination database from monitoring. The TOXSWA software programme was used to conduct a preliminary risk assessment for pesticides at the early stage of the MAMAS project.

Chapter 4 details the development of laboratory and *in situ* bioassays, specifically determining prawn density in the testing jar, the amount of food for the feeding test and number of replications to use for post-exposure feeding test. This chapter also discusses the environmental factors that affect survival and post-exposure feeding rates of freshwater prawns. In addition, the results of tests with three parameters (hardness, pH and temperature) compared with the control treatment are also reported.

Chapter 5 shows the results of using post-exposure feeding inhibition as the endpoint of toxicity tests under laboratory conditions, with carbendazim, chlorpyrifos, dimethoate, profenofos and zinc as test chemicals. This chapter also describes the recovery tests of *M. rosenbergii* after exposure to chlorpyrifos.

Chapter 6 explains the deployment of prawns in microcosms and the use of post-exposure feeding as indicators of effect of pesticides in this set-up.

Chapter 7 gives the details on deployment of prawns *in situ* at reference (uncontaminated) and contaminated sites, and the use of post-exposure feeding as an indicator of the effect of pesticides. The sites used are the same areas where the preliminary risk assessment was conducted (Chapter 3).

Finally, Chapter 8 discusses the whole research and presents general discussion, implications of the research, and recommendations for further study.

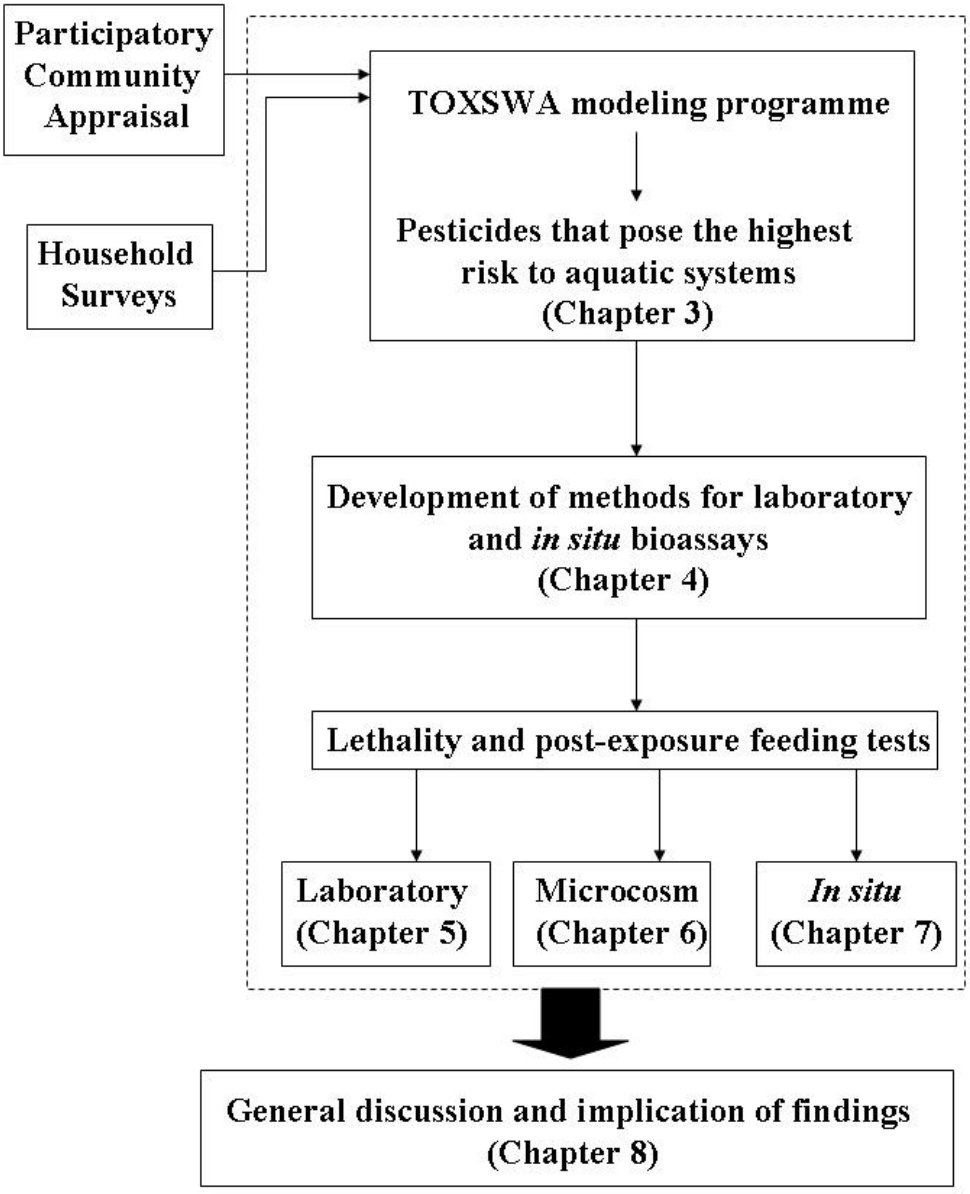


Figure 1.4 Research framework

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Test animals

Macrobrachium rosenbergii postlarvae (PLs) were obtained directly from commercial hatcheries located Supanburi and Nakornprathom provinces and were used for all experiments in this study. At this stage, the prawns are able to swim horizontally. They have a rostrum with dorsal and ventral teeth and their behaviour is predominantly benthic, as in adults. This is the stage when they are ready to be stocked in earthen ponds. PLs used in the experiments were 9-10 mm long (Figure 2.1) and had uniform size. Age was around 40 days from hatching.

Figure 2.1 *M. rosenbergii* post-larvae (PLs) under microscope (10X)

The animals were acclimated using the same method for all experiments, except for the hardness experiment. They were acclimated in glass tanks (30 L) at 30 PLs/L in soft ASTM. They were fed with *Artemia* (first-instar nauplii) at 7

nauplii/mL, at 0800 and 1600 hrs. After 48 hours, animals were transferred to the experimental set-up.

2.2 Test medium

Prawn growth depends on an ability to moult (Hartnoll, 1982), availability of food and water quality. High alkalinity in culture media stimulates moulting (Latif, 1994). Soft ASTM water was used in all experiments to reduce the frequency of moulting during the tests. McWilliam and Baird (2002a) reported that the moulting of *Daphnia magna* could interfere with feeding, resulting in error. Thus any bioassays should be completed within a single moult cycle.

Soft ASTM water (APHA, 1995) was used for acclimation and experimental work. It was a synthetic medium, consisting of four inorganic salts dissolved in distilled water (Table 2.1). Salt stock solutions of 50 mL each of KCl, $NaHCO_3$ and $MgSO_4$ $7H_2O$ and 500 mL $CaSO_4 \bullet ½ H_2O$ were added to a 10-L container and the final volume was made up to 10 L with distilled water.

Table 2.1. Composition and water quality of freshwater soft medium (ASTM)

Composition	**Concentration**
Calcium sulfate ($CaSO_4 \bullet ½ H_2O$)	0.5057 g / L
Potassium chloride (KCl)	0.2 g / 500 mL
Sodium bicarbonate ($NaHCO_3$)	9.6 g / L
Magnesium sulfate ($MgSO_4$ $7H_2O$)	7.595 g / 500 mL
Water quality parameters	**Concentration**
pH	7.2-7.6
Hardness	40-90 mg $CaCO_3$/L

2.3 Feeding

Animals were fed with *Artemia* (first-instar nauplii), hatched from cysts in the laboratory. Ten g of cysts were placed in 5 L of seawater (25-35 ppt) with aeration and a light source. Highly-concentrated seawater from a salt farm was mixed with de-chlorinated tap water to obtain a 25-35 ppt seawater for *Artemia* cyst incubation. After 24 hours, *Artemia* nauplii were harvested. Before harvesting, aeration was removed and the solution was left untouched for approximately ten minutes. This allowed the unhatched cysts to float and the freshly-hatched nauplii to sink to the bottom of the container or swim toward a light source as described by Treece (1993). *Artemia* were then siphoned off from the container into a beaker and the volume adjusted to 100 mL by adding distilled water.

The density of *Artemia* in the beaker was high and the amount varied in each batch that was hatched. It was therefore necessary to calculate the amount of *Artemia* every time they were hatched. This was done by taking 1 mL of *Artemia* from the concentrated beaker and diluting it to 100 mL by distilled water in a second beaker. Then 1 mL of *Artemia* from the second beaker was placed in a counting cell (Sedgwick-Rafter) and counted under the microscope using low power (10X). The number obtained was multiplied by 100. This estimated the number of *Artemia* in the original concentration in the first beaker (# *Artemia* in 1 mL). This procedure was done for every batch hatched and calculated according to feeding concentration in acclimation units needed at each feeding time. *Artemia* were added into the acclimation units at 7 *Artemia*/mL based on New and Valenti (2000).

2.4 Post-exposure feeding rate calculation

M. rosenbergii PLs were fed with *Artemia* after exposure to any media, i.e. with and without chemical. To determine the post-exposure feeding rate, the initial and final quantities of *Artemia* should be known, including the duration of feeding and the number of test animals being fed. Since each replicate used only 1 PL, the feeding rate equation used was simplified from Gauld's equation (Allen *et al.*, 1995):

$$F = \frac{(C_o - C_t)}{T} \qquad \text{…….. Equation A}$$

Where : F = feeding rate of a single animal (*Artemia*/PL/hr)

C_o = initial amount of food (100 *Artemia*)

C_t = final amount of food (remaining *Artemia*)

T = time animals were allowed to feed (4 hours)

Initial and final counts of *Artemia* (C_o and C_t) were recorded. At the end of the feeding period, the remaining *Artemia* were put into a 15 mL glass tube per replicate and preserved with Lugol's solution. The contents of the tubes were left to settle down for at least half an hour. After this, the *Artemia* were removed with a glass dropper and placed in the counting cell and counted using a microscope Model Olympia, at low power (10X).

2.5 Fixing solution

These studies used small and active organisms such as prawn and *Artemia*. Procedures such as counting initial and final numbers to determine survival or mortality and feeding rate involved meticulous work. Lugol's solution was used to fix the organisms after each test to facilitate ease in counting, helping to preserve and colour the test organisms. The use of Lugol's solution for this purpose is common (Unesco,1968).

Lugol's solution procedure:

Solution A: dissolve 50 g potassium iodide (KI) and 25 g iodine (I_2) in 100 mL boiled distilled water (hot)

Solution B: dissolve 25 g sodium acetate (NaOAc) in 250 mL distilled water

When solution A cools, mix the two solutions and store in a cool, dark place.

2.6 *In situ* chamber design

The *in situ* chamber (Figure 2.2) was made from 3.4 cm external diameter plastic PVC tubing cut into 14.5 cm length. Total volume is 89.3 mL. Each chamber had two rectangular windows to allow water to pass through the chamber. The windows are covered with 508 µm nylon mesh. Before placing the nylon mesh, the chambers were coated by enamel or epoxy which had penguard enamel (comp A) mixed with penguard hardener (comp B) at the ratio 4 parts comp A to 1 part comp B. Enamel prevents chemicals binding on the chamber surface.

During deployment, the chambers were held in wire rack (Figure 2.3) to keep them at least 15 cm. above the sediment.

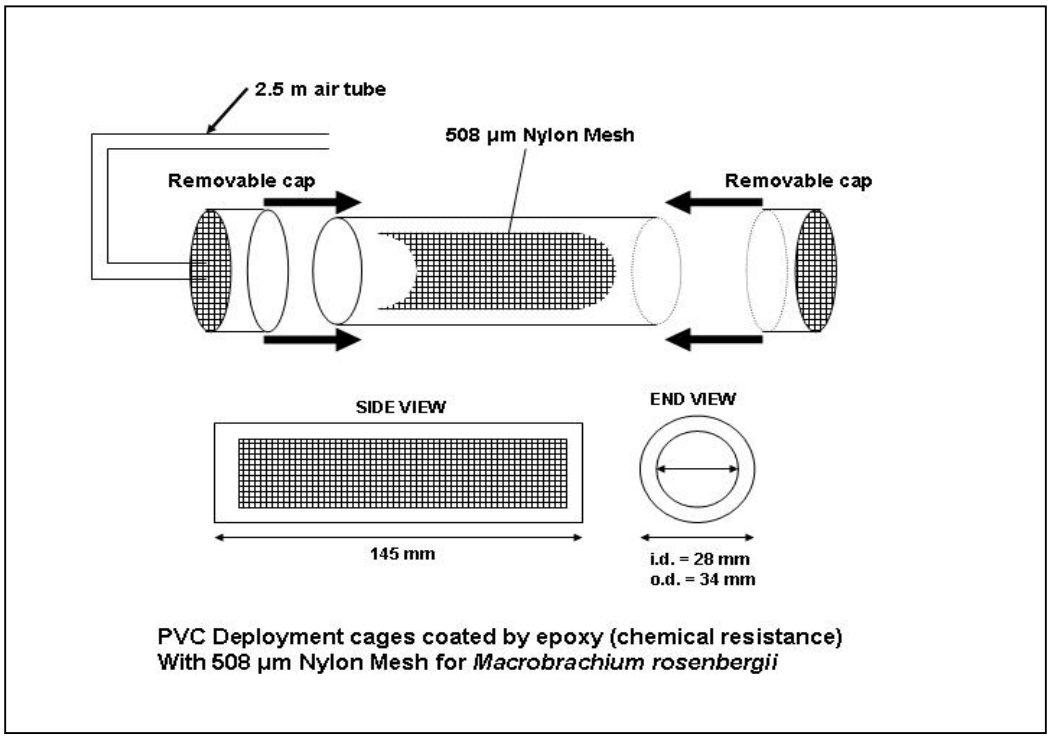


Figure 2.2 Diagram of the *in situ* chamber used in microcosm and field deployment.

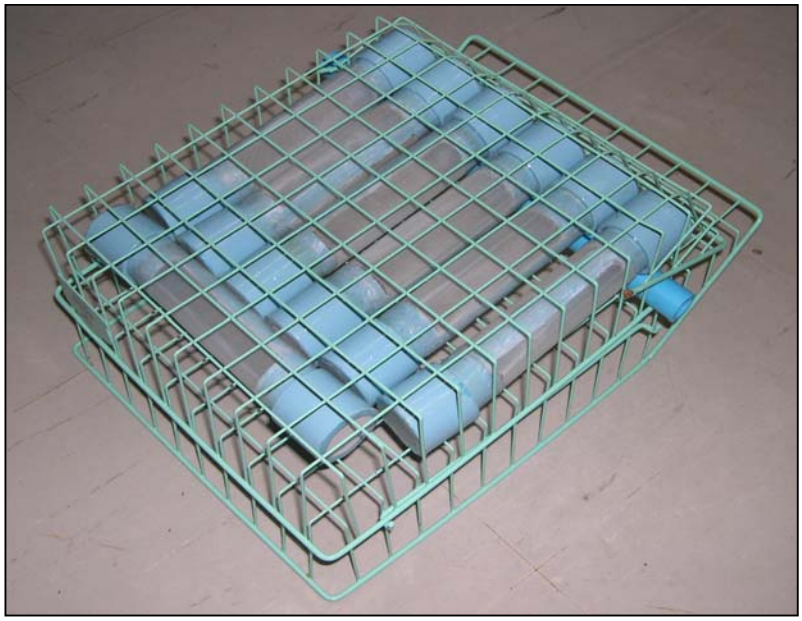


Figure 2.3 Rack used for holding chambers during field deployment

2.7 Water quality parameters

For the toxicity tests in the laboratory, dissolved oxygen, pH, temperature, conductivity and hardness were measured at the beginning and every 24 hrs.

For microcosm and *in situ* bioassays the following water quality parameters were measured at 9:00 a.m. on the day of deployment.

1) Dissolved oxygen was measured by using a Consort C534 DO meter.
2) pH values were measured with a Consort C534 pH meter.
3) Water temperature was recorded using the same DO meter while measuring dissolved oxygen.
4) Conductivity was measured by using a Consort C532 conductivity meter.
5) Total hardness (as $CaCO_3$) was measured by the EDTA titrimetric method. The water sample was titrated by standard EDTA with the presence of Erichrome Black T as the indicator in the solution (APHA, 1995).
6) Ammonia Nitrogen (NH_3-N) was determined by the phenate method using spectrophotometer at 630 nm (APHA, 1995)

7) Nitrite Nitrogen (NO_2-N) was measured by the Sulfanilamide method and using spectrophotometer, (APHA, 1995).

8) Total suspended solids (TSS)

Glass fiber filters were dried at 110 - 550 ˚ C and weighed. Water sample was filtered through the GFC filter paper and then sample was dried again in an oven at 110 ˚C for 3 hours and weighed. (APHA, 1995).

9) Total volatile solids (TVS)

The GFC filter paper containing the residue from the TSS analysis was placed in a muffle furnace at 550^{o}C for 30 minutes, cooled in the desiccators and weighed (APHA,1995).

10) Chlorophyll a was analyzed by filtering 5 ml of water sample through a GF/C paper and put in 5 ml of methanol for 24 hours in refrigerator and measured by a Turner Designs Fluorometer.

2.8 Pesticide analysis : chlorpyrifos, dimethoate and profenofos

Chlorpyrifos, dimethoate and profenofos concentrations in the study sites were determined by gas chromatography (GC/MS model HP 5973). Duplicate 500-mL samples of the solution to be analyzed were concentrated on OASIS HLB solid phase extraction cartridges. Cartridges were preconditioned by letting 6 mL of a mixture of 10 % methanol/90 % MTBE (methyl t-butyl ether) pass through them, with a flow rate between 6 and 12 mL/min. The cartridges were rinsed with 6 mL methanol and 6 mL of distilled water. Then, the water sample was passed through the cartridge with a maximum flow rate of 15 mL/min. After this, the cartridge was washed with 5 mL of 20 % methanol/80 % water, and then left under vacuum for 5 to 10 minutes to remove any remaining water. The cartridge was then eluted with 8 mL of 10 % methanol/90 % MTBE to remove any remaining pesticide in the cartridge, with the flow rate maintained at 6 to 12 mL/min. The eluent was collected into 10 mL test tubes. The samples were evaporated to dryness under nitrogen in a fume cupboard. The pesticide was then re-dissolved with 1 mL of methanol. Then samples were injected into HP-5MS, 0.25 mm X 30 m X 0.25 µm column in gas chromatography. Flow rate was maintained at 1.3 mL/min, with initial temperature at 50 ˚C and increasing to 190 ˚C

with at a rate of 30 ˚C/min for 3 minutes, and increasing it to 300 ˚C with a rate of 10 ˚C/min and holding it for 5 minutes. The results were corrected for volume extracted and the percent recovery for each sample was also determined, given in Table 2.2. These methods were based on Bailey *et al*., (2000).

Table 2.2 Details of retention time and mean recovery of organic chemicals.

Test substance	Retention Time (minutes)	Mean recovery (%) (± STD, n = 6)
Profenofos (GC/MS)	11.83	100.7 (18.32)
Dimethoate (GC/MS)	6.92	77.18 (0.4)
Chlorpyrifos (GC/MS)	9.88	101.9 (3.32)
Carbendazim (HPLC)	4.54	97 (1.2)

2.9 Pesticide analysis : carbendazim

Water samples (approximately 300-mL) from microcosm tanks were taken using a Perspex tube to analyse carbendazim. After filtering through Whatmann GF/C filters, 250-mL water was extracted with octadecyl (C-18, supelco) solid phase extraction columns. The extraction columns were conditioned with 5 mL methanol and 5 mL distilled water. After extraction, carbendazim was eluted from the column with 2 successive portions of 1.25 mL acetonitrile into glass test tubes. The samples were then diluted with water to a fixed volume of 5 mL and analysed with high performance liquid chromatography. Sub samples of 100 µL were injected with a Hitachi model L-7200 autosampler. The mobile phase (water:acetonitrile = 40:60) was set at a flow rate of 0.7 mL/min. The analytical column used was a ZORBAX ODS (length 250 mm, width 4.6 mm) provided with a guard column of the same origin. The column was mounted in a Hitachi model L-7300 oven, which was set at 40 ˚C. Carbendazim was detected using a Hitachi UV detector model L-7400 set at a wavelength of 220 nm. Under these conditions, the retention time for the carbendazim peak was 8 min and the detection limit in water was 2 µg/L. Carbendazim recovery

from water was 97 ± 1.2 % (mean ± sd, n = 6). Carbendazim concentrations were calculated using a calibration series based on external standards. Present carbendazim concentrations were corrected for recovery concentration by mean recovery in table 2.2 (multiple by % recovery). These methods were based on van Wijngaarden *et al*., (1998).

2.10 Statistical analysis

Post-exposure feeding rates and mortality of test animals were compared to feeding rates and mortality at control or reference sites using a one-tailed Bonferroni multiple comparisons test in SPSS version 10 and one way ANOVA in MINITAB version 13 (Zar, 1999). Raw data were checked for normality using MINITAB version 13 before proceeding to comparison between the treatments and different study sites. Post-exposure feeding rate data was transformed as Ln (post-exposure feeding rate) when raw data did not pass the normality test. Pearson product moment coefficients were used to explore the correlation between post-exposure feeding rate and mortality.

2.11 Toxicology laboratory

The toxicology laboratory at the Asian Institute of Technology (AIT), Thailand has the space and facilities to conduct toxicity tests on aquatic organisms. Temperature inside the laboratory was maintained at 25 ± 2 °C through use of two air condition units. Facilities in the laboratory included four 30-litre glass aquaria, aeration system, water supply system, refrigerator, shelves, glass jars of varying sizes, microscope and glasswares. The toxicology laboratory is attached to the main Aquaculture and Aquatic Resources Management laboratory under the School of Environment, Resources and Development, AIT, where additional facilities, equipment and laboratory materials and supplies were used when required.

2.12 Study areas

All the four study sites were in the central region of Thailand (Figure 2.4). The two main study sites were characterised by diverse cropping (mainly of vegetables) at Kokprajedee sub-district, Nakhon Chaisri district, Nakhon Pathom province and the mono-crop (mainly tangerine) at Salakru sub-district, Nong Sua District, Pathum Thani province. The other two study sites (Lamlukka and AIT) were reference sites for *in situ* bioassay comparison.

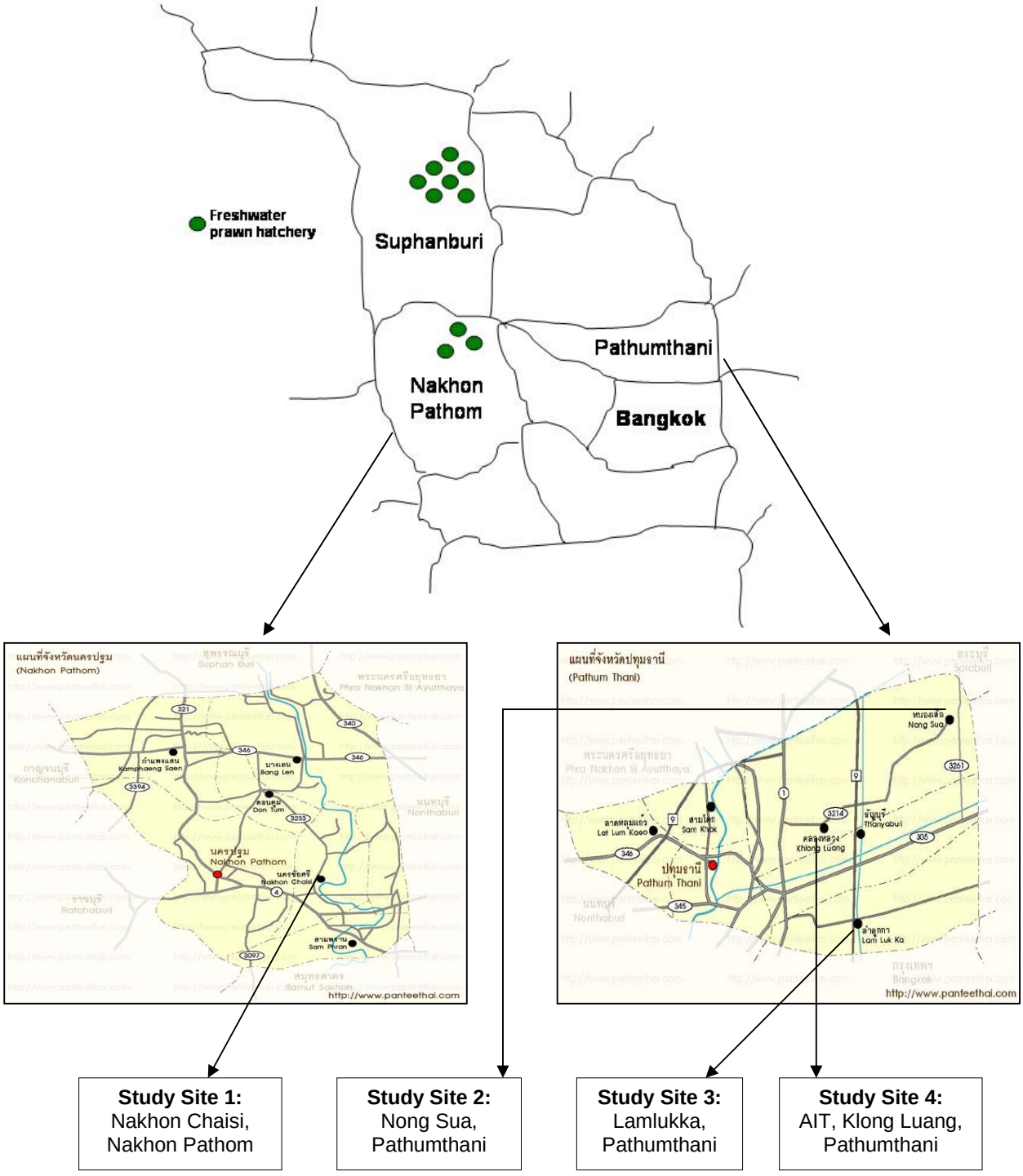


Figure 2.4 Maps showing the four study sites. These four study sites are located in Nakhon Pathom and Pathum Thani provinces, central region of Thailand.

Study site 1 (Figure 2.5) : Kokprajadee (Nakorn Chaisri District, Nakhon Pathom province) is a multi-crop farming area. Many kinds of fruit and vegetables are grown such as celery, Chinese cabbage, Chinese kale, guava, lettuce and rose apple. Local people use canals and ponds as a source of food-fish through fishing and aquaculture. The area was formerly rice fields and was converted to raised dikes and ditches to produce fruit and vegetables in the early 1980s (Prices, 2003). Pesticides are used in large quantities. Some target pesticides used in this area include profenofos and carbendazim. This study site is located 130 Km from AIT, west of Bangkok. Five vegetable farms (Chanchai, Chuchart, Chumpon, Somjit and Surat) and one guava farm (Samnieng) were used as deployment sites.

Figure 2.5 Study site 1 Kokprajadee sub-district, Nakorn Chaisri District, Nakhon Pathom province.

Study site 2 (Figure 2.6): Nong Seua (Nong Seua district, Pathum Thani province) is a tangerine farming area where the pesticide dimethoate is widely used. Local people use various canal systems as a source of food fish through culture and fishing activities. In the past, rice was the main crop in the Salakru sub-district, Nong Sua District, Pathum Thani province area. Tangerine farming was later introduced by new comers and has formed the vast proportion of high intensity agricultural production in this area since the early 1980s (Jungbluth, 2000). Recently, however, an unidentified disease has been having significant constraints on tangerine production. Longan, sweet corn, mango and mushrooms are now additionally produced. Pond and cage-in-canal fish culture systems have also appeared in the last few years in Salakru. This study site is located 70 Km from AIT and north-east of Bangkok. Shortly before the planned bioassays, a large decrease in tangerine price made farmers abandon the crop since it was not profitable anymore. Tangerine price dropped many times until it reached 2 Baht/kg, approximately 0.04 euro/kg. Farmers decided to either culture other crops in between tangerine trees or destroy the crops by burning the trees. Two bioassays (Mali and Tew farms) were deployed in this area during the wet season and one (Add farm) during the dry season.

Figure 2.6 Study site 2 Salakru sub-district, Nong Sua District, Pathum Thani province.

Study site 3 (Figure 2.7): The organic farm in Lam Luk Ka district, Pathum Thani province is a vegetable and fruit farm, although it is surrounded by rice fields. This farm had not used pesticides and other agricultural chemicals since 1999. This study site was designated as the reference study site. The purpose of having a reference study site is to have a basis of comparison for the results of deployment at contaminated sites (study sites 1 and 2). This farm is located 40 km from AIT.

Figure 2.7 Study site 3 - Organic farm (Lam Luk Ka district, Pathum Thani province).

Study site 4 (Figure 2.8): Asian Institute of Technology (AIT) is located at a large site (625 ha) 42 km. north of Bangkok, Thailand (16 km. north of the Don Muang International Airport) on Phaholyothin Road. The site which is a purpose-designed campus with recreational parkland and agricultural research facilities is supplied by an external irrigation source. Input water is regularly monitored for pesticide contamination before being pumped in through the Environmental Engineering Laboratory. In addition, AIT does not allow the use of chemicals in its agricultural research facilities.

Figure 2.8 Study site 4 - AIT water supply canal.

2.13 Water irrigation system in the study sites

The study sites are located in irrigated areas, supplied by open, usually earthen canals irrigation system. Main on-farm canals are supplied from the main irrigation canal system. As there are activities during the whole year in the agricultural areas, the main canal needs to have water all the time. Farmers pump the water from the supply canal to the farm canals (Figure 2.9). During the dry season, farmers maintain water level in the farm canal by pumping in from the supply canal or main canal directly. However, during the rainy or wet season, farmers need to pump water out from the farm canal into the supply canal (outside the farm) to avoid flooding in the farm which can destroy their standing crops. These water canal systems are near the crops and the possibility of contamination from pesticides is high when farmers apply pesticides to their crops. *In situ* bioassays were deployed in farm canals, considered the most impacted area in terms of pesticide exposure.

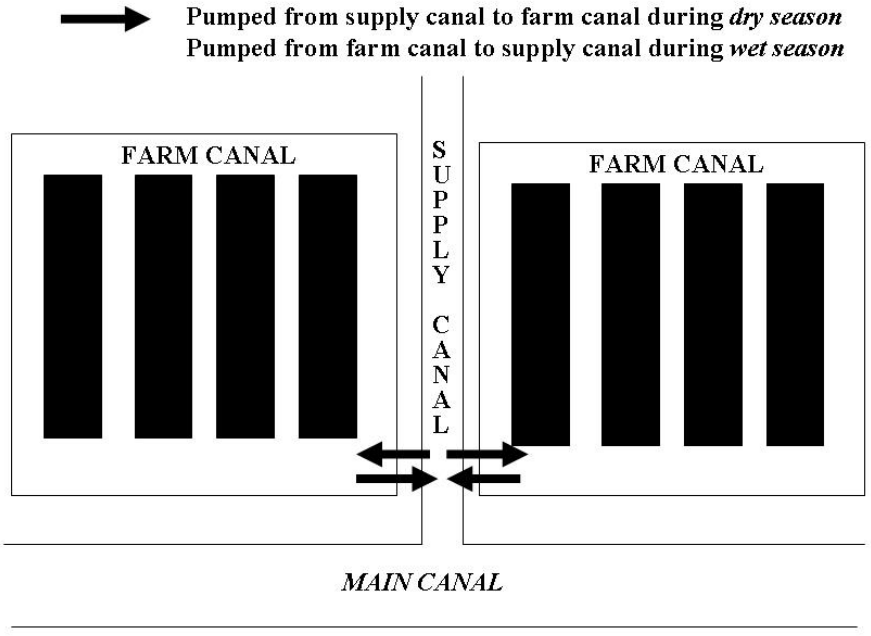


Figure 2.9 Diagram of farm canal and supply canal or main canal (not to scale) in study sites 1 and 2. The difference from these two study sites were farm canal depth and size of the crops (vegetable or fruit trees).

CHAPTER 3

PRELIMINARY RISK ASSESSMENT OF PESTICIDE USE IN STUDY AREAS

3.1 Introduction

There are many variables or parameters that influence the occurrence of pesticide residues in aquatic systems. Pesticide characteristics in combination with environmental parameters make residue studies more complex. Pesticides currently used in many countries could reach aquatic ecosystems during their use. Penetration of pesticide into aquatic biota, substrates and water are likely via pesticide runoff from agriculture and forestry, considered the most significant route and from rainfall, accidental spraying of water bodies, accidental spills and continuous release from industrial wastewater (Landa and Soldan, 1988). Preliminary risk assessment using a tiered approach is capable of providing basic information needed for screening the level of risk from pesticide and could be applied to a large number of pesticides that could endanger a natural ecosystem. A tiered approach is defined as a risk assessment starting from simple risk screening (Tier 1) to more complex site specific risk assessments (Tier 2). Also, it should not be assumed that no risk exists when experimental monitoring findings are negative. Villa *et al.* (2003) stated that sub-lethal effects even at very low concentrations could occur from highly toxic chemicals such as insecticides.

Active ingredients and brands of pesticide in use often differ among countries. The farmer's decision regarding which to use depends on crop type and the specific pest to eliminate. Farmers and local extension officers often lack information on pesticide types and the relative risk of their use in specific contexts. As well, it is important for farmers to know risk mitigation measures, such as how pesticides can be managed to reduce risks to users, consumers and the environment. Researchers, policy-makers and the public need to know this information to promote public and environmental health awareness and the development of improved management practices. The first step is to assess the risk of a ecosystem becoming contaminated by

a given pesticide. In assessing the ecological risks of a new pesticide, its fate and effects are investigated under realistic field conditions, taking into account good agricultural practices and the spatial and temporal variability of the ecosystems potentially under stress (Satapornvanit *et al*., 2004). The time, costs and logistics necessary for this approach, however, make it impossible to evaluate all active ingredients and formulated products. Thus, in Europe, for example, a tiered approach has been adopted.

The first, relatively simple, tier of aquatic risk assessment is based on the estimation of a PEC/NEC (Predicted Environmental Concentration/No Effect Concentration) ratio (EU, 1991 and 1997). In this ratio, the calculated concentration of the pesticide in surface water (PEC) is compared to the expected NEC. If the PEC does not exceed the NEC, the pesticide is not expected to have an effect on the aquatic community.

The first tier PEC is calculated with the help of a simplified scenario for a standard freshwater system (stagnant; water depth to 30 cm overlying sediment of 5 cm depth) on the basis of the recommended dose used for pest control and the expected drift percentage and runoff or drainage fractions (FOCUS, 2001). The NEC is based on concentration-effect relationships studied in the laboratory with a limited number of “standard” species, viz., an alga, *Daphnia* and fish. These species are chosen because of their ease in handling and rearing in the laboratory. Test procedures have been adapted as a standardised protocol and are well described in, for instance, the Organisation for Economic Co-operation and Development guidelines (OECD, 1993). To protect sensitive indigenous aquatic populations, the NEC is usually calculated by multiplying the toxicity value of the most sensitive standard test species by an assessment factor (e.g. a factor of 1/100 for acute EC50s or a factor of 1/10 for chronic NOEC’s in the uniform principles) (EU, 1997).

In the first tier, the PEC is based on an ‘extreme worst case loading’ scenario. If, based on this PEC compared with the NEC, the use is considered safe, no further risk assessment is required. If, however, the result indicates that use is not safe, it is necessary to do a second or third or fourth tier assessment.

Preliminary risk assessment of pesticide using the tiered approach would help to assess which pesticide poses the highest risk to environment. This chapter describes this method conducted with many pesticides being used in Thailand for fruit and vegetable farming. Assessment was based on the quantities applied, the frequency of pesticide application, pesticide characteristics and the no effect concentration.

The aim of this chapter was to assess the type of pesticide which had the highest impact on the environment, and the crop that received it the most; thereby its farming and consumption could also pose a risk to farmers and consumers. Results from this chapter were used to inform the lethal and sub-lethal experiments in Chapter 5. Figure 3.1 presented the flow chart of the risk assessment in this chapter.

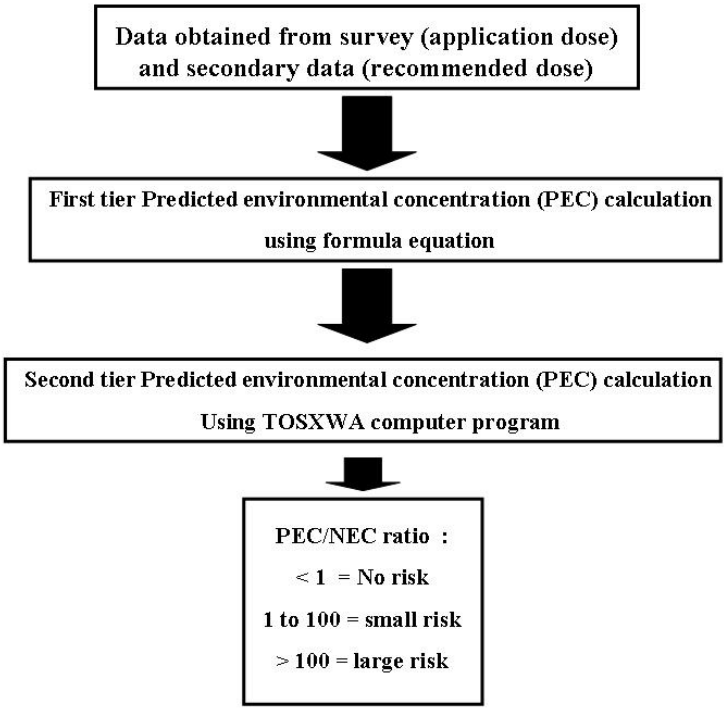


Figure 3.1 Flow chart of the preliminary risk assessment

3.2 Materials and methods

3.2.1 Study sites

According to the diversity of agricultural systems in Thailand, two different study sites in area of diversified and intensive agriculture were selected, namely, an area in which, more diverse cropping (vegetable/fruit) occurred (mixed crop) in Kokprajedee sub-district, Nakorn Chaisi district, Nakhon Pathom province (site 1 in chapter 2) and another site where a single commercial crop dominated, the 'mono-crop' (tangerine) site in Salakru sub-district, Nong Sua District, Pathum Thani province (Site 2 in chapter 2)

Three villages from each study site were selected for both participatory community appraisals (PCA) and a follow up household survey (Van den Brink, *et al.*, 2003a). Twenty households were randomly sampled in each village (i.e. 60 households in each study site) and were interviewed using questionnaires to obtain more information about the types of pesticide used in their farms, amounts of pesticide per cultured area, frequency of pesticide application and basic information of households such as number of family members and sources of daily food.

3.2.2 First tier PEC calculation

First tier PEC calculation involved a preliminary estimation of the risks posed by each pesticide to the aquatic environment. The total quantity of each pesticide applied to each crop during the crop cycle was used to very simply calculate a first tier PEC. These PECs allow the identification of pesticide-crop combinations that may cause the highest risks. The PEC equation below is calculated assuming a one-time pesticide application.

$$PEC = \frac{Dose \times Number \cdot of \cdot appliction \times Drift \cdot fraction \times 0.1}{Volume \cdot of \cdot water \cdot body}$$

Where,

PEC (μg/L)

PEC is the Predicted Environmental Concentration, in this case, the peak concentration. This was calculated from instantaneous input of cumulated pesticide loading during the crop cycle. PEC presents the estimated concentration of individual active ingredient in the pesticide in the water body.

Dose (g a.i./ha)

The loading of active ingredients for one application was calculated from the applied dose of the formulated product accounting for the % of active ingredient of the pesticide product. Concentrations of active ingredients in solid form were provided as an expression of weight per weight (W/W) whilst liquid forms were expressed as weight per volume (W/V), where in the latter case 1% = 10g / 1000 mL. This was taken into consideration in calculations.

Number of applications (days)

The highest number of applications in a crop during its crop cycle was gathered from the household survey. A crop cycle of 45 days was used for both vegetable (mixed-crop) and fruit (mono crop). For the other crops in the mono crop site, the crop cycle varies between 28 and 182 days. The worst case situation was considered to occur by using the highest values of number of pesticide applications.

Drift fraction (%)

This is the percentage of the pesticide dose (as active ingredient) applied to 1 m^2 of soil and deposited on 1 m^2 of surface water. The estimation of this percentage was based on the assumption that the pesticide was either applied from a boat or by knapsack spray. Boats used for irrigation of the crops are also used to apply pesticides, moving through the irrigation ditches spraying water behind them. In this way the pesticide is diluted with the irrigation water. The resulting spray pattern falls on both the water and the crops on the adjacent strips of land. Based on these observations and as a worst case scenario, a drift percentage of 100 % is assumed for boat applications. For knapsack spraying, the water directly next to the soil receives the same amount of pesticide as the soil, since the crops are grown directly next to the water course, which has a width of 1-2 m. When both sides of the water course are

sprayed using a knapsack spray an average pesticide deposition via spray drift of 30 % is assumed. The percentage is divided by 100 to obtain a fraction.

0.1: This factor is added to convert g/ha to mg/m^2.

Volume of water body (m^3)

The volume is calculated assuming a canal length of 1 m, a width of 1 m and a water depth 0.5 m considered worst case which is 1 m x 1 m x 0.5 m = 0.5 m^3. In practice canals are varying dimension.

3.2.3 Second tier PEC calculation

The second tier PEC was calculated to obtain a more realistic estimate of the concentrations of pesticide in surface waters. The first tier PEC was calculated assuming all loadings enter the water body instantaneously, causing a peak concentration which is the PEC. In calculating the second tier PEC, the processes that determine the fate of the pesticide in surface water were taken into account and a realistic application scheme was considered. The difference between the first tier PEC and the second tier PEC is due to the breakdown through various processes between applications resulting in lower PECs. The second tier PEC also takes into account successive applications. In this study, the TOXSWA modelling software (Beltman *et al.*, 1999) was used to calculate the second tier PEC.

Second tier PEC calculations for risk assessment involves the input of data relating to pesticide use, pesticide properties and various environmental parameters to the TOXSWA model. Only the pesticide-crop combinations from the first tier assessment which revealed the highest PEC values were selected for the second tier PEC calculation.

For this calculation, the dry season scenarios were chosen as the worst case scenarios. Concentrations are highest in the dry season because the water is at its shallowest. The values entered for each parameter in TOXSWA are shown in Table

3.1. The parameter values of pesticides used in TOXSWA are given in Table 3.2. The drift deposition on the water surface for each application was calculated according to the equation below:

$$Drift\ deposition\ on\ water\ surface\ (mg/m^2) = Dose\ (g\ a.i./ha) \times Drift\ \ fraction\ (\%) \times 0.1$$

Table 3.1. TOXSWA scenario parameters for Thailand

	Country	Thailand		Comments
	Location	Farm Canals		
	Crop Type	Mixed Fruit & Vegetables		
	TOXSWA Scenarios Parameters	Scenario 1: Dry Season	Scenario 2: Wet Season	Worse case scenarios considered in each case
	Slope	0.0001	0.0001	Assumed vertical slope
	Bottom width	1 m	1 m	For 1 m^3 water
	Suspended solids			
	- concentration (g/m^3)	50	50	Assumed from local knowledge
	- mass ratio organic matter	0.5	0.5	Assumed to be low
	Water Layer Segments			
	- length water body (m)	1	1	For 1 m^2 water surface
	- No. segments	1	1	Insignificant
	Macrophytes	0	0	Assumed worst case
	Sediment Segments	1	1	Assumed 1
	- thickness (m)	0.05	0.05	Assumed depth
	- bulk density (kg/m^3)	800	800	Assumed low bulk density
	- porosity			Default – insignificant
	- tortuosity			Default – insignificant
	- mass ratio	0.085	0.085	Assumed low organic matter
Hydrology	Flow velocity water body	0	0	Assumed no flow
	Water depth water body (m)	0.50	0.75	Assumed lowest from expert judgement
	Temp. (water & sediment °C)	33	30	Assumed average of seasonal temp.
	Dispersion coefficient in water (m^2/d)	1	1	Dummy value when flow is zero
	Dispersion length in sediment (m)	0	0	Assumed no dispersion in sediment
	Upward seepage & concentration of pesticide in incoming water			
	- seepage (mm/d)	0	0	Assumed no upward seepage
	- conc. (mg/L)	0	0	Assumed no upward seepage
	Initialisation			
	Segments	Water layer	Water layer	Assumed pesticide stays in water
	- position (m)	0.00-1.00	0.00-1.00	Insignificant
	- initial conc. (µg/L)	0	0	

Table 3.2 Pesticide properties for calculation of second tier PEC

Active ingredient name	Molecular Mass (g/mole)	Psat (mPa)	Temp. Psat (°C)	S (g/L)	Temp. S (°C)	DT_{50}-water (d)	DT_{50}-sedim. (d)	$*K_{om}$ (L/kg)
Abamectin	873.1	2E-07	22.5	0.005000	20	56	10000	2860
Captan	300.61	1.10E-05	25	0.005100	22.5	1	1	75
Carbaryl	201.23	1.60E-04	24	0.120000	30	14	14	34
Carbendazim	191.19	6.50E-08	20	0.008000	20	90	10000	76
Carbofuran	221.25	8.00E-05	22.5	0.351000	25	50	50	13
Carbosulfan	380.5	4.10E-05	25	0.000300	25	7.6	10000	1000
Chlorfenapyr	407.6	1.00E-15	20	1.000000	20	10000	10000	1000
Chlorfluazuron	540.7	1.00E-08	20	0.000010	20	42	10000	1000
Cypermethrin	416.3	1.90E-07	20	0.000004	20	14	10000	2137
Dicrotophos	237.2	9.30E-03	20	1.000000	20	20	20	1000
Difenoconazole	406.3	3.30E-08	25	0.015000	25	145	10000	1000
Diflubenzuron	310.69	1.20E-07	25	0.000080	20	10	10000	104
Dimethoate	229.2	1.10E-03	25	23.800000	20	21	10000	17
EPN	323.3	4.10E-05	23	0.00000092	24	15	10000	96700
Fipronil	437.2	3.70E-07	25	0.001900	25	28	10000	1000
Glyphosat	169.1	1.00E+00	25	12.000000	25	30	10000	3200
Malathion	330.3	5.30E-03	30	0.145000	25	1	10000	1000
Mancozeb	330	1.00E+00	25	0.006000	25	70	10000	1143
Metalaxyl	279.3	7.50E-04	25	8.400000	22	56	10000	27
Methamidophos	141.1	2.30E-03	20	200.000000	20	23.5	10000	5
Methomyl	162.2	6.70E-03	25	58.000000	25	30	10000	12
Mevinphos	224.15	1.70E-02	20	600.000000	22.5	20.5	10000	17
Profenofos	373.6	1.24E-04	25	0.028000	25	8	10000	13965
Propineb	289.8	0.0001	22.5	0.010000	20	1	10000	1000
Prothiofos	345.2	6.00E-04	22.5	0.000070	20	280	10000	1000
Tetradifon	356	3.20E-08	20	0.000078	20	52	10000	455
Zineb	275.8	1.00E-05	20	0.010000	22.5	37	10000	571

Note:

Psat means saturated gas pressure.
DT_{50}-water means time to 50% breakdown of original concentration or half-life in water.
DT_{50}-sedim. means time to 50 % breakdown of original concentration or half-life in sediment.
$*K_{om}$, sorption coefficient for organic matter, can be used for sorption to suspended solids and for sorption to sediment.

Mean frequency of pesticide application and the time interval between applications were extracted from the household survey. A period of 45 days for vegetable and fruit in the mixed-crop site and 28 to 182 days depending on crop type for mono-crops was simulated, being the crop cycle period in which the series of pesticide applications occurred.

The half-life estimates used for the simulations in the TOXSWA model are found in Tomlin (1997 and 2000) and Linders *et al.* (1994). When available, the DT_{50} of the water-sediment study was used, but if the data was not available, the hydrolysis, photolysis and degradation times in water at pH 7 were used which can be found in Tomlin, (1997 and 2000) and Linders *et al.* (1994). As a last choice the DT_{50} of soil was used. If it was still not available, the value for DT_{50} water and sediment of 10,000 days was used as default values. With the molar enthalpy the transformation rate can be calculated for the temperature considered in the scenario. For the activation energy for transformation, the default value of 55,000 J/mole was used for all pesticides (Beltman *et al.*, 1999).

K_{om} and K_{oc} sorption constants were normalised for organic carbon. The sorption coefficient K_{om} for suspended solids and for sediment were both taken from the sorption coefficient for soil according to Tomlin (1997 and 2000) and Linders *et al.* (1994). If not available the K_{om} was calculated from the K_{oc} using the equation below,

$K_{om} = 1.7 \text{ X } K_{oc}$ (FOCUS, 2001)

If both K_{om} and K_{oc} were unavailable, the default value of 1,000 L/kg was used. The Freundlich exponent was set at the default value of 0.9 (Calvet *et al.* 1980).

Saturated gas pressure was obtained from Tomlin (1997 and 2000) and Linders *et al.* (1994). The conversion from m Hg to Pa is 1 m Hg = 133322 Pa and 1 mm Hg = 133.322 Pa. If the references did not have a value for saturated gas pressure, the value was set at 1 E-15 Pa. The temperature at which the saturated gas pressure was measured is needed as well. With this temperature and the molar enthalpy the saturated gas pressure can be calculated for the temperature considered in the scenario. For the molar enthalpy of vaporization the default value of 95,000 J/mole was used (Beltman *et al.*, 1999).

Solubility in water was also obtained from Tomlin (1997 and 2000) and Linders *et al.* (1994). With the temperature at which the solubility is measured and the

molar enthalpy the solubility can be calculated for the temperature considered in the scenario. For the molar enthalpy of solution, the default value of 27,000 J/mole was used (Beltman *et al.*, 1999).

Molecular mass was also found in Tomlin (1997 and 2000) and Linders *et al.* (1994). When not available the molecular mass data was set at 100 g/mole.

All calculations are done for all combinations of crop and pesticides that were found in the household surveys.

3.2.4 No Effect Concentration (NEC)

The preliminary risk assessment was based on the estimation of a PEC/NEC ratio. For the calculation of the ratio, the second tier PEC was calculated according to the procedures as described in section 3.2.3.

First tier NEC calculation

The calculation of the NEC is based on laboratory toxicity data (LC_{50}/ EC_{50}/NOEC's) gathered for a limited number of 'standard species': viz. algae, *Daphnia* and fish. These species have been chosen because of the ease in handling and rearing in the laboratory. A list of standard test species was derived from OECD (1993). Toxicity data from laboratory tests only were taken into account. Furthermore, values obtained from laboratory tests with non-reported endpoints or endpoints that were not considered relevant were excluded, including toxicity values from laboratory tests with a duration that is considered out of range. Table 3.3 lists relevant endpoints and duration for laboratory toxicity tests.

When more than one EC_{50} or NOEC values were found for the same species, the geometric mean was calculated. If no EC_{50} or NOEC data were available within one of the three species groups, the database of the RIVM (RijksInstituut voor Volksgezondheld en Milleu = National Institute for Public Health and the Environment, The Netherlands) was used (De Zwart, 2002). If toxicity values were

still missing for the most relevant species groups for a certain pesticide (i.e. insecticide and acaricide: *Daphnia*, fish; herbicide and plant growth regulator: algae and macrophytes; fungicide: *Daphnia*, fish, algae and macrophytes), the Pesticide Manual (Tomlin, 2000) was checked. For all pesticides an NOEC or EC_{50} value was obtained from at least one species within the most relevant species group(s). Due to the uncertainties associated with the extrapolation from one species to another and to protect sensitive indigenous aquatic populations, the NEC is calculated by multiplying the toxicity value by an assessment factor (Table 3.4) (EU,1997). After application of the assessment factors, the lowest NEC for each pesticide was taken.

Table 3.3 Relevant endpoints and duration for laboratory toxicity tests (EU, 1997)

Species group	Toxicity measure	Relevant endpoints	Relevant duration of test (days)
Daphnia / fish	acute EC_{50}	Mortality Behaviour Intoxication	1-4
	chronic NOEC	See EC_{50+} Reproduction	> 4
Macrophytes	acute EC_{50}	Growth Population	2-14
	chronic NOEC	See EC_{50}	
Algae	acute EC_{50}	Growth Population	1 – 4
	chronic NOEC	See EC_{50}	1 – 4

Table 3.4 Assessment factors for extrapolation of toxicity values for standard test species to NEC (EU, 1997)

Short term exposure
0.01 x acute EC50 fish, *Daphnia*
0.1 x acute EC50 algae and macrophyte
Long-term exposure
0.1 x chronic NOEC fish, *Daphnia*, algae and macrophyte

3.3 Results and Discussion

The first and second tier PECs for crop-pesticide combinations in the study sites based on the dose information obtained from HH surveys are presented in Appendix 3. Appendix 4 shows the NECs in µg/L of all pesticides that have been evaluated. The second tier PEC/NEC ratios for crop-pesticide combinations in the study sites based on application dosages are presented in Appendix 5. The first and second tier PECs for crop-presticide combinations in the study sites based on the recommended dosages are shown in Appendix 6.

The top ten pesticides of the first tier in the mixed crop site were all insecticides. These chemicals do not only affect the insects but also kill crustaceans and many other organisms present in the water system. FAO (1988) reported that even very low concentrations of mevinphos (1.46 µg/L) could kill *Daphnia magna*.

Of the top ten pesticides in the mono-crop site, eight were insecticides and two were fungicides. Dimethoate was the most commonly used pesticide on tangerine farms, posing the highest risk to farmers and consumers (Jungbluth, 2000). However, there are no existing studies on the impact of this pesticide on tropical aquatic animals.

In the mixed crop site, mevinphos posed the highest risk among the pesticides used. For vegetable farms, the tiered approach showed abamectin as having the highest risk followed by profenofos.

During risk analysis, a standard dose approach, i.e. the dose recommended by the company or extension service or used for the registration of the pesticide in the local market, is recommended. Through this approach, the variations between years and between farmers do not influence the results of the risk assessment of pesticide. The PEC/NEC results based on the recommended dose are then compared with the doses given by respondents in the HH survey. Appendix 2 shows the PEC/NEC risk quotients that were lower than actual doses used by farmers (from the HH survey) except for metalaxyl in the mixed crop and captan, carbaryl and carbofuran in the

mono-crop. The values were lower than the actual application dose. This shows that the quantity of pesticides used by farmers far exceeded recommended levels.

The major source of uncertainty of the preliminary risk assessment (PRA) conducted in this study is the fact that pesticide properties used for this assessment were obtained from databases originating from Europe and North America. It is questionable whether these are representative of the Asian situation. It is possible that the breakdown of pesticides may be different under warmer and more eutrophic conditions compared to the sediment water system used to establish the DT_{50} for registration purposes in Europe. The same can be argued regarding the toxicity of the chemicals towards tropical species although Maltby *et al.* (2002a) could not demonstrate differences in sensitivity between temperate and tropical species for a few pesticides (carbofuran, chlorpyrifos and fenitrothion). Brock *et al.* (2000a; 2000b) also found that there were no systematic differences in threshold levels derived from semi-field experiments conducted in temperate and warmer conditions although temperature may considerably influence the extent and types of secondary effects if safe threshold levels were exceeded (Van Wijngaarden *et al.,* 2003). Therefore it is important that more studies be done to determine the differences in the fate and effects of pesticides under temperate and tropical conditions.

The results of this tiered approach show the pesticides (Appendices 2 and 5) which have an impact on organisms in the aquatic system. The first ten pesticides in both application dosages and recommendation dosages had PEC/NEC ratios of more than 1, showing that they were all posing a risk to the environment. Dimethoate and profenofos were selected to represent the organophosphates as test chemicals for the development of procedures for *Macrobrachium rosenbergii* toxicity tests.

To obtain a more relevant and wider range of data, additional pesticides, carbendazim (a fungicide from the carbamate group) and chlorpyrifos, and a heavy metal, zinc, were also selected as test chemicals. Chlorpyrifos, also an organophosphate, is considered more toxic than dimethoate and profenofos and affect insects even at its lowest concentrations (Extoxnet, 1996a).

Dimethoate was being used in large quantities in tangerine farms in Thailand (Ratanamaneechat and Cherdchoo-ngarm, 2000). From the field survey, results showed that dimethoate were popular and used in many farms in difference quantities (Appendix 6). Profenofos was also used in large amounts in vegetable farms in Thailand. The details of these four pesticides and zinc are described in Chapter 5.

CHAPTER 4
DEVELOPMENT OF LABORATORY AND *IN SITU* BIOASSAYS WITH *Macrobrachium rosenbergii*

4.1 Introduction:

The effect of pesticides on living organisms can be assessed by using a standard toxicity test or single-species bioassay exposed to different concentrations. The performance of such bioassays can be evaluated using selected criteria. Calow (1993) stated that relevance, reliability, repeatability/sensitivity, reproducibility and robustness are the major criteria for successful toxicity tests. Toxicity tests should be standardised and carried out according to precisely defined protocols. That provides the possibility of comparing studies undertaken at different time or in different location. This is both cost effective and by limiting the number of organisms used shows concern for animal welfare.

Standardisation of bioassay procedures aims to produce a test that provides reliable and repeatable results. Reliability is the ability to make observations in a controlled way and with confidence (Calow, 1996). Repeatability with less variability must be standardised to ensure that changes in biological change caused by stress from contaminants can be detected. Abiotic conditions and biotic factors in laboratory bioassays must be considered to minimize the variables affecting biological activity of the test species. In addition, the sample size should be large enough to ensure that any statistical analysis performed on biological end point is sufficient to detect the changes (Calow, 1996).

Sensitivity of any toxicity test also depends on the chosen organism. For example, younger/smaller animals are generally more sensitive than larger individuals of the same species (Law and Yeo, 1997). Because of the cost and length of time required to perform a full life cycle test, scientists have been encouraged to search for sensitive species and sensitive life stages (Adams, 1995).

M. rosenbergii was suggested by Castillo *et.al.* (1997) as a potential test species for aquatic toxicity studies. *M. rosenbergii* culture is distributed in many countries in Asia-Pacific such as Bangladesh, China, India, Malaysia, Philippines, Vietnam and Thailand (New, 2005). *M. rosenbergii* can grow in wide range of water types not only in the freshwater. Jain and Diwan (2002) also suggested that *M. rosenbergii* can be cultured in saline water wastelands in India. *M. rosenbergii* is also cultured in a range of different culture systems such as in polyculture with tilapia in Jamaica (Aiken, *et.al.*, 2002), in pen culture within natural rivers in southern Vietnam (Son, *et.al.*, 2005), in rice fields (Kurup and Ranjeet, 2002; Giap, *et.al.*, 2005; New, 2005) and grow with wild tiger shrimp (*P. monodon*) in Bangladesh (New, 2005).

M. rosenbergii shows early individual differences in growth rate mainly because of differences in the ability to compete for food (Brown, *et.al.*, 2003). The tendency to cannibalism, moulting performance, density of prawn, feeding behaviour and availability of food can all affect amount of food consumed by prawn (some details already mentioned in Chapter 1). Water quality also effect the growth and food consumption rate.

Availability of oxygen also impacts on the feeding activity of prawn. Smith (1982) stated that most aquatic organisms show a maximum activity or active metabolism at oxygen concentrations of 3 to 7 mg/L. Prawns feed normally within this range of dissolved oxygen. *M. rosenbergii* has poorer survival and less tolerance to low dissolved oxygen than other organisms. Dissolved oxygen concentration was lower than critical level of 2 ppm at early morning (6:00 a.m.) in rice-prawn culture in Vietnam for example (Giap, *et.al.*, 2005).

Chen (2003) studied the effect of pH on survival, growth, moulting and feeding of prawn. The minimum acceptable pH levels were 6.2 and 7.4 based on growth and feeding, respectively.

Temperature is another factor affecting feeding activity of prawn. Arana-Magallon and Ortega-Salas (2005) reared the prawns for 120 days under laboratory conditions at 20˚C and 33˚C. The growth was significantly greater at 33˚C than at 20˚C. The higher temperature gave a higher feeding rate, resulting in better growth.

Niu *et al.* (2003) also carried out an experiment on the effect of temperature on food consumption, growth and oxygen consumption of freshwater prawn, *M. rosenbergii* postlarvae at 23, 28 and 33˚C in the laboratory. The results showed that the animal's initial body weight had a closer linear relationship with food consumption and growth. Food consumption increased directly with temperature.

In terms of water hardness, Venugopal *et al.* (2003) found that high total hardness (530 ppm as $CaCO_3$) hampered the growth of postlarvae during a two months rearing period. Hardness is one of the factors that could affect feeding activity of PLs.

Other water quality parameters such as ammonia, nitrate and nitrite had a potential to increase especially in intensive culture systems. Ammonia and nitrite should be kept at concentrations of <0.5 and <0.1 mg/L, respectively (Correia *et al*., 2000). Nitrate, however, is not a limiting factor in prawn larviculture (Mallasen *et al.*, 2004).

Physical parameters also have an effect on the growth and food consumption of prawn. Lin *et al.* (1998) indicated that long periods of daylight, especially continuous light could increase growth rate, gross growth efficiency and assimilation efficiency of prawn larvae. *M. rosenbergii* has nocturnal behaviour and prefers locations under the shade or bottom of the culture tank during the day (Ismael and New, 2000).

The quantity of available food in culture system also affects feeding rate. Valenti *et al.* (1998) recommended that the quantity of *Artemia* to be fed to the larvae be based on the tank volume and the previous day's consumption. For intensive culture techniques, a concentration of 5 to 15 nauplii/mL is recommended, to be given immediately after the larvae start to feed. Niu *et.al.,* 2003 reported that maximum food intake for prawns reared individually required high levels of food and dissolved oxygen.

Feeding activity may be reduced in the presence of pesticide or other stressors due to its effect on the senses of the organism. Prawns catch food based on sight,

smell and taste (Baond, 1996) and when these senses are affected by chemicals, they will not be able to feed. The use of feeding as an endpoint has many advantages. Maltby *et al.* (2002a) developed *Gammarus pulex* feeding inhibition for short-term sublethal biomonitoring of water quality. Feeding inhibition occurred under low water quality conditions and in contaminated sites. Feeding inhibition was also affected by season, pH and temperature levels (Maltby *et al.*, 2002b). McWilliam and Baird (2002a) indicated that post-exposure feeding inhibition of *D. magna* was more sensitive to toxicity than effects measured at the community level. This endpoint was linked to effects measured by other bioassays (chironomid emergence) and in other trophic levels (phytoplankton). Castro *et al.* (2004) showed that biomarker responses (acetylcholinesterase) were related to feeding inhibition. Their short-term bioassay (post-exposure feeding inhibition in guppies, *Poecilia reticulate*) was able to detect contamination of heavy metals.

Environmental parameters may negatively or positively affect the feeding activity of prawns thereby affecting the reliability and repeatability of bioassays using feeding as an end point. In the laboratory, these effects could be minimized because all environmental parameters could be controlled and kept within optimal ranges. However, during field deployments, animals are not exposed to constant water quality conditions. Several factors might affect prawn feeding behaviour in the field, but their subsequent effect on post-exposure feeding rates has not yet been identified. If the ideal ranges of water quality (table 1.2) have an effect on post-exposure feeding rates, then comparison between feeding rate data at contaminated and non-contaminated sites may not be possible. Therefore, for the purpose of this *M. rosenbergii* bioassay it is important that optimum environmental factors or ideal environment factors should have minimal or no effect on post-exposure feeding rates. This would allow the bioassay to be compared between pesticide-contaminated sites and control sites (non-contaminated).

No research has been done on the effect of specific environmental parameters on feeding rates of *Macrobrachium* during and after their exposure to chemicals. Most studies have focused on acceptable or optimal ranges of environmental parameters in hatcheries, nurseries and culture ponds to increase production and improve management, such as avoidance or elimination of chemical contamination,

and health and water quality management (Correia *et al.*, 2000; New and Valenti, 2000; Cheu and Jiann-Chu, 2003 and Arana-Magallon and Ortega-Salas,2005).

Conducting *in situ* bioassays involves environmental factors that could affect test organisms as they are exposed to the pesticide and other potentially toxic substances. Thus, before doing field and even microcosm trials, it is necessary to determine the effects of some environmental parameters on the survival and post-exposure feeding rate of the test animals. Knowing this information could help to determine whether the responses of the test animals during *in situ* deployment relate to exposure to the pesticide alone or to environmental factors.

4.2 Aims :

The main objective of this section was to develop and standardise a basic and practical *M. rosenbergii* bioassay which can be applied under laboratory and field (*in situ*) conditions. This standardisation should minimise post-exposure feeding rate variations under clean (non-toxic) conditions which should also be confirmed by statistical analysis in terms of response variability. General materials and methods used in each experiment in terms of test animal, test medium and food chosen are described.

Specifically, the experiments described in this section were conducted to:

- observe frequency of moulting of *M. rosenbergii* under soft media (ASTM)
- determine the optimal density of *M. rosenbergii* in test glass jars (laboratory) and in chambers (*in situ*)
- determine the suitable size of *M. rosenbergii* to be used for the bioassays
- develop a procedure for measuring *Artemia* concentrations that does not require specialised equipment, i.e. easy and low-cost.
- determine the period of feeding and the amount of *Artemia* to be fed to the prawn after exposure to any contaminant
- determine the need for aeration during post-exposure feeding experiments

- determine the level of replication required for the post-exposure feeding experiments for a statistically sound result
- determine the effect of water pH, temperature and hardness on prawn mortality and control post-exposure feeding rates. These factors were chosen as they are likely to be the most important factors affecting feeding rate and survival under laboratory and field conditions.

To achieve these objectives, the following experiments were conducted and are described in this chapter:

- Development of a laboratory bioassay with M. *rosenbergii*: exposure phase
    - Observations on moulting under normal culture conditions
    - Prawn density in test glass jars

- Development of a laboratory bioassay with *M. rosenbergii*: post-exposure phase
    - Influence of prawn body size on post-exposure feeding
    - Influence of feeding schedule and food quantity (using *Artemia* as the food)
    - Replication required for post-exposure feeding tests
    - Requirement for aeration during post-exposure feeding tests

- Development of an *in situ* bioassay with *M. rosenbergii*
    - Influence of prawn density in *in situ* chambers on post-exposure feeding rate
    - Effect of water pH, temperature and hardness on survival and post-exposure feeding rate

4.3 Development of laboratory bioassay with *Macrobrachium rosenbergii* : Exposure phase

4.3.1 Observations on moulting of *Macrobrachium rosenbergii* under normal culture conditions

Moulting or ecdysis is an energy demanding process. When crustaceans moult there is a decrease in feeding rate (Anger, 2001). The occurrence of moulting increased variability in feeding rates, with greatest variation when moulting occurred among many animals. When feeding measurements were being recorded, moulting could be kept to a minimum by completing the bioassay process during a single moult cycle. The aim of this experiment was to determine the period of one moulting cycle for *M. rosenbergii* at the postlarval stage.

Methodology

Twenty glass jars containing one animal per jar and fed with *Artemia* (three times per day at 7 nauplii/mL) were set up for 16 days. Fifty percent (50%) of the water was changed every day. Before water exchange, moulted exoseletons were recorded then removed. The mean and standard error of moulting period (days) of individual prawns were calculated.

Results

Table 4.1 indicates the moulting activity of the prawns. Prawns in 2 out of the 20 replicates died before the 16-day experiment ended. From the remaining 18 replicates, it was calculated that intermoult intervals ranged from every 3 to 8 days, with an average of every 4.5 (± 0.22, n = 43) days in soft water.

Section conclusion

Moulting cycle for postlarval size 9-10 mm. was 4.5 (± 0.22, n = 43) days in soft (ASTM) water.

Table 4.1 Moulting time of *M. rosenbergii* is indicated by shaded boxes

	NUMBER OF CULTURE DAYS																Interval
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
Rep. 1		■								■			■			■	7,3,3 days
Rep. 2					■				■				■				4,4 days
Rep. 3		■					■			■				■			5,3,4 days
Rep. 4										■			■				3 days
Rep. 5			■								■				■		8,4 days
Rep. 6			■				■				■				■		4,4,4 days
Rep. 7	■								■				■				8,4 days
Rep. 8		■				■				■							4,4 days
Rep. 9	■				■				■								4,4 days
Rep. 10									■				■				4 days
Rep. 11				■				■				■					4,4 days
Rep. 12		■								■					■		8,5 days
Rep. 13			■				■				■					■	4,4,5 days
Rep. 14		■						■				■				■	6,4,4 days
Rep. 15				■				■				■					4,4 days
Rep. 16		■				■				■				■			4,4,4 days
Rep. 17								■				■					4 days
Rep. 18		■					■					■					5,5 days
Rep. 19			■							■			■		■		7,3,2 days
Rep. 20		■								■				■			8,4 days
Moulting No.	2	8	4	2	2	2	4	4	4	8	3	5	6	3	4	3	

4.3.2 Prawn density in test glass jars

Methodology

Animals were placed in jars containing 500 mL water at 5, 10, 15 and 20 PLs/jar. The bottom area of the jar is 113 cm^2 and densities were equivalent to 442, 884, 1327 and 1770 PLs/m^2, respectively. Each treatment had four replicates. Survival and water quality parameters such as dissolved oxygen, temperature, pH and conductivity in each jar were recorded at 0, 24 and 48 hours. Bonferroni Multiple comparisons test in SPSS version 10 was used to compare survival from different densities of prawns.

Results

Figures 4.1 and 4.2 present the levels of dissolved oxygen (% saturation) and conductivity, respectively, during the density test without aeration. Temperature and pH were not different at all densities throughout 48 hours. pH was between the range of 7 to 8.5. Temperature was between 25-32 ˚C. Dissolved oxygen was not less than 3 mg/L, although it decreased on the second and third days for densities 10 and 20 PLs/jar. In jars with a higher density (20 PL/jar), dissolved oxygen decreased more than that of the other densities. Conductivity increased with density of prawns and ranged from 169 - 299 µS. At high densities more animals are excreting, resulting in more charged particles.

Table 4.2 presents the performance of prawns after 24 and 48 hrs exposure. All prawns in treatments 5 and 10 prawns/jar survived, while a low mortality was recorded in higher density jars in days 1 and 2. Survival in all treatments however was not significantly different ($F_{3,12}$ = 2.36; P = 0.123). Thus, 10 animals per jar was chosen for the feeding test as there would be more remaining animals to use for the subsequent post-exposure feeding test.

Section conclusion

Ten prawns per jar was chosen as the appropriate density for the 24 and 48 hours exposure time as this would provide sufficient number of prawns to continue the post-exposure feeding test.

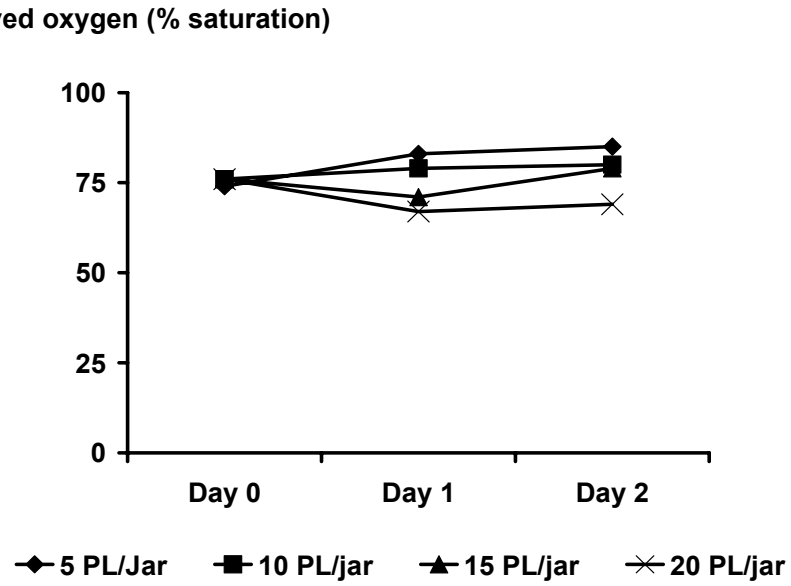


Figure 4.1 Dissolved oxygen (% saturation) in exposure jars with different densities of post-larvae (PLs) from day 0 until day 2 (48 hr).

Table 4.2 Mean number of dead prawns in preliminary culture test

Treatment	Day 0	Day 1	Day 2
5 post-larvae/jar	0	0	0
10 post-larvae/jar	0	0	0
15 post-larvae/jar	0	0	1
20 post-larvae/jar	0	1	1

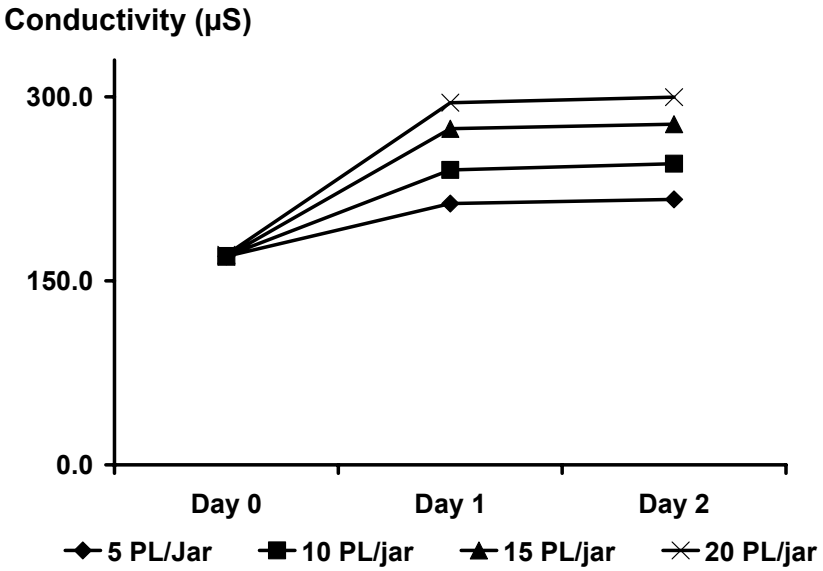


Figure 4.2 Conductivity in exposure jars with different densities of post-larvae (PLs) from day 0 until day 2 (48 hr).

4.4 Development of laboratory bioassay with *Macrobrachium rosenbergii* : Post-exposure phase

4.4.1 Influence of prawn body size

The experiment was set up to determine the relationship between size (length) of prawn and the amount of feed consumption after exposure to non-contaminated media with good water quality conditions. The density of prawn used was obtained from the experiment in section 4.3.2 (10 prawns per glass jar).

Methodology

Post-larvae used in this experiment were prepared in the same way as in section 2.1 with 10 PLs per jar. PLs of different lengths (9 to 14 mm) were exposed to clean water without feeding for 24 hours, after which survival was recorded. Then for the post-exposure feeding rate test, all remaining animals were randomly placed in 100 ml plastic jars containing 80 mL soft ASTM at 1 PL/jar. One hundred Artemia were also added into each jar. The animals were allowed to feed for 4 hours under dark conditions. After this period, feeding was stopped by adding a few drops of

Lugol's solution into each jar. Feeding rate was calculated as in section 2.4. The total length of the PLs was also measured. Frequency dot plots were used to present the relationship between total length and post-exposure feeding rate.

Results

Figure 4.3 presents the results of the post-exposure feeding test which show the effect of the size of prawns (total length) on feeding rate. The bigger the prawn, the higher was their feeding rate. Larger individuals could eat up to 100 *Artemia* per hour. Using bigger prawns for experiments would necessitate feeding more *Artemia,* increasing time needed to prepare the food. If replication was increased, this would also involve more time to count the *Artemia*. Thus prawns at 9-10 mm were considered most appropriate for the feeding tests as their post-exposure feeding rate was less than 20 *Artemia* per hour.

Section conclusion

Results of this experiment showed that the amount of feed required increases in proportion to body size. The suitable size of test animals for this study was 9-10 mm with a post-exposure feeding rate of less than 20 *Artemia* per hour. Thus the amount of feed set at 100 *Artemia* per replicate from this section's tests would be enough to calculate the post-exposure feeding rate.

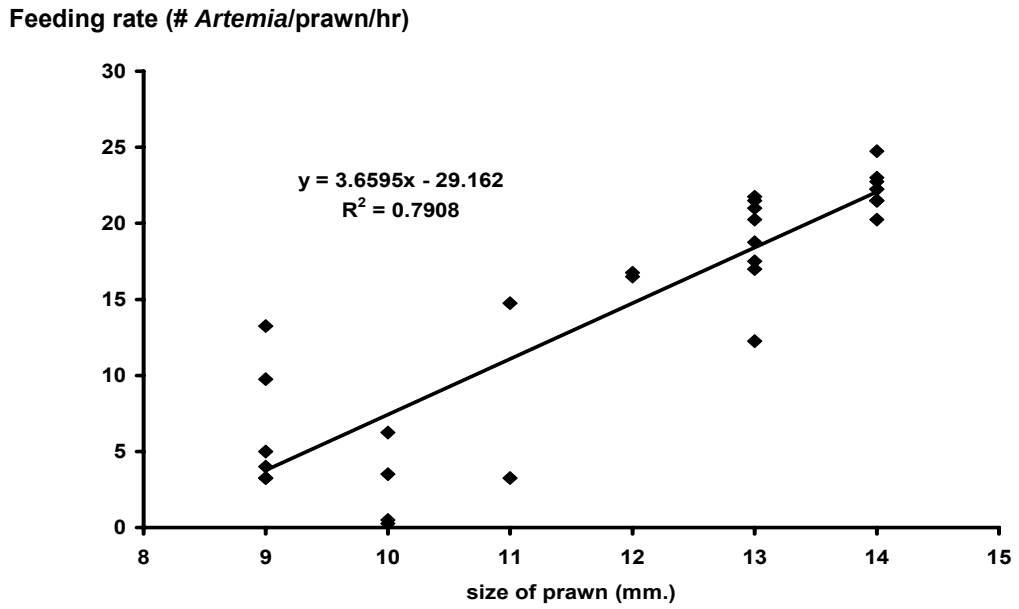


Figure 4.3 Post-exposure feeding rate of prawns with total length from 9 mm. to 14 mm.

4.4.2 Influence of feeding schedule and food quantity

Food is a fundamental aspect in the larval development of decapod crustaceans (Correia *et al.*, 2000). There is a danger in underfeeding postlarvae in experiments which could result in errors in feeding calculations. Thus there was a need to determine the amount of food that would be sufficient present to prawns of specific sizes during the feeding tests and the appropriate feeding period to observe food consumption in prawns.

Methodology

This experiment began with prawns at 10 PL/glass jar in clean media (soft ASTM) without feeding. After 24 hours of exposure to clean media, prawns were fed with 50, 70 and 100 *Artemia* at different feeding periods of 2, 4, 6 and 8 hours. Each treatment had 1 PL and three replicates. After each feeding time, the remaining *Artemia* were collected, fixed with Lugol's solution and placed in 15 mL glass tubes. Sample variability relative to the sample mean was expressed as coefficient of variation (CV). Gomez and Gomez (1984) stated that the CV indicates the degree of

precision to which the treatments are compared and is a good index of the reliability of the test. ANOVA with more than one observation for each factor combination (with replication) from MINITAB version 13.1 was used to analyse interaction between feeding period and density of *Artemia*. Bonferroni multiple comparisons test in SPSS version 10 was used to compare the post-exposure feeding rates from different densities of *Artemia* (50, 70 and 100 *Artemia* per jar) and feeding period (2, 4, 6 and 8 hours).

Results

Figure 4.4 shows a general decreasing trend in the coefficient of variation (CV) for 2 and 4 hours feeding duration, with 50, 70 and 100 *Artemia*. It was expected that the CV would decrease with increasing amount of feed and feeding duration. However, for 6 and 8 hours feeding duration, the CV showed an opposite trend. *Artemia* were all consumed within 6 to 8 hours in the treatment with 100 *Artemia*. But for the treatment with 50 and 70 *Artemia*, there were still some remaining after 6 and 8 hours. The feeding rate was low for 6 and 8 hours with 50 and 70 *Artemia* which could be caused by other factors. However, these could not be detected during this experiment.

When only 2 and 4 hours were considered, the former gave the lowest CV. There was a significant difference between 2 and 4 hours feeding period ($F_{3,23}$ = 13.18; P <0.001). However, both densities of *Artemia* (70 and 100 *Artemia*) and their interactions between feeding period (2 and 4 hours) were not significantly different ($F_{2,23}$ = 0.32 ; P = 0.728 and $F_{6,23}$ = 1.19; P = 0.344, respectively).

Section conclusion

This experiment concluded that feeding duration for post-exposure feeding test should be 4 hours and the number of *Artemia* to be fed was 100. Even though the treatment with 2 hours feeding period showed a lower coefficient of variation, 4 hours was considered better because it gave more time for feeding.

The number of *Artemia* to be fed was another point to consider to ensure that there was enough food for each prawn for a four hour period and still have some remaining for post-exposure feeding rate calculation. Both 70 and 100 *Artemia*

treatments were not significantly different. With 100 *Artemia* per replication there was confidence that the prawn would not be stressed from lack of feed during the tests.

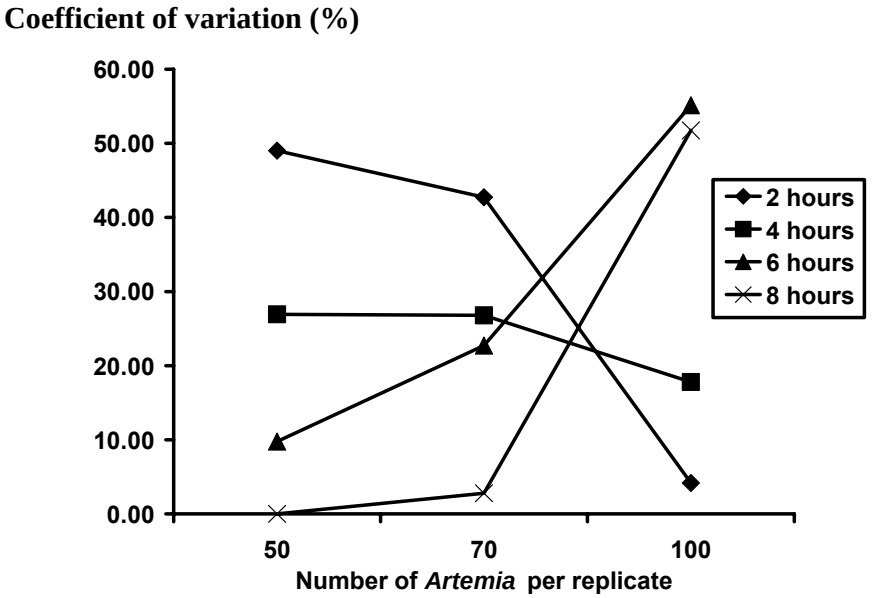


Figure 4.4 The coefficient of variation of *Macrobrachium rosenbergii* feeding rates on *Artemia* offered at different densities over different durations.

4.4.3 Number of replicates

The aim of this experiment was to establish the number of replicates required to get a minimum difference of 40 %, 20 %, 15 %, 10 % and 5 % between treatments during the feeding period (from Experiment in 4.4.2). The power of a statistical test (1-β) is defined as the probability that the test will correctly reject the null hypothesis when it is false, avoiding type II errors (β). Increasing the power of a statistical test minimizes the chances of committing type II errors and can be increased by increasing the number of replicates (Zar, 1999). A power of 80 % was chosen as this is traditionally the level used for statistical testing.

The power (1-β) calculation was simplified from sample size estimation in one-sample test (Zar,1999):

$$n = (S^2/\delta^2) \times (t_{\alpha,\upsilon} + t_{\beta(1),\upsilon})^2$$ .....Equation B

where

n = the sample size estimate

$t_{\alpha,\upsilon}$ = t value at confidence level (95 %) and that sample size (υ)

$t_{\beta(1),\upsilon}$ = t value at confidence level (95 %) and in that sample size (υ)

S = standard deviation
δ = the expected difference between the two means

Source : Zar, 1999 (Power of One –Sample Testing)

Methodology

The PLs used in this experiment were placed in clean water (soft ASTM) for 24 hours prior to the experiment. Then, twenty 100 ml plastic jars containing 80 mL of soft ASTM were stocked with 1 PL/jar. Animals in each jar were presented with 100 *Artemia* for 4 hours, after which, the remaining *Artemia* were removed and feeding rates calculated as in equation A.

A test was performed on feeding rates to estimate the minimum sample size required to detect at least a 40 %, 20 %, 15 %, 10 % and 5 % difference between 2 or more means when using ANOVA with a power of at least 80 % (at the $\propto$ = 0.05 significance level), (Zar,1999).

Results

Figure 4.5 presents the changes in the power of one set of post-exposure feeding test data, performed on a 4 hours feeding rate data, with increasing number of replicates, when the minimum detectable difference between treatments were 40 %, 20 %, 15 %, 10 % and 5 % (at the alpha = 0.05 significance level).

Power increased as the number of replicates increased. At 40 % difference between means the desired power of 0.8 was reached with 5 replicates. But the ability of the bioassay to detect small differences in feeding rates would be more important than high statistical power. Increasing replication from 5 to 10 showed an increase in

power, but when the number of replicates was increased above 19, power decreased. Ten replicates with power of 0.58 increased power from 0.17 at 5 replicates to 0.58 at 10 replicates with a 20 % difference between means. The number of replicates must be more than 16 to increase the power value to 0.8 and still have a 20 % difference between means.

Section conclusion

Ideally, the number of replicates could be 16 per treatment to give the test more sensitivity to detect a 20 % difference between means and 80 % to reject the null hypothesis when it is false, avoiding type II errors. Aiming for a 20 % difference between means was impractical for the post-exposure feeding test because more resources would be required to process the feeding test (time). Use of 10 replicates was the compromise replication level for the post-exposure feeding test, giving a 58 % possibility to avoid type II errors and to detect 20 % difference between means.

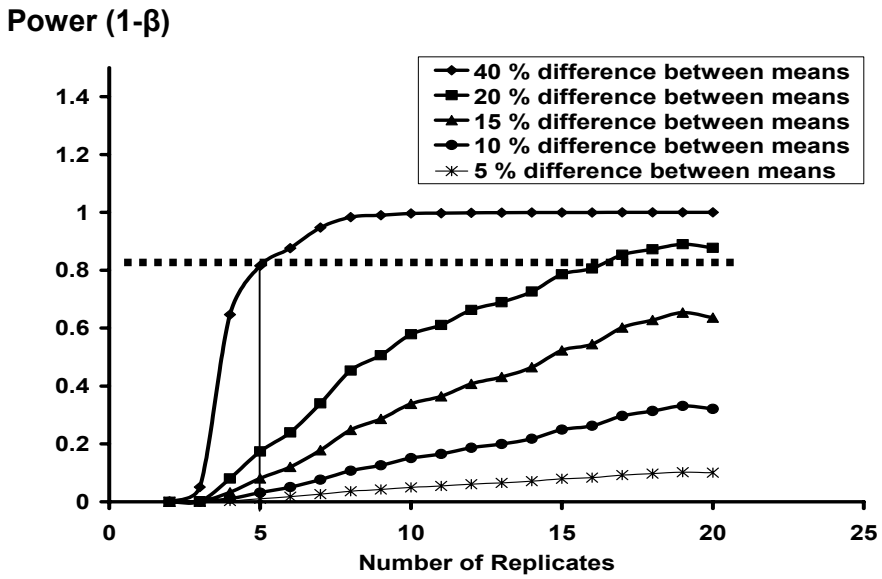


Figure 4.5 The number of replicates required to give at least an 80 % probability of detecting a minimum difference of 40 %, 20 %, 15 %, 10 % and 5 % between treatment means ($\alpha = 0.05$).

4.4.4 Requirement for aeration during feeding tests

Methodology

The PLs used were exposed to clean media (soft ASTM) for 24 hours prior to the feeding experiment. Individual PLs was then stocked in fifty 100 ml jars containing 80 mL of soft ASTM with 100 *Artemia*. Twenty five of the jars were aerated gently, with air blowing slowly on the surface of water. The jars were covered with paraffin. The other 25 jars did not have aeration and were covered with their own plastic caps. The animals in each jar were fed with 100 *Artemia* for 4 hours. After feeding, the remaining *Artemia* were removed and feeding rates calculated as in equation A (chapter 2).

A test was performed to estimate the feeding rate in aerated and non-aerated conditions. A one way ANOVA was performed on feeding rate data obtained from both aerated and non-aerated jars to observe whether there were significant differences in feeding rates between the two conditions.

Results

Figure 4.6 presents the post-exposure feeding rate with aeration and without aeration. The feeding rate obtained with aeration averaged 8.45 (±0.46 SE) *Artemia* per hour per animal while without aeration, it was 10.41 (±0.76 SE) *Artemia* per hour per animal. There was a significant difference in the post-exposure feeding rate between these two groups. ANOVA confirmed the difference in feeding rate between aerated and non-aerated conditions ($F_{1,60}$ = 3.83; P = 0.055). Aerating the water appeared to stress the test animals and affect their feeding rate.

Section conclusion

It is not necessary to move the water through aeration during the post-exposure feeding test as it could cause more stress to test animals.

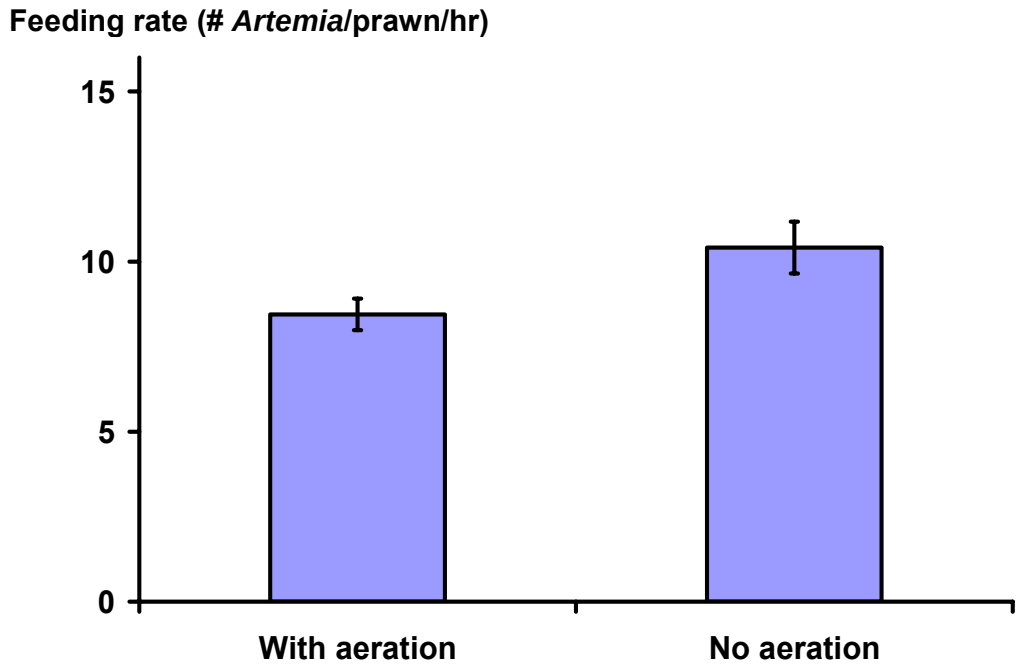


Figure 4.6 Post-exposure feeding rate of prawns in aerated and non-aerated conditions. There was significant effect on the feeding rate ($p < 0.05$). Bars indicate standard error.

4.5 Development of *in situ* bioassay with *Macrobrachium rosenbergii*

4.5.1 Influence of prawn density in chambers on the post-exposure feeding rate

In the field deployment, it was necessary to use cages or chambers to hold the test animals and protect them from predators. This also facilitated easy retrieval after deployment. Chambers were made from PVC and 508 µm nylon mesh and coated with chemical-resistant epoxy (Figure 2.2). The total water volume inside the chamber was 89.3 ml. The mesh was sealed to the chamber with white thermal glue which was non-toxic to cladocerans, according to Pereira *et al.* (1999). To develop procedures for *in situ* testing using the chambers, an experiment was initially conducted in the laboratory to determine the suitable density of prawns to be placed in the chambers.

Methodology

Prawns were placed in the chambers at different densities (5, 10, 15 and 20 prawns per chamber) for exposure testing. The animals were randomly assigned, with 3 replicates for each treatment. Chambers with prawns were put in a 30 L aquarium with aeration for 24 hrs. After 24 hrs, a feeding test was performed. Ten replicates were set up for each treatment during the post-exposure feeding test. Results of the feeding test were evaluated by calculating the coefficient of variation (CV) for each treatment. Bonferroni multiple comparisons test in SPSS version 10 was used to compare the post-exposure feeding rates from different densities of prawns (5, 10, 15 and 20 prawns per chamber).

Results

Table 4.3 presents the feeding rate of prawns and coefficient of variation from each treatment. Post-exposure feeding rate ranged from 8 (±0.66 SE) to 12 (±0.57 SE) *Artemia* per hour per animal. Survival ranged from 73 to 100 %. The average survival in all treatments was 82.9 % (± 2.15 SE). Bonferroni testing revealed no significant difference in survival between treatments with different prawn densities ($F_{3,8} = 0.355$; $P = 0.787$). But for feeding rate, a significant difference between 10 and 20 prawns

per chamber was found ($F_{1,17}$ = 17.44; P = 0.001). Ten prawns per chamber had the lowest coefficient of variation (14.84 %); this density was therefore chosen for further field deployment studies.

Section conclusion

All treatments were deployed in a controlled environment considered to be optimal for deployment. The average mortality was less than 20 % which indicated good environment during exposure, causing no stress for the animals in the chamber. Results from this experiment concluded that 10 animals per chamber was the optimal number for the *in situ* bioassay based on statistical data giving the lowest CV.

Table 4.3 Mean post-exposure feeding rate, standard deviation (SD) and coefficient of variation (CV) on different density of prawns in deployment chambers

Density of prawn (PLs/89.3 mL)	**Survival during exposure (%)**	**Post-exposure feeding**		
		Feeding Rate (No. *Artemia*/PL/hr)	**SD**	**CV (%)**
5	87	10.58	3.74	35.37
10	83	12.15	1.80	14.84
15	80	11.56	2.22	19.19
20	82	8.53	1.98	23.20

4.5.2 Effect of environmental parameters on post-exposure feeding rates

4.5.2.1 Test animals and acclimation

All animals used were post-larvae (PLs) obtained from a commercial hatchery. They were acclimated as in 2.1 and after 48 hours animals were ready to be used for the experiment.

4.5.2.2 Effect of pH on survival and post-exposure feeding rate

In the natural environment, there is a wide range of pH levels depending on many factors such as temperature, oxygen saturation and photosynthesis. If pH was higher or lower than the optimal range (7.0-8.5), survival and feeding rate of the prawns could be affected. But within the optimal range, it was expected that pH would have little or no effect on survival and feeding rate.

Laboratory experiments were conducted to expose animals to pH levels of 3.0, 5.0, 7.0, 7.5, 8.0, 8.5, 9.0 and 11.0. The pH solutions were prepared using soft water ASTM and hard water ASTM which had pH values of 7.0 and 8.0, respectively. Solutions with pH 3.0 and 5.0 were adjusted by adding 69% HNO_3 drop by drop using a 3 ml plastic pipette while stirring. pH was monitored using a pH meter. Solutions with pH 7.5, 8.5, 9.0 and 11.0 were adjusted by dissolving NaOH accordingly.

For each pH treatment, 10 prawns were randomly assigned to 1,000 mL glass jars containing 500 mL of the pH solution. Each treatment had five replicates. Animals were exposed to the treatments for 24 hours. After 24 hours exposure, mortality was recorded. Remaining animals were used for the post-exposure feeding test.

For the post-exposure feeding test, the remaining animals were randomly placed in 100 ml plastic jars containing 80 mL soft ASTM at one PL/jar, with 10 replicates per treatment. One hundred *Artemia* were individually counted and added

per jar as feed. Feeding duration was 4 hours under dark conditions. After 4 hours, feeding activity was terminated by adding a few drops of Lugol's solution. Remaining *Artemia* were collected and counted. Post-exposure feeding rate was calculated as in section 3.4.

Bonferroni multiple comparisons test in SPSS version 10 was used to determine which treatments were significantly different from the control (pH 7:soft water)

Results

Figure 4.7 shows the effect of pH on post-exposure feeding rate of prawns. Low pH resulted in a higher mortality. Survival at pH 5 was 42% (± 3.74 SE) while that at pH 9.0 was 64% (± 2.45 SE). At pH 7.0-8.5, survival ranged from 73 to 95%. Survival at pH levels range between 7.0 to 8.5 were not significantly different ($F_{3,12}$ = 4.26; P = 0.029). However, survival at pH 5 and 9 were significantly different ($F_{2,11}$ = 46.18; P < 0.001) from that of the control (pH 7.0).

The post-exposure feeding rates generally decreased with pH outside the optimal pH range of 7 to 8. From a feeding rate of 14 (± 1.51 SE) *Artemia* per prawn per hour at pH 7, it decreased to 3.6 (±0.51 SE), 5.0 (±2.22 SE) and 4.8 (±0.86 SE) *Artemia* per prawn per hour at pH 5, 8.5 and 9, respectively. Bonferroni test showed that there were significant differences between feeding rates of prawns exposed to pH 5, 8.5 and 9 and the control (pH 7) (P < 0.05). ANOVA revealed that the post-exposure feeding rates obtained at pH 7.0 to 8.0 ($F_{2,10}$ = 0.01; P = 0.986) were not significantly different

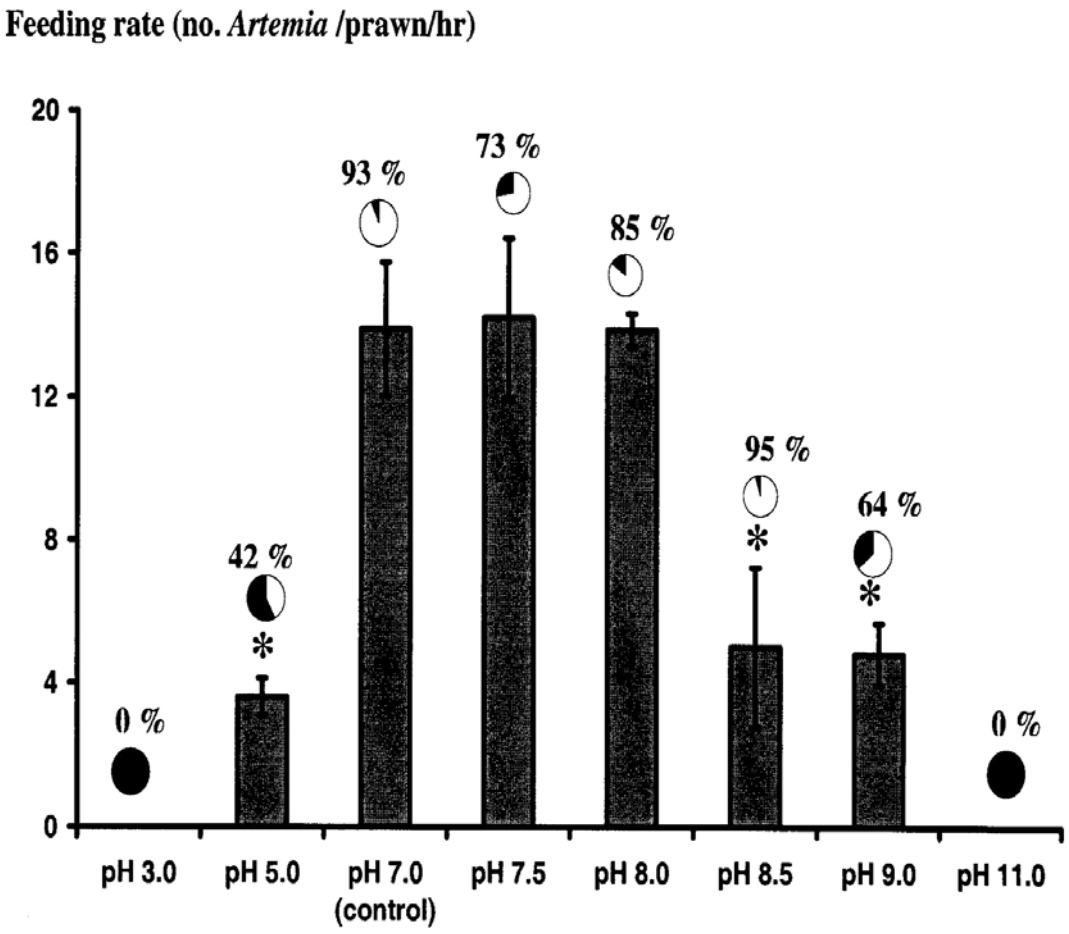


Figure 4.7 Effect of pH on post-exposure feeding rates in *M. rosenbergii*. Error bars indicate standard error. Asterisks indicate post-exposure feeding rates that are significantly different from the control ($P < 0.05$). Pie charts and percentages present average survival of animals during 24 hours exposure.

4.5.2.3 Temperature effects on survival and post-exposure feeding rates

Mortalities occur only when animals are suddenly transferred to a temperature largely different from that of their medium (New and Valenti, 2000). Rapid transports can also cause stress and affect their feeding activity. During this particular trial, animals were transferred directly from the inside AIT laboratory to the outdoor jars where the temperature was higher.

Temperature in the laboratory during the test was 25 ˚C while that in the field (outside the laboratory) was 36 ˚C. Animals were transferred to outdoor jars immediately without acclimation.

Ten animals were randomly assigned to 1,000 mL glass jars containing 500 mL soft ASTM. The two treatments used were treatments: inside (25˚C) and outside (36˚C) the laboratory. Each treatment had five replicates. After 24 hours exposure to these two locations with different temperature conditions, the number of surviving animals was counted. Then 10 remaining animals from each treatment were again randomly placed in 100 mL plastic jars at 1 PL/jar for the post-exposure feeding test. Each jar contained 80 mL soft ASTM and 100 *Artemia*. This feeding test was conducted in the dark. After 4 hours, feeding activity was terminated by adding a few drops of Lugol's solution. Remaining *Artemia* was collected and counted. Feeding rate was calculated as in 2.4.

A one way analysis of variance (ANOVA) was performed on feeding rate data obtained to observe whether there were significant differences in feeding rates for different treatments. A t-test was used to compare treatments and to determine significant differences between them.

Results

Figure 4.8 shows the effect of temperature on post-exposure feeding rate of prawns. A significant difference in mortality inside (12%) and outside (2%) the laboratory was detected ($F_{1,8} = 12.5$; $P = 0.008$). Conversely, survival inside (25 ˚C)

and outside (36 ˚C) laboratory were 88 % (± 2) and 98 % (± 2), respectively. The post-exposure feeding rates inside (25˚C) and outside (36˚C) the laboratory were 15 (± 1.20 SE) and 14 (± 1.38 SE) *Artemia* per prawn per hour, respectively. A one-way ANOVA demonstrated no difference in feeding rates between treatments ($F_{1,8} = 0.04$; P = 0.842). Temperature has an effect on survival but not on feeding rates. The outside temperature of 36 ˚C is more representative of the normal temperature for prawns.

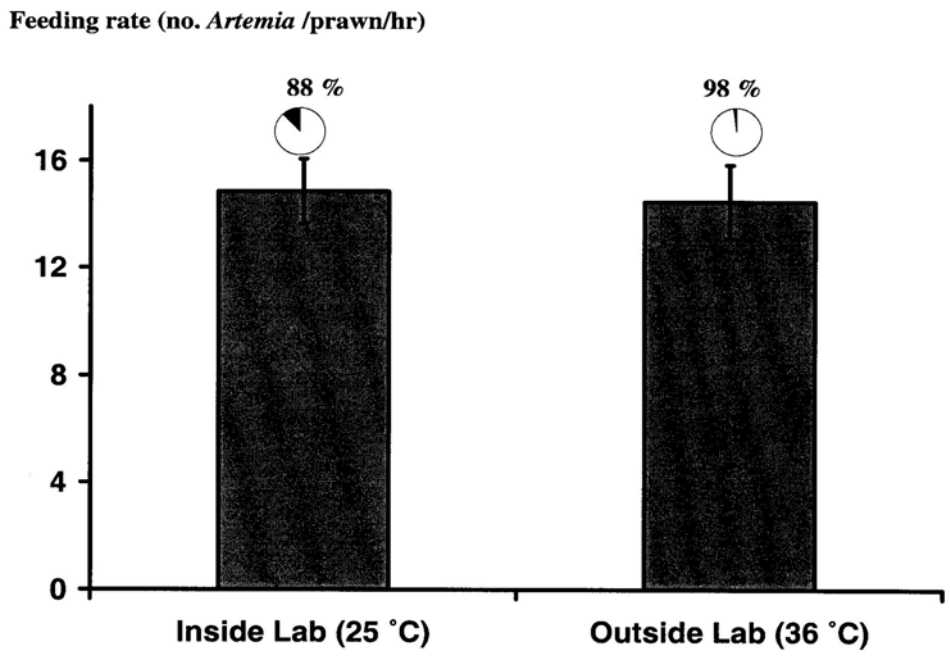


Figure 4.8 Effect of temperature on post-exposure feeding rates in *M. rosenbergii*. Error bars indicate standard error. Pie charts and percentages present average survival of animals during 24 hours exposure.

4.5.2.4 Effect of hardness on survival and post-exposure feeding rates

M. rosenbergii postlarvae are mostly nursed in water with a neutral pH (7) but in this research were cultured in the laboratory in soft water ASTM. During field exposure prawns may be affected by changes in water hardness, thereby affecting survival and feeding during post-exposure. This study determined the effect of hard and soft water acclimation in combination with differing hard and soft water exposures on *M. rosenbergii*.

The soft water used during this experiment was soft water ASTM (with a hardness of 40 – 90 mg/L $CaCO_3$). The procedures for soft water ASTM preparation could be found in section 2.2. The hard water used was hard water ASTM. Preparation for hard water ASTM involved mixing four inorganic salts (KCl -1.6 g in 1 L, $MgSO_4$. $7H_2O$ – 49.1 g in 1 L, $NaHCO_3$ – 38.4 g in 1 L and $CaSO_4$ – 12 g in 5 L). From each stock solution, 100 ml KCl, 100 ml $MgSO_4$. $7H_2O$, 100 ml $NaHCO_3$ and 1000 ml $CaSO_4$ were added to a 20 L jar container and the final volume made up to 20 L by adding distilled water. The hard water ASTM had a pH of 8 and a hardness of 160-180 mg/L $CaCO_3$. Treatments for acclimation, exposure and post-exposure water hardness used during the experiment are shown in Table 4.4 and schematic diagram in Figure 4.9.

Table 4.4 Types of ASTM water used during acclimation, exposure and post-exposure periods to determine the experiment on the effect of water hardness on prawn survival and post-exposure feeding rate.

	Acclimation	**Exposure**	**Post-exposure**
Treatment 1 (Control)	SOFT	SOFT	SOFT
Treatment 2	SOFT	HARD	SOFT
Treatment 3	HARD	HARD	SOFT
Treatment 4	HARD	SOFT	SOFT

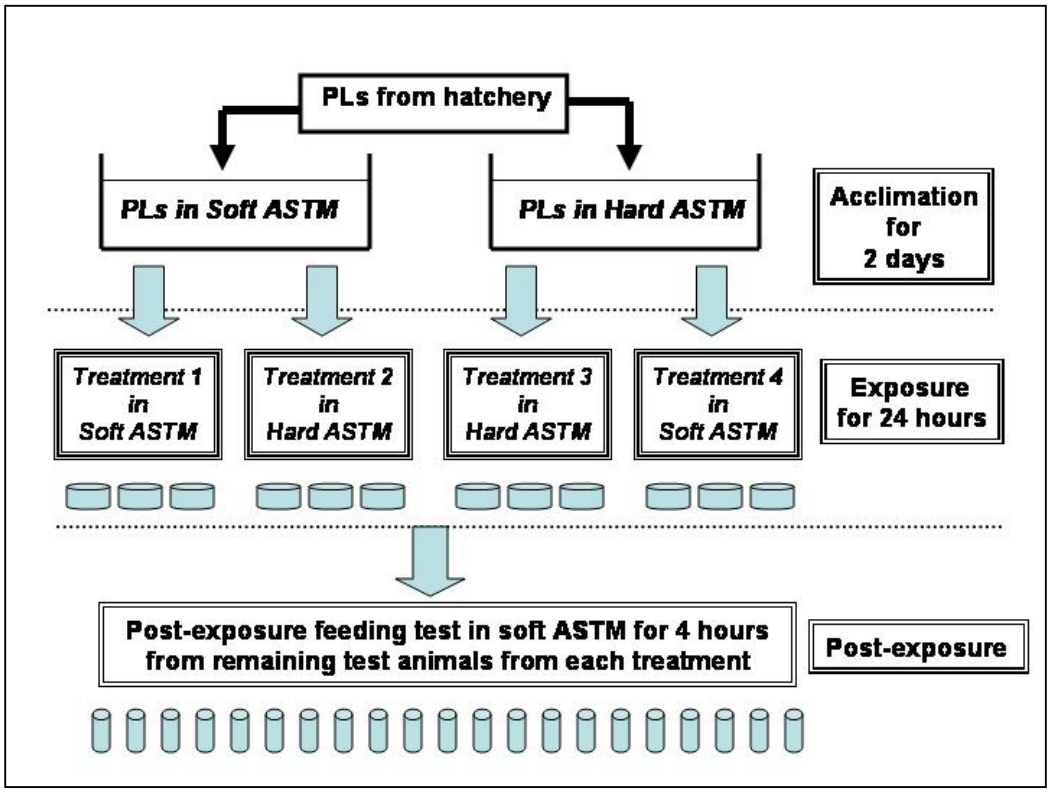


Figure 4.9 Schematic diagram of hardness experiment.

Treatment 1 was used as the control as soft water was normally used to hold the animals prior to trials. In this experiment, the prawns were acclimated as soon as they arrived from the hatchery. Prawns were acclimated for 2 days (48 hours). Those to be used for Treatments 1 and 2 were acclimated in soft ASTM (as described in 2.2) while those for Treatments 3 and 4 were acclimated in hard ASTM. After the 2-day acclimation period, prawns were exposed to different levels of hardness for 24 hours.

For each treatment 10 animals were randomly placed in a 1000 mL glass jar containing 500 mL of water with the assigned hardness. Each treatment had five replicates. After 24 hours exposure, survival was recorded and the remaining animals were again individually placed at random in 100 mL plastic jars with 80 mL of soft water ASTM and 100 *Artemia*, with 10 replicates. They were left undisturbed for 4 hours for feeding in the dark. After 4 hours, feeding activity was stopped by adding a drop of Lugol's solution. Feeding rate was calculated as in 2.4.

Bonferroni Multiple Comparisons test in SPSS version 10 was used to compare treatments and determine significant differences from the control (acclimation, exposure and post-exposure in soft water).

Results

Figure 4.10 shows the effect of hardness on post-exposure feeding rates of prawns. The test animals were acclimated and exposed to soft and hard water. This experiment aimed to find the flexibility of the bioassay at different levels of water hardness. Bonferroni test showed no significant differences in post-exposure feeding rates in all treatments when compared with control (soft water acclimated/soft water exposed). Even though prawns in Treatment 2 (acclimated in soft water and exposed to hard water) had a higher feeding rate (13 ± 1.42 *Artemia* per prawn per hour ± SE) than those in other treatments (average 10 ± 1.14 *Artemia* per prawn per hour ± SE), ANOVA revealed that there was no significant difference ($F_{1,9} = 4.72$; $P = 0.058$). Post-exposure feeding rate of prawns acclimated in hard water and exposed to soft water (9 ± 1.48 *Artemia* per prawn per hour ± SE) was not different from that of prawns acclimated in hard water and exposed to hard water (10 ± 1.31 *Artemia* per prawn per hour ± SE) ($F_{1,15} = 0.06$; $P = 0.817$).

Mortality was less than 10 % in all treatments. ANOVA revealed that there was no significant difference ($F_{3,8} = 2.0$; $P = 0.193$) in mortality. Prawns exposed were active and did not show that they were affected by exposure to different levels of hardness.

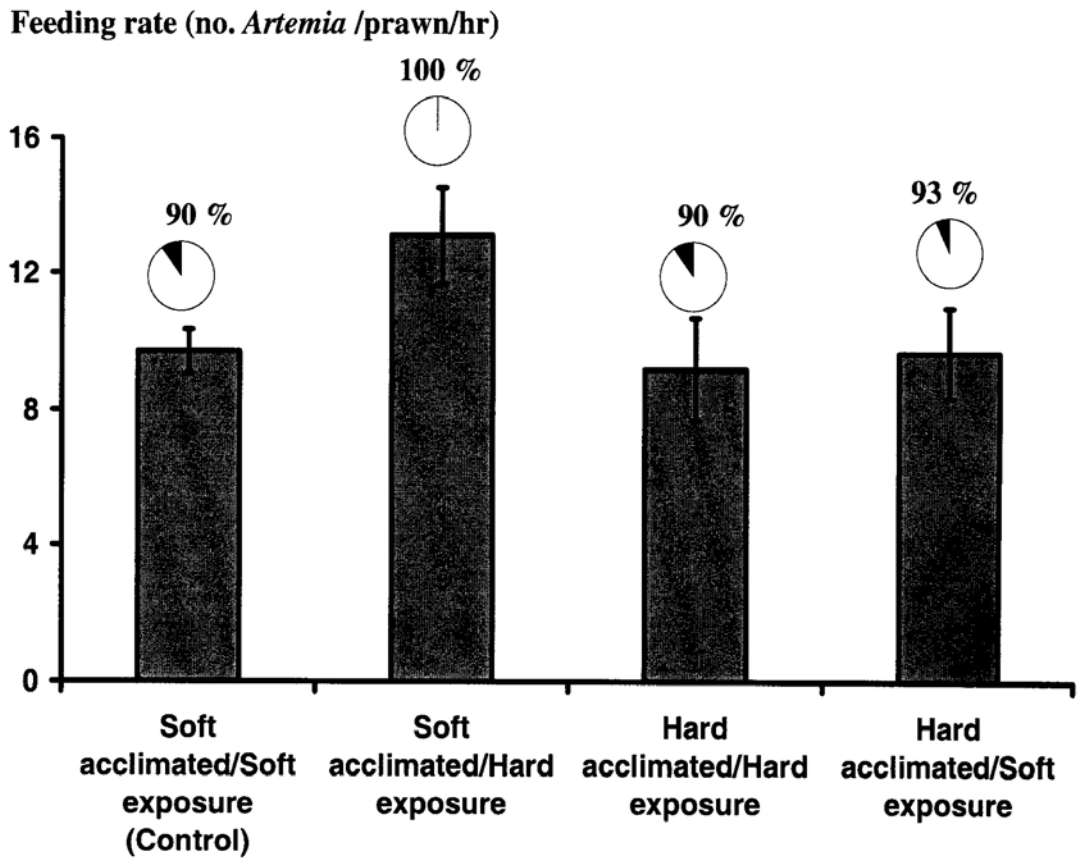


Figure 4.10 Effect of hardness on post-exposure feeding rates in *M. rosenbergii*. Error bars indicate standard error. Pie charts and percentages present average survival of animals during 24 hours exposure.

4.6 Discussion

The aim of the experiments detailed in this chapter was to develop a method for *M. rosenbergii* bioassay that could produce repeatable results while minimising factors that may contribute to variation in feeding rates. Replication should also be sufficient to detect differences between post-exposure feeding rates. In the *M. rosenbergii* bioassay, feeding rate variability may be affected by factors such as moulting, density, amount of food (*Artemia*), feeding duration, prawn length, number of replicates and aeration. These factors were taken into consideration during the experiments and the best ways to minimise variation in the post-exposure feeding rates were determined.

The growth of crustaceans depends on moulting (Hartnoll, 1982). Prawns require a medium that has high calcium content to permit shell calcification after moulting, thus a high alkalinity concentration in culture media stimulates moulting (Latif, 1994). Therefore, soft ASTM was used as the water medium for all experiments in this research because it is low in alkalinity, which was expected to prevent frequent moulting of test animals. It was necessary to determine the moulting cycle of *M. rosenbergii* in soft ASTM since there are no papers describing this topic. There was a concern in this study that the moulting activity of PLs would affect the experiments. Since PLs from hatcheries are normally produced from different broodstock, i.e. one cycle of production in hatcheries requires 40 to 60 females with fertilized eggs per 300 L tank (New and Valenti, 2000), their individual moulting cycles are unlikely to be synchronized. PLs could moult everyday after the hatching period until the post-larvae stage. During the moulting period, PLs are vulnerable to attack by their own kind and by predators. Even in toxicity tests it would be difficult to avoid this situation. If more than one or two prawns in the control moulted, the experiment should be set up again. From the tests on moulting of PLs in soft ASTM, the range of moulting period was from 3-8 days with an average of 4.6 (± 0.28 SE, n = 30) days. Four to five days should then be long enough for the bioassay which requires 24 hours of exposure to test substances and 4 hours for post-exposure feeding test.

The next point of concern for standard bioassay was the density of prawns in the test containers. The density of prawns could affect the conditions in the test containers due to their cannibalistic and territorial behaviour. Simple water quality monitoring showed that there was a slight decrease in dissolved oxygen concentration (% saturation) at the highest density (20 PLs/jar) but it was still within the acceptable range. All parameters were within the optimal range for prawns as described by Zimmermann (1998). Dissolved oxygen remained about 3 mg/L. pH was 7 - 8.5. Temperature was 25 - 32˚C. Conductivity increased according to time and stocking density. It might have more ions as time passed by in higher density jars, such as ammonia and total nitrogen through excretion. Conductivity measures the ability of water to carry an electrical current and is directly related to the dissolved ions (charged particles) present in the water. In natural waters, such as a lake, pond or

river, conductivity often increases when there is an increase in pollutants (Yasouri and Foster,1991; Isidori *et al.*, 2004)

In terms of survival after 24 and 48 hrs exposure, the treatments with 5 and 10 prawns/jar did not have any mortality, thus, 10 animals/jar was chosen as the density for the tests using exposure jars as there would be more animals left to continue on to the post-exposure feeding tests. Survival in all treatments were not significantly different ($F_{3,12}$ = 2.36; P = 0.123). Prawns are cultured at even higher densities, ranging from <200 to >6,000 PL/m^2 of bottom area (New and Valenti, 2000). The density chosen for the experiments in this research (884 PL/ m^2) was almost 4 times less than the average density (3,100 PL/m^2) of culture systems.

In prawn hatcheries, the number of *Artemia* given as food has to be increased at every stage of the prawn's life cycle (New and Valenti, 2000). Results from trials on the relationship between prawn size and post-exposure feeding rate showed that more *Artemia* were needed when prawns were longer or larger. Barros and Valenti (2003) found the same results. They mentioned that in the last larval stages (IX-XI), the regression equation gave very high maximum ingestion rates. At these stages, the functional response is type I (ingestion increases linearly with increasing number of prey until it reaches saturation point), with *Artemia* nauplii as food. Using prawns at these stages for the bioassay would require more time and increased cost. If the prawns were larger, food could be depleted and the feeding rate would be biased. Thus, this study concluded that the size of prawn to be used should be between 9-10 mm.

The coefficient of variation (CV) was used to demonstrate variability in feeding rates with differing amounts of food (*Artemia*) and feeding duration. It was expected that the CV would decrease with increasing food and time spent feeding. An increase in the time spent feeding should have produced a lower CV as it allowed more time for feeding rates to settle and become more uniform. The CV for prawn feeding at 2 and 4 hours followed the expected trend, i.e. CV decreasing with increasing food and time spent feeding. However, for the 6 and 8 hours feeding duration, the CV increased with increasing food which left only 2 and 4 hours feeding duration for consideration (Figure 4.4). Two hours feeding duration produced the

lowest CV (4.17 %), however, feeding for this length of time was too short because prawns need to adjust to a new environment in the feeding jar. So the treatment using 4 hours with 100 *Artemia* was chosen even though the CV (17.79 %) was higher. However, this CV is still much lower than those obtained by McWilliam (2001) and Crichton (2003) with 24 % for baseline *D. magna* feeding rates and 23 % for *Lymnaea peregra* (freshwater snail), respectively.

The average post-exposure feeding rates for 4 hours at all three densities of feed were not significantly different with average of 11.59 ± 0.85 *Artemia*/prawn/hr (± SE). However, there was a significant difference in feeding period between 2 and 4 hours ($F_{3,23} = 13.18$; $P < 0.001$). This experiment concluded that the optimal number of *Artemia* per replicate was 100 with 4 hours feeding period for post-exposure feeding test.

It was expected that statistical power would increase with increasing number of replicates. However, results obtained in the tests did not reach the desired power of 0.8. It was only at 40 % difference between means that the desired power of 0.8 was reached, with 5 replicates. By increasing the number of samples tested, it was found that 16 replicates were required to reach the 0.8 power level when 20 % was the detectable difference between feeding rate means. But the ability of the bioassay to detect small differences in feeding rates will be more important than high statistical power. A power of 0.58 with 10 replicates was chosen with a 20 % difference between means. The number of animals required to give higher levels of replication became impracticable to produce as it would mean too much time would be spent in counting *Artemia*.

The experiment to determine the requirement for aeration during feeding tests indicated that aeration was not necessary. Niu *et al.* (2003) found that food consumption increased directly with temperature and oxygen consumption increased significantly with temperature. Normally in the feeding procedure in hatcheries, aeration is turned off during feeding time and resumed later when observing all larvae are actively feeding (Chowdhury, *et al.*, 1993). In this study, it was found that feeding decreased in jars with aeration. Figure 4.6 shows the differences in results, and ANOVA revealed that there was a significant difference in feeding rates between

aerated and un-aerated containers ($F_{1,60}$ = 3.83, P = 0.055). Aeration might have strongly disturbed the water in the small jars and affected feeding activity, making the prawns more stressed and finding food more difficult. The jars were small enough for the PLs to find the *Artemia* without the aid of aeration; each jar contained 80 mL water, 1 PL and 100 *Artemia*).

The average post-exposure feeding rate without aeration was 10.4 (± 0.76 SE) *Artemia*/prawn/hr. In comparing the feeding rate from this experiment with the experiment comparing densities of feed (section 4.4.2) with average feeding rate of 11.59 (±0.85 SE) *Artemia*/prawn/hr, ANOVA revealed that there was no difference in the feeding rate ($F_{1,70}$ = 0.01, P = 0.922). This shows that the feeding measuring process developed and used in this study is repeatable and can give the correct measurement for the feeding of prawn under the laboratory condition.

The *in situ* bioassay test chamber requires to retain animals during deployment in the field. Animals should be exposed to field conditions so that lethal or sub-lethal effects are detected after exposure. The design of the test chamber to be used in the *in situ* bioassay is important. It should be cylindrical to avoid dead corners, and secure enough to allow the test animals to be held for a specified period of time. It should have enough space so as not to stress them and the material should be non-toxic (Meletti, 2002). Laboratory trials on densities showed that 10 prawns per chamber (i.e. 1 prawn/8.93 mL) had the lowest CV, which was then used as the prawn density during *in situ* bioassays. Studies with other species have used varying densities in chambers deployed in aquatic systems, such as *Ceriodaphnia dubia* neonates at 1 individual/10 mL (Pereira *et al*., 1999), *Chironomus tentans* (midge) 2^{nd}-3^{rd} instar at 1/48.2 mL (Chappie and Burton, 1997), *Daphnia magna* neonates at 1/10 mL (Pereira *et al*., 1999) and 1/20.6 mL (Maltby *et al*., 2000), 4-day old *Daphnia magna* at 1/17.5 mL (McWilliam and Baird, 2002), *Gammarus pulex* size 7.52 mg DW at 1/98 mL (Maltby *et al*., 2000) and size 8.24 mg DW at 1/3.27 mL (Maltby *et al*., 2002a), and *Hyalella azteca* (amphipod) of < 2 weeks old at 1/48.2 mL (Chappie and Burton, 1997). For *M. rosenbergii*, the chosen density of 1/8.93 mL, obtained from initial tests in laboratory (optimal) conditions, is comparable to the densities used by other studies with different species mentioned, especially with *G. pulex* which had nearly the same size with the *M. rosenbergii* PLs used in this study (in terms of length).

The determination of the effect of environmental factors on survival and feeding rate of prawns was also necessary. Post-exposure feeding rates of *M. rosenbergii* exposed to a pH range of 7.5 to 8.0 did not significantly differ from the control post-exposure feeding rate at pH 7.0 ($P>0.05$) (Figure 4.10). These ranges are within the optimal pH values for *M. rosenbergii* growth of pH 7.0-8.5 (New, 1995). Post-exposure feeding rate decreased in prawns exposed at pH levels outside this optimal range (pH 5.0, 8.5, 9.0). All prawns exposed to pH 3 and 11 died thus no post-exposure feeding data could be obtained. Results of feeding inhibition were similar to the ones obtained by Su-Mei Chen and Jiann-Chu Chen (2003), however, they used juveniles rather than post-larvae. They reported that feeding rate was reduced in *M. rosenbergii* juveniles (2.06 ± 0.10 g) exposed to pH 6.8 and lower.

At less than pH 5.0 mortality occurred which corresponded with the findings of other authors who also reported prawn mortalities at pH 6.8 and lower. With *M. rosenbergii* juveniles (0.13 ± 0.01 g), Su-Mei Chen and Jiann-Chu Chen (2003) reported that the 24-, 48-, 72- and 96-h LC50s (median lethal concentrations) of pH were 4.00, 4.05, 4.07 and 4.08, respectively.

The results of this experiment have implications for the type of environment where *M. rosenbergii* bioassays could be carried out. It is possible to conduct the bioassay with differing pH levels although they should be between 7.0 to 8.0 and compare the data obtained with that of the reference site data. Any decrease in post-exposure feeding rate or survival would not be due to changes in pH as long as the pH levels are within this range. The bioassay would not be suitable for use in acidic (such as acid-sulphate water) and strongly alkaline (such as highly eutrophic) environments i.e. below pH 7.0 and higher than pH 8.0 for detecting the effects of pollutants on survival and post-exposure feeding rate. However, this bioassay could be used to study the effects of acidic and strong basic environments on feeding rate of prawns if they have not been exposed to any contaminant.

Post-exposure feeding was largely unaffected by a difference of almost 10˚C in temperature. The feeding rate after exposure to a higher temperature (36˚C) showed that there was no effect of increasing the temperature during the exposure period. *M. rosenbergii* bioassay can be conducted at a higher temperature without any effect on

post-exposure feeding rate. Even though the change in temperature was rapid (from 25˚C in the laboratory suddenly transferred to the field with 36˚C, without acclimation), the prawns were able to tolerate it which showed that they are a suitable test species, as their normal temperature range is between 25 and 32 ˚C over 24 hours in their tropical habitats (Zimmermann, 1998).

One concern about feeding trials is the excretion of waste. Chen and Kou (1996) reported that the amount of nitrogenous waste excreted by prawns increased at temperatures between 17 and 32 ˚C, and with time between 6 and 24 hours. In this study, feeding period was only 4 hours so the tests were not affected by wastes.

However, there was a significant difference in survival ($F_{1,8}$ = 12.5 ; P = 0.008) wherein at a higher temperature (36˚C), survival was higher. Less than 10 % mortality occurred at this exposure, suggesting that the field temperatures in the range observed had no effect on survival. This tolerance allows the bioassay to be conducted at a range of sites with different temperatures throughout the year.

For the purpose of the *M. rosenbergii* bioassay, it would be useful to acclimate animals to soft water even if they are going to be exposed to hard water during field trials. These reasons were explained in chapter 2. As could be seen from Figure 4.10, soft water acclimated animals exposed to soft or hard water for 24 hours did not have significantly altered post-exposure feeding rates from hard water-acclimated and soft or hard water-exposed animals (P > 0.05). This implies that animals acclimated in soft water could be exposed to hard or soft water in the field without any effects on baseline post-exposure feeding rate. This will allow comparison of feeding rates between sites with differing water hardness. Boyd and Zimmermann (2000) supported the low hardness for acclimatization stating that prawn can tolerate a wide range of calcium hardness concentrations (20 to 200 mg/L).

Additionally, survival during exposure period was high and ranged from 90 % to 100 % which were not significantly different ($F_{3,8}$ = 2.0 ; P = 0.193). It is unlikely that any mortality occurring in the field during the bioassay would be due to variation in water hardness. Thus, survival can be compared between sites which do not have similar water hardness.

The procedures developed for *M. rosenbergii* bioassay described in this chapter will be useful for other tropical species chosen for standard aquatic toxicity tests (lethal test). The protocol provided the standard size, density, appropriate media and number of replications required for test animals. pH could likely be one of the main potential limiting factors which should be taken into account when interpreting data from field bioassays. Hardness and temperature may not be limiting as the acceptable ranges are wider. The bioassay is beneficial in assessing pesticide contamination not only of water supply for *M. rosenbergii* hatcheries and culture ponds, but also of other culture species which depend on natural water such as rivers or storage pond. All these water sources have a potential to be contaminated from pesticides used in agriculture.

4.7 Summary for the bioassay protocol

Figure 4.11 presents all the processes of laboratory and *in situ* bioassay protocol developed from the experiments described in this chapter. There are three main periods, namely, acclimation, exposure and post-exposure, which requires 5 days to complete the *M. rosenbergii* bioassay procedures. Time consumed does not include materials preparation and travel time in the case of the *in situ* bioassay.

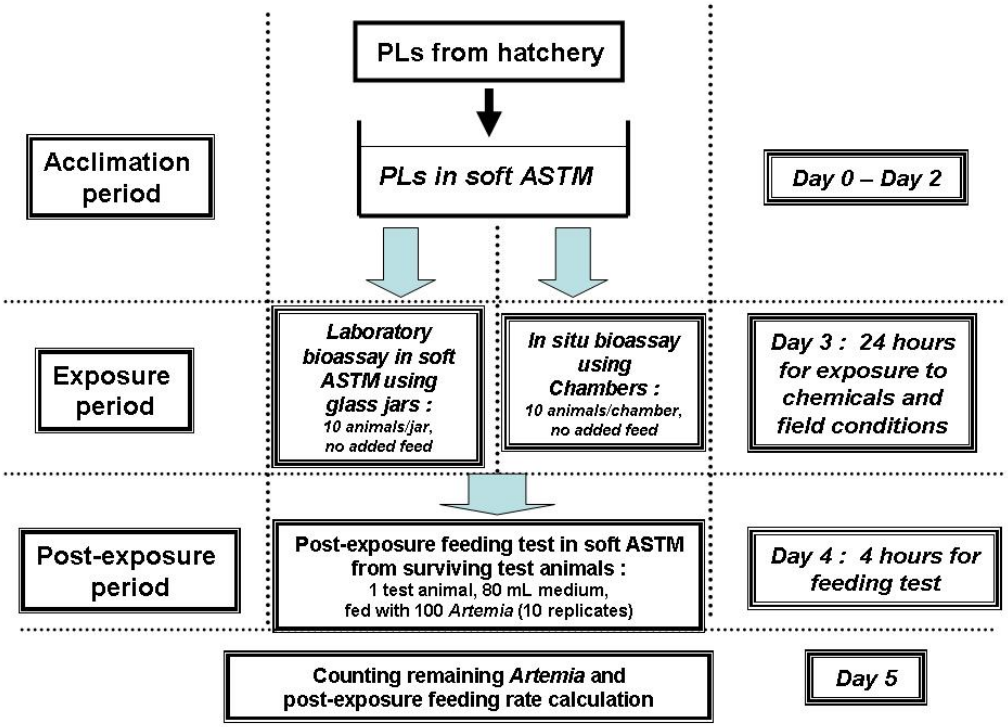


Figure 4.11 Schematic diagram for the *M. rosenbergii* bioassay protocol showing the different periods of the bioassay, number of test animals in laboratory and *in situ* bioassay, number of *Artemia* in the feeding test and the time duration to process this protocol.

CHAPTER 5

LABORATORY TOXICITY TEST OF POST-EXPOSURE FEEDING INHIBITION AND RECOVERY AFTER EXPOSURE TO SELECTED CHEMICALS

5.1 Introduction

An aquatic toxicity test is conducted to determine the relationship between chemical contaminants and an aquatic organism, and the extent of the chemicals' effects on the organism. A wide range of effects may occur, from lethal (rapid death) to sublethal effects, or, in some cases, no effect may be seen at all (Moriarty, 1990). Sublethal effects can be seen in the inhibition of an organism's physiological functions such as feeding, growth and reproduction, or effects on its behaviour, biochemical and histological functions.

According to Leeuwen and Hermens (1996), there are three common ways to derive $LC_{50.}$ The most common way is by observation, when 50% of the test organisms can be seen dying at a given test concentration. It can also be derived by interpolation, i.e. test organisms are exposed to a range of concentrations, with more than 50% dying at one concentration and less than 50 % at a lower test concentration, and the LC_{50} is estimated by interpolation between these two data points. The third method is by statistically deriving the LC_{50} through analyzing mortality data from a series of test concentrations through probit analysis.

Sublethal concentrations are those that are below the lethal concentration, producing less obvious effects on the behaviour, biochemical and/or physiological function and histology of test organisms.

Generally, negative effects on behavioural and physiological sublethal endpoints have been observed during exposure to toxicants. For the post-exposure response to be used as an endpoint, effects observed during exposure should be persistent even after such exposure. Brent and Herricks (1998) found that after the freshwater organisms *Ceriodaphnia dubia*, *Hyalella azteca* and *Pimephales promelas*

were exposed to a range of zinc concentrations, there was a delayed effect, evident in increased immobility for up to 172 hours after exposure. Taylor *et al.* (1998) and McWilliam and Baird (2002a) have also demonstrated the use of feeding inhibition in *Daphnia magna* after exposure to cadmium and other organic chemicals.

Taylor *et al.* (1998) studied the cadmium absorption to algae cells which were fed to *D. magna*. Their results obtained for the 4-hour period following exposure showed that the feeding rate of animals exposed to algal-bound cadmium was greatly reduced compared with that of animals fed clean algae. Starved animals also showed a significant, though lesser, reduction of feeding rate in the presence of dissolved cadmium compared with uncontaminated medium. Another experiment by Taylor *et al.* (1998) studied the presence of dissolved cadmium in the medium at 2.5 µg/L with the algal-bound cadmium and found that it had little or no apparent effect on reducing the feeding rates of animals, since the same effect was achieved by maintaining the animals under starvation conditions in clean medium.

Since mortality and post-exposure feeding responses of *M. rosenbergii* were proposed as endpoints, it must be proven that they are suitable for use in the bioassay. Aquatic toxicity tests have been used for many years to estimate the hazard from exposure to chemicals. However, the extrapolation of hazard in the laboratory to risk of damage to the natural environment is often difficult. There are various other endpoints such as feeding, growth and reproduction that may be manifested at lower exposure levels. Sublethal physiological endpoints are generally recognized to be more sensitive than lethal endpoints since physiological endpoints can be detected before organisms die, and this is often the initial reaction of organisms to stress (Gerhardt, 1996).

The following section will illustrate the use of procedures developed for a *M. rosenbergii* bioassay under laboratory conditions to study the effects of zinc, profenofos, dimethoate, chlorpyrifos and carbendazim exposure on the feeding rate of the giant prawn. Experiments using zinc and four pesticides (chlorpyrifos, dimethoate, profenofos and carbendazim) at different levels for this *M. rosenbergii* bioassay were expected to effect a reduction in post-exposure feeding rate and survival.

5.1.1 Zinc in aquatic environment

Zinc is used in a variety of industrial processes. It enters aquatic environments through domestic, industrial and mining effluents. Leaching of rocks and other natural processes can release small amounts of zinc into inland water, but zinc levels may often be greatly increased by human activities, ranging from mining to industry (Birch *et al.*, 1996). Many zinc salts are highly soluble in water. Zinc compounds such as zinc chloride, zinc oxide, zinc sulfate and zinc sulfide may also be found at hazardous waste sites.

Zinc is one of the most commonly used metals in the world with major uses such as for galvanizing steel, producing alloys and as an ingredient in the manufacture of ceramics, paints and rubber. Zinc compounds are also used as wood preservatives and in manufacturing and dyeing fabrics. Likewise, it is used by the drug and pharmaceutical industries as a component in the manufacture of common body and health care products.

Zinc level in the natural water depends on water hardness. Its binding property with particulate matter is variable since it depends on the physico-chemical characteristics of the aquatic system. Acceptable maximum concentration of zinc for freshwater aquatic life ranges from 33 to 265 µg/L, depending on water hardness (Ministry of Environment, 1999). Increasing pH from 6.5 to 8.4 increased the toxicity of zinc to the Cladoceran *Ceriodaphnia* cf *dubia* (Hyne *et al.*, 2005). Depletion of oxygen also increased the toxicity of zinc for fish (Hattink, *et al.*, 2006). The study of bull trout (*Salvelinus confluentus*) and rainbow trout (*Oncorhynchus mykiss*) to acute exposure of zinc showed that higher hardness and lower pH water produced lower toxicity and slower rates of toxicity in both species (Hansen *et al.*,2002). Table 5.1 shows the relative sensitivities of invertebrates and aquatic animals to zinc, with the adult vertebrates more tolerant to very high concentrations except for trout. The table also indicates the relative sensitivity of crustaceans to zinc.

Zinc, as an essential element in the fish body, can be tolerated by the organism over a wide range of concentrations as long as it is within the required level. In this

study, zinc was chosen as a standard test substance because it is widely used as a toxic reference substance and it is not as highly toxic as cadmium, mercury and lead. Zinc was chosen as a representative of heavy metals to determine its impact on post-exposure feeding rate. Moreover, it was a safer choice for health reasons.

Table 5.1 Relative sensitivities of aquatic animals and invertebrates to zinc

Species	Effect	Duration (days)	Reported Effect Concentration (µg/L)	Reference
Daphnia magna	LC_{50}	2	1220	Balch *et al.* (2000)
Ceriodaphnia	LC_{50}	2	500	Balch *et al.* (2000)
C. dubia	EC_{50}	2	382 (pH 5.5) 413 (pH 6.5) 200 (pH 6.5-7.5)	Hyne *et al.* (2005)
D. magna	EC_{50}	21	112-536	Schamphelaere *et al.* (2005)
Strongylocentrotus purpuratus (purple urchin)	EC_{50} (Larval development)	4	97.2	Phillips, *et al.* (1998)
Salvelinus confluentus (Blue trout)	LC_{50}	6	56.1	Hansen *et al.* (2002)
Oncorhynchus mykiss (rainbow trout)	LC_{50}	6	34.7	Hansen *et al.* (2002)
O. mykiss	LC_{50}	30	337-1970	Schamphelaere *et al.* (2005)
Cottus bairdi (Mottled sculpin)	EC_{50}	5 6 7 8 9 13 21 30	94 57 48 42 38 33 32 32	Woodling *et al.* (2002)
C. bairdi (Mottled sculpin)	LC_{50}	4 5 6 7 8 9 13	156 92 62 45 41 38 38	Woodling *et al.* (2002)
Poecilia reticulata (guppy)	LC_{50}	0.5	6400-9100	Widianarko *et al.* (2001)
Limanda limanda (Dab : marine fish)	LC_{50}	4	4000-100000	Taylor *et al.* (1985)
Chelon labrosus (grey mullet)	LC_{50}	4	4000-100000	Taylor *et al.* (1985)
Callianasa australiensis	LC_{50}	4	10200	Ahsanullah *et al.* (1981)
C. australiensis	LC_{50}	14	1150	Ahsanullah *et al.* (1981)

5.1.2 Carbendazim, chlorpyrifos, dimethoate and profenofos in aquatic environment

Chlorpyrifos, dimethoate and profenofos belong to the organophosphate insecticides. Carbendazim is a carbamate fungicide. Some organophosphate and carbamate pesticides are comparatively short-lived in aquatic systems. Kumar and Chapman (2001) studied concentrations of profenofos in water of lagoons, creeks and some irrigation canals of the Wee Waa district of New South Wales, Australia where profenofos was heavily used in cotton-growing areas. They showed that six weeks after spraying, profenofos levels in water had declined significantly to undetectable levels. Residue concentrations in natural waters were very low but because these chemicals acted rapidly through interference with cholinergic nerve transmission, even a short-term exposure could incapacitate or kill a wide spectrum of non-target animals (Hill, 1995). Kumar and Chapman (2001) also addressed the increasing accumulation of profenofos residues in fish and associated it with reduction of acetylcholinesterase (AChE) which causes nerve disorder.

Pesticide levels are likely to vary both temporally and spatially in time with seasonal and management difference. Pesticide concentrations in natural waters may be low in some seasons (Shigehisa and Shiraishi, 1998), whilst occurring at high levels in farm canal systems, especially immediately after application. Aquatic animals in these canals may be affected after a short exposure and also in the longer term which can be detected through post-exposure tests.

Dimethoate and profenofos were chosen as test pesticides because of the high risk they present to the water body and the organisms in the farm canal, as shown by the results of the preliminary risk assessment for pesticides reported in chapter 2. Chlorpyrifos and carbendazim were additional pesticides considered. Chlorpyrifos is now considered one of the world's leading insecticides in terms of the amount used. Carbendazim is a fungicide that is extensively used worldwide.

5.1.2.1 Carbendazim

Description:

Carbendazim is a fungicide with systemic function (benzimidazole group) and is considered an important chemical for disease control in plants. There have been concerns regarding its use due to its potential to disrupt hormone functions, harming both humans and the environment (PAN, 2002).

Carbendazim is used on crops, fruits, ornamentals and vegetables to control a wide range of diseases, in addition to being used in food storage after harvest and during seed pre-planting treatment (PAN, 2002). In Thailand, fungicides are used in a large range of crops including corn, fruit trees, legumes, pineapple, rice, rubber and tobacco (Sirisingh, 2000). Imports of fungicides into Thailand reached 4,960 t in 1999, up from 1,483 t in 1980 as their use in farms intensified, with carbendazim ranking 4th in terms of quantity imported (580 t of active ingredients), valued at US$ 2.88M (Sirisingh, 2000).

Use:

PAN (2002) reported that carbendazim has been popular especially in Europe and Asia, as it was effective in disrupting fungal growth by interfering with spindle formation during cell division. Recently its use has decreased as there are other fungicides which are more effective.

Carbendazim is registered to be used for foliar spraying, post-harvest treatment and soil drenching (JMPR, 2004). In Thailand, carbendazim is also sprayed directly on vegetables and mangoes.

In other countries such as Australia, Germany and the Netherlands, carbendazim is used in many ways, including dipping of plant material, overall spraying, post-harvest dipping of fruit, post-harvest shower and spraying (JMPR, 2004). In the UK, it is also the most commonly used fungicide (Papiya-Sarkar, 2005).

Toxicity:

Carbendazim can penetrate the surface of plants and fruit so it cannot be completely removed by washing (Cook, 2004). In Germany it was considered as a hormone disrupting chemical and the USEPA has classified it as a carcinogen. PAN (2002) suggested that the primary source of exposure to carbendazim for the consumer was dietary. Residues of carbendazim were found in fruit and vegetables presenting risks to consumers. In 2003, Cook (2004) reported that in the UK, the government found over 12 % of pears had detectable levels of carbendazim residues. These levels were considered acceptable by the government leading to the conclusion that this might not pose any risk to human health. However, in the long term, this may not be true.

People working with carbendazim can be exposed through the manufacturing process or when they use it on-farm. Risks from exposure through dermal contact or inhalation can be reduced if workers wear protective gear such as clothing and masks. It is highly toxic to aquatic organisms including invertebrates and fish. LC_{50} levels found in laboratory tests in aquatic organisms ranged from 0.087 mg/L for aquatic invertebrates to 5.5. mg/L for bluegill sunfish.

Appendix 7 presents the range of invertebrate and animal sensitivity to carbendazim.

5.1.2.2 Chlorpyrifos

Description:

Chlorpyrifos is an organophosphate insecticide with a broad range of efficiency. Oregon University's Extoxnet (1996a) described chlorpyrifos as being used not only for mosquitoes, although this was its original function, but also for controlling other pest organisms such as cockroaches, corn rootworms, cutworms, fire ants, flea beetles, flies, grubs, lice and termites. A wide range of plants are being sprayed with chlorpyrifos, namely cotton, fruit, grain nut and vegetables, and even ornamental plants. In addition, it is also directly used on sheep and turkeys, and for

treating areas such as commercial establishments, dog kennels, domestic dwellings, farm buildings, horse sites and storage bins.

The USEPA (2004a) has described chlorpyrifos as a broad-spectrum insecticide, used on fruit and vegetables to control pests, as well as a mosquiticide, termiticide, a treatment for areas such as lawns and turfs, pasture, woodland and farmsteads, around homes and non-residential settings, on pet collars and cattle eartags. The USA, EPA (2004a) stated that use of chlorpyrifos was unrestricted, with 50% being used in agriculture and 50% in non-agriculture functions. In Thailand, chlorpyrifos is used in rice fields and, together with cypermethrin often contributes to pesticide cocktails.

Chlorpyrifos comes in the form of granules, powder (dustable and wettable) and emulsifiable concentrate (Extoxnet, 1996a). It appears as an amber to white crystalline solid with a mild sulfur odour. Water solubility is 2 mg/L at 25°C. It is also soluble in other solvents such as acetone, benzene, carbon disulfide, chloroform, diethyl ether, methanol, methylene chloride and xylene.

Use:

The USEPA (2004a) reported that chlorpyrifos is applied in many ways, such as aerial, aerosol sprayer, airblast sprayer, chemigation, compressed air sprayer, groundboom, hydraulic hand-held sprayer, low and high pressure hand wands, pet collars, shaker can and tractor-drawn granular spreader.

Toxicity:

Chlorpyrifos is easily absorbed by humans, going directly into the bloodstream through the intestine when ingested, through the lungs when inhaled or through the skin when exposed to it (Extoxnet, 1996a), although skin absorption is limited.

Chlorpyrifos is moderately toxic to humans (Extoxnet, 1996a). The central nervous, cardiovascular and respiratory systems may be affected from chlorpyrifos poisoning. It might also irritate the skin and eye. With a repeated or prolonged exposure, the above symptoms would occasionally appear, in a delayed manner. Some

other symptoms observed in workers exposed to this pesticide include confusion, severe depression, disorientation, drowsiness, headache, impaired memory and concentration, insomnia, irritability, nightmares and speech difficulties. There is no evidence that chlorpyrifos is carcinogenic, or has an effect on mutation and reproduction.

The USEPA (2004a) reported that chlorpyrifos can cause cholinesterase inhibition in humans, wherein the nervous system is over stimulated, leading to confusion, dizziness and nausea. At high exposures, it can lead to respiratory paralysis and eventually death. In the US, mitigation on its use was implemented, especially on apples, grapes and tomatoes. Use of this pesticide in non-agricultural areas such as homes and non-residential settings had been stopped to eliminate risks to residential areas. However Extoxnet (1996a) states that this pesticide does not have the potential to bioaccumulate significantly. In humans, a portion may be stored in fat tissues with a half-life of about 62 hours. When cows were fed with chlorpyrifos, it was found unchanged in the feces but not in urine or milk. In tests using rats, it was found only in the body fat and not in any other tissue.

Extoxnet (1996a) reported that chlorpyrifos affects pests on contact with the skin and poisons them when ingested. The pesticide is very highly toxic to freshwater fish and other aquatic organisms such as invertebrates, estuarine and marine organisms, with 96-hour LC_{50} ranging from 0.009 mg/L in mature rainbow trout to 0.331 mg/L in fathead minnow; at 0.002 mg/L for a 30-day period, offspring of fathead minnows had decreased survival and growth and several deformities were found. Furthermore, the pesticide has the potential to accumulate in tissues of aquatic organisms with smaller organisms more likely to be affected and its persistence in sediments may pose a risk to sea bottom dwellers.

The USEPA (2004a) also confirmed that risk to birds, fish and mammals was high, and that for aquatic invertebrates it was very high. Likewise, chlorpyrifos is toxic to some plants such as lettuce. It accumulates in certain crops, with residues remaining on the plants for 10 to 14 days.

As mentioned earlier, chlorpyrifos is persistent in soils. Depending on soil type, climate, moisture, oxygen, pH and other conditions, half-life may range from 2 weeks to over a year, and on the average, from 60 to 120 days. It is less persistent in soils with a higher pH, although the half-life is not affected by soil texture and organic matter content. It also strongly attaches on soil particles and is insoluble in water, making it less likely to leach or contaminate groundwater (Extoxnet, 1996a). However, the USEPA (2004a) reported that some localized application as termiticide within 30 meters of wells resulted in drinking water contamination between 1992-1997.

Chlorpyrifos concentrations and their persistence in water depend on the type of formulation. Emulsifiable concentrations and wettable powders are likely to increase concentrations in the water when added into water in these forms more than using granules. However, when adding the former, persistence is less than when using granules. This insecticide appears to be unstable in water and decreasing by about 3-fold for every 10°C drop in temperature. In contrast to its persistence in soil, hydrolysis increases when pH becomes alkaline. At neutral pH and 25°C, its half-life is from 35 to 78 days (Extoxnet, 1996a).

The USEPA (2004a) has classified chlorpyrifos as moderately toxic, and proper care especially in its application should be taken. Workers should wear protective clothing and retreatment interval should be 24 hours.

Appendix 8 shows the range in invertebrate and animal sensitivity to chlorpyrifos.

5.1.2.3 Dimethoate

Description:

Dimethoate is an organophosphate insecticide/acaricide/miticide classified by the USEPA (2004b) as a systemic or general chemical, used mainly on a variety of agricultural and ornamental crops. Other uses are on non-crop items such as equipment, land, outdoor wastes, residential area and sewage treatment.

Extoxnet (1996b) reports that dimethoate is used to kill insects and mites on contact. Other insects affected by it include aphids, plant hoppers and whiteflies which could be found on a number of crops such as apples, corn, cotton, grapes, lemons, melons, oranges, tangerines, tomatoes, watermelons and other vegetables. Further, dimethoate is used to spray buildings and to control botflies on livestock. This pesticide comes in the form of aerosol spray, dust and emulsifiable concentrate.

In a survey of agricultural pesticides in Thailand (Ratanamaneechat and Cherdchoo-ngarm, 2000), it was reported that dimethoate had 171 trade names. They considered it as one of the organophosphate insecticides which had relatively high toxicity to humans and suggested that its use should be carefully monitored.

Use:

Dimethoate is applied by airblast, backpack sprayer, groundboom, low pressure handwand or soil drench (USEPA, 2005). The USEPA does not allow the use of chemigation, high pressure handwand and sprinkler cans. In Thailand, dimethoate is used in tangerine farms.

Chilli farms in both temperate and tropical countries spray dimethoate on chilli plants to eliminate aphids and red spider mites (AsiaRecipe.com, 2000). In Malaysia, folia spraying and trunk injection of dimethoate on palm pants are done during severe infestation of pests on coconut palms (Sivapragasam and Loke, 2004).

Toxicity:

The USEPA (2004a) reported that dimethoate causes cholinesterase inhibition in humans. At very high exposures such as accidents or major spills, there will be respiratory paralysis and death. Extoxnet (1996b) reported that no cholinesterase inhibition occurred in adults ingesting 18 mg dimethoate/day for 21 days. There were also no toxic effects and cholinesterase inhibition in humans ingesting 2.5 mg/day for 4 weeks. Cholinesterase inhibition was observed only when ingesting 30 mg/day and higher.

Extoxnet (1996b) also mentioned that dermal absorption, ingestion and inhalation of dimethoate is moderately toxic to humans. Experiments conducted

revealed that oral LD_{50} values ranged from 180-330 mg/kg for rats, 160 mg/kg in mice, 400-500 mg/kg in rabbits, 550-600 mg/kg (pure and laboratory grade) and 350-400 mg/kg (technical grade) for guinea pigs. For dermal LD_{50} values, it ranged from 100-600 mg/kg in rats. It was also reported that dimethoate is not irritating to the skin and eyes of animals in the laboratory but for humans, severe eye irritation could occur.

Workers handling dimethoate are exposed to high risks (USEPA, 2004b). Effects of overexposure to dimethoate are similar to those effects by other organophosphates, namely convulsions, dizziness, headache, incontinence, incoordination, numbness, respiratory depression, slow heartbeat, tingling sensations and unconsciousness.

Extoxnet (1996b) also reported that dimethoate is moderately toxic to fish and more toxic to aquatic invertebrates such as stoneflies and scuds (*Gammarus pseudolimnaeus*). LC_{50}s are 6.0 mg/L in bluegill sunfish and 6.2 mg/L in rainbow trout. The USEPA (2004a) mentioned that dimethoate poses acute risks to aquatic invertebrates. Their exposure might come from surface run-offs to rivers and streams.

Appendix 9 demonstrates that invertebrates and animals range in their sensitivity to dimethoate.

5.1.2.4 Profenofos

Description:

Profenofos is an organophosphate insecticide/miticide (USEPA, 2004c), called phosphoric acid ester functioning as a broad spectrum insecticide and acaricide (Calderon and Hare, 1986).

In the USA, it is used only on cotton crops (USEPA, 2004c) with a total of about 352,000 kg used annually. Profenofos is a major component in the Integrated Pest Management programmes for cotton.

According to Calderon and Hare (1986), profenofos was found to be effective not only against cotton pests but also against the diamondback moth (*Plutella xylostella* L.) at 0.5 kg AI/ha, which is a destructive pest of vegetables. Thus in Southeast Asia, it is used widely in vegetable farms to avoid losses caused by this pest which ranged from 50-100% of vegetable production.

The USEPA (2004c) mentioned that profenofos could cause cholinesterase inhibition among humans at high doses. At very high doses 9.7mg/kg/day, it could lead to respiratory paralysis and death.

How applied:

Profenofos is applied aerially and by ground equipment in the US (USEPA, 2004c). In studies done by Calderon and Hare (1986), a high volume spray was used to evaluate the effectiveness of profenofos in Malaysia, the Philippines, Taiwan and Thailand.

Toxicity:

According to USEPA (2000), there is a low risk of profenofos poisoning from food and drinking water. Occupational risk concerns exist for those conducting pesticide application through aerial mixers/loaders and aerial applicators. Risks are high for workers who re-enter the field after treatment, especially if they do not use personal protective equipment (USEPA, 2004c). Because of these risks, USEPA had set up risk mitigation measures for handlers and workers as well as for the environment. For handlers and workers, these measures included reducing maximum application rate to 15.3 g of active ingredient per ha with a higher rate of 20.25 g active ingredient per ha only for lepidopteran pests (caterpillar) for only twice per season, closing aerial mixing/loading systems as well as cockpits and cabs used for pesticide applications, prohibiting mixing and loading of pesticide on the same day as application, reducing seasonal application rate from 122.85 g to 102.15 g active ingredients per ha per season, and requiring manufacturers to put a notice on the label of pesticide containers on the need for protective clothing and equipment for workers re-entering treated fields. For environmental risk mitigation, requirements for a 90 metre buffer zone around bodies of water for aerial applications and a 30 metre buffer zone around bodies of water for ground applications were imposed. The reduction in

application rates mentioned above was also expected to decrease the risks to the environment.

There is a concern for profenofos toxicity to fish (USEPA, 2004c). A number of fish kills occurred in the US from profenofos exposure, even when following the recommended dose stated on the label of profenofos container (maximum of 20.25 g active ingredient per ha). Kumar and Chapman (2001) reported that profenofos residues were found in tissues of fish (bony bream, carp and mosquito fish) collected from cotton-growing areas where profenofos was used. Persistence of residues in fish was longer than in the soil or water. They used acetylcholinestarase (AChE) activity as an indicator of profenofos poisoning in fish. LC_{50} levels found in laboratory tests in aquatic organisms ranged from 0.005 mg/L for aquatic invertebrates to 30 mg/L for Japanese eel (Yokoyama *et al.*, 1988)

Appendix 10 demonstrates the ranges in invertebrate and animal sensitivity to profenofos.

There are no published studies regarding the toxicity of the above mentioned chemicals on *M. rosenbergii*. There have been some studies on the lethality of other pesticides on *M. rosenbergii*, such as paraquat, propanil, glyphosate and 2,4-D (Utayopas, 1983), endrin and carbofuran (Siripatrachai, 1984), metamidophos (Lorenzo and Sanchez, 1989), malathion (Lamarrei and Edwards, 1991), copper sulphate, endosulfan and malathion (Natarajan *et al*., 1992) and ametryne, copper oxychloride and endosulfan (Lombardi *et al*., 2001). However, these studies used different sizes and ages of *M. rosenbergii* using freshwater (Lombardi *et al*., 2001) and brackishwater (Utayopas, 1983; Siripatrachai, 1984; Lorenzo and Sanchez, 1989; Lamarrei and Edwards, 1991; Natarajan *et al*., 1992) as test media.

5.2 Aims

The aims of this section are:

- To conduct lethal toxicity tests of pesticides (carbendazim, chlorpyrifos, dimethoate and profenofos) and heavy metal (zinc) on *M. rosenbergii*

- To study the feeding behaviour of *M. rosenbergii* after exposure (as a sub-lethal effect) to the pesticides and heavy metal under laboratory conditions by calculation of post-exposure feeding rates

- To study recovery in feeding rates during the post-exposure period

5.3 Materials and methods

5.3.1 Animals and acclimation

All animals used were post-larvae (PLs) with sizes ranging from 9-10 mm obtained from a commercial hatchery. They were acclimated as in section 2.1 for 48 hours, after which time animals were ready to be used in the experiments.

5.3.2 Test media

For each chemical tested, a stock solution was prepared in soft water ASTM. Preparation was the same for lethality tests and post-exposure feeding tests. The pesticides used were in a commercial form. Stock solutions for chlorpyrifos, dimethoate and profenofos were prepared using the “change sequence” technique (diluting the high concentration to lower concentration). Carbendazim was diluted directly with soft water ASTM.

The lack of published studies on the impacts of test chemicals on *M. rosenbergii* meant that range finding tests were required to find the concentration

range that has effects on *M. rosenbergii* post-larvae. The LC_{50} of these test chemicals on other crustacean species could be initially used as the basis for assigning ranges of test concentrations (G. Taylor, personal communication).

Zinc (as $ZnSO.7H_2O$, BDH, Analar Grade): A 850 µg/L stock solution was prepared and 5 concentrations (53.1, 106.2, 212.5, 425 and 850 µg/L) were used for lethality tests, and 4 concentrations (53.1, 106.2, 212.5 and 425 µg/L) for post-exposure feeding tests. The concentrations used for lethality tests were based on the 48 h LC_{50} of zinc on crustaceans *Daphnia magna* and *Ceriodaphnia* sp. (Balch *et al*., 2000) which was between 500 and 1,220 µg/L (Table 5.1). From the average of 860 µg/L, the concentration of 850 µg/L was chosen for ease in calculation, and a 50% dilution was done until 53.1 µg/L. For post-exposure feeding tests, concentrations were chosen based on the concentrations that had survivors from the lethality tests.

Chlorpyrifos (40 % W/V): A 10 µg/L stock solution was prepared, with 7 concentrations (0.15, 0.3, 0.6, 1.2, 2.5 and 5 µg/L) used for lethality tests, and 4 concentrations (0.15, 0.3, 0.6 and 1.2 µg/L) for post-exposure feeding tests. The concentrations used for lethality tests were based on the 48 h LC_{50} of chlorpyrifos on *Penaeus vannamei* juvenile or white leg shrimp (Galindo *et al*., 1996) which was 4.8 µg/L (Appendix 8). Starting from the rounded off number of 5 µg/L, a 50% dilution was done until 0.15 µg/L. For post-exposure feeding tests, concentrations were chosen based on the concentrations that had survivors from the lethality tests.

Dimethoate (40 % W/V): A 1,250 µg/L stock solution was prepared, with 5 concentrations (78.125, 156.25, 312.5, 625 and 1250 µg/L) used for lethality tests, and 3 concentrations (78.125, 156.25 and 312.5 µg/L) for post-exposure feeding tests. The concentrations used for lethality tests were based on the 48 h LC_{50} of dimethoate on *Daphnia magna* neonate (Beusen and Neven, 1989) which was between the ranges of 830 and 2,000 µg/L (Appendix 9). The average of the range was rounded off to 1,250 µg/L, a 50% dilution was done until 78.125 µg/L. For post-exposure feeding tests, concentrations were chosen based on the concentrations that had survivors from the lethality tests.

Profenofos (50 % W/V): A 50 µg/L stock solution was prepared, with 5 concentrations (3.125, 6.25, 12.5, 25 and 50 µg/L) used for lethality tests and 3 concentrations (3.125, 6.25, and 12.5 µg/L) for post-exposure feeding tests. The concentrations used for lethality tests were based on the 96 hours LC_{50} of profenofos on *Callinectes sapidus* or blue crab (USEPA, 2000) which was 33 µg/L (Appendix 10). Since the tests conducted were only for 24 hours, a higher concentration of 50 µg/L was chosen, and a 50% dilution was done until 0.15 µg/L. For post-exposure feeding tests, concentrations were chosen based on the concentrations that had survivors from the lethality tests.

Carbendazim (50 % W/V): For the first experiment, a 10 mg/L stock solution was prepared and 5 concentrations (3.3, 33, 100, 1,000 and 10,000 µg/L) were made for lethality and post-exposure tests. For the second experiment, a 200 mg/L stock solution was prepared and 4 concentrations (10, 25, 50 and 100 mg/L) were made for the lethality and post-exposure tests. These concentrations were based on those derived by Van den Brink *et al*. (2000).

5.3.3 Experiments for lethality and post-exposure feeding

Experiments for lethality and post-exposure feeding were performed following the methods below for each pesticide and heavy metal. Each chemical stock solution was diluted with soft water ASTM.

5.3.3.1 Lethality tests

Each treatment concentration of 500 ml was contained in a 1000 ml glass jar (exposure glass jar). Three replicates were assigned for each concentration. Using a spoon (made by cutting in half the bulb of a 3 ml plastic pipette), ten prawns were randomly selected and carefully added into each exposure glass jar by placing the spoon gently below the water surface and allowing the prawns to swim from the spoon to the medium. Control jars were similarly set up. No food was given during the exposure time. Prawns were added into each jar from the lowest to the highest concentration to minimize the risk of cross-contamination. The test began when half

of the organisms were in the exposure jars. The mortality of prawns was recorded after 24 and 48 hours.

5.3.3.2 Post-exposure feeding rate tests

Ten plastic jars with caps were filled with 80 ml of soft ASTM freshwater. One hundred *Artemia* nauplii were added into each jar. The remaining prawns from each concentration in 5.3.2.1 (lethality tests) were randomly added into the jars at one prawn per jar. Three jars were also set up without prawns to be used as the basis for evaluating the initial number of *Artemia* nauplii. A black plastic bag was laid on top of the jars to prevent disturbance from external stimuli. Prawns were then left for 4 hours to feed. After 4 hours, mortality and survival of prawns were recorded, then three drops of Lugol's solution were added into each jar. After one hour, the dead prawns were removed and their lengths measured. The jars were then shaken gently clockwise to concentrate the remaining *Artemia*. They were then siphoned out by glass dropper and placed in 25 ml test tubes, one for each replicate. They were left to stand for another hour. Then, using a glass dropper, the *Artemia* were taken out from the test tubes and placed into a Sedgwick-Rafter counting cell. They were counted under the microscope at 10X (low power). The number of remaining *Artemia* was recorded and feeding rates were calculated as in Chapter 2.

5.3.4 Chemical analysis

5.3.4.1 Zinc analysis

For zinc analysis, duplicate water samples of 50 ml were collected from the highest concentration and second highest concentration in each series after 24 and 48 hours during the lethality tests to observe whether concentrations decreased during the experiment. The concentration of zinc was analyzed using a flame atomic absorption spectrometer (Perkin elmer-Model 5100zl Graphite Furnace Atomic Absorption System).

5.3.4.2 Pesticide analysis

Chlorpyrifos, dimethoate, profenofos and carbendazim concentrations were determined by methods described in Chapter 2. The glass bottles for pesticide analysis were rinsed using Acetonitrite 3 times and then with distilled water (5 times) before using to collect the samples.

5.3.5 Statistical analysis

The feeding inhibition concentration (EC_{50}) was the concentration that reduced feeding rate to 50 % of the control feeding rate over a 4 hour post-exposure feeding period. The EC_{50} was calculated using a linear model for all chemicals. Before linear regression was determined, the percentage of feeding inhibition was transformed to natural log. Regression significance was determined with analysis of variance and model fit and accuracy were determined by examining standardized residuals versus fitted values, normality tests of residuals and adjusted co-efficient of determination (r^2). Confidence intervals for linear regression of EC_{50} estimates were calculated using an inverse prediction technique (Zar, 1999).

From each lethality test, 24 and 48 hour LC_{50} were calculated using the standard probit procedure (Finney, 1971). Analysis for both tests used actual concentration data. Actual concentrations were normalised for recovery rates of test substances (Table 2.2 in Chapter 2).

The Bonferroni Multiple Comparisons test in SPSS version 10 was used to compare treatments and to determine significant differences from the control (no organic chemical, only soft water).

5.3.6 Experiment on recovery from pesticide exposure

Chlorpyrifos was selected to conduct the recovery experiment. The recovery experiment was conducted to determine whether the prawns would be able to recover when they are placed in clean water. The experiment started with exposing the prawns to chlorpyrifos for 24 hours, after which 10 prawns that remained from each concentration were put in normal soft water ASTM (no pesticide) for more than 24

hours. Prawns were added into each jar from the lowest to the highest concentration to minimize the risk of cross-contamination. Prawn mortality was recorded after 24 hours. The remaining prawns were then subjected to post-exposure feeding rate test as in 5.3.3.2.

5.4 Results

Actual concentrations of all test substances were calculated from the percentages of recovery in Table 2.2. Actual concentrations were used to calculate lethal concentration at 50 % (LC_{50}) and effect concentration at 50 % (EC_{50}) using post-exposure feeding rates. All concentrations in the graphs are presented as nominal concentrations. Actual concentrations of carbendazim differed by less than 10 % from nominal concentration. This difference was considered small enough to regard nominal and actual concentrations as equivalent (McWilliam and Baird, 2002a).

Table 5.2 presents the 24 and 48 hours LC_{50} values and 24 hours EC_{50} values (post-exposure feeding inhibition) obtained for *M. rosenbergii* exposed to carbendazim, chlorpyrifos, dimethoate, profenofos and zinc.

Table 5.2 shows that only chlorpyrifos and zinc had LC_{50} values greater than the post-exposure feeding EC_{50}. The LC_{50} values of profenofos and dimethoate were lower than the post-exposure feeding EC_{50} both at 24 and 48 hours. In this study, carbendazim concentrations from 3.3 µg/L to 100 mg/L did not show any effect on survival and feeding rates. There were high variations in the model for post-exposure feeding rate with exception of chlorpyrifos and zinc. R^2 of chlorpyrifos and zinc were 0.61 and 0.44, respectively, and regressions were significant ($p < 0.05$).

Table 5.3 presents the results of one way ANOVA to compare the effects of chemicals and recovery in soft ASTM for 24 hours on *M. rosenbergii* post-exposure feeding rate. These statistical results are also reflected in Figures 5.1(a) to 5.1(h) to show significant and no-significant differences from the control.

Table 5.2 Acute 24 and 48 hours toxicity values for *M. rosenbergii* EC_{50} of feeding rate after 24 hours exposure

Chemical	EC_{50} 24 hrs With 95 % C.I (µg/L)	Adjust R^2	LC_{50} 24 hrs With 95 % C.I (µg/L)	LC_{50} 48 hrs With 95 % C.I (µg/L)
Profenofos	19.8 (-6.49 – 73.90)	0.23	11.6 (11.2 – 11.9)	9.8 (can not be calculated)
Dimethoate	190.6 (-318.17 - 796.06)	0.28	142.1 (141.4 – 142.8)	102.7 (96.9 – 108.4)
Chlorpyrifos	0.27 (-0.40 – 0.92)	0.61	0.7 (0.6 – 0.8)	0.3 (0.1 – 0.6)
Carbendazim	> 100 mg/L	-	> 100 mg/L	-
Zinc	132.4 (-252.43 – 481.86)	0.44	439.7 (438.8 – 440.6)	329.0 (327.9 – 330.1)

Table 5.3 Results of one-way ANOVA to compare the effects of chemicals and recovery in soft ASTM for 24 hours on *M. rosenbergii* post-exposure feeding rate

Experiment	Source of error	Sum of squares	d.f.	F-value	p-value
(a) Zinc	Between groups	284.175	4	7.11	0.001
	Within groups	199.825	20		
	Total	484.000	24		
(b) Chlorpyrifos I	Between groups	392.416	4	8.261	<0.001
	Within groups	225.644	19		
	Total	618.060	23		
(c) Dimethoate	Between groups	105.909	3	2.830	0.072
	Within groups	199.625	16		
	Total	305.534	19		
(d) Profenofos	Between groups	103.525	3	2.293	0.108
	Within groups	316.065	21		
	Total	419.590	24		
(e) Carbendazim I	Between groups	35.281	5	0.759	0.586
	Within groups	325.588	35		
	Total	360.869	40		
(f) Carbendazim II	Between groups	149.967	4	2.963	0.030
	Within groups	556.701	44		
	Total	706.668	48		
Experiment on recovery from pesticide exposure :					
(g) Chlorpyrifos II	Between groups	285.600	4	6.180	0.001
	Within groups	381.257	33		
	Total	666.857	37		
(h) Recovery from Chlorpyrifos II	Between groups	38.359	4	0.795	0.535
	Within groups	506.388	42		
	Total	544.747	46		

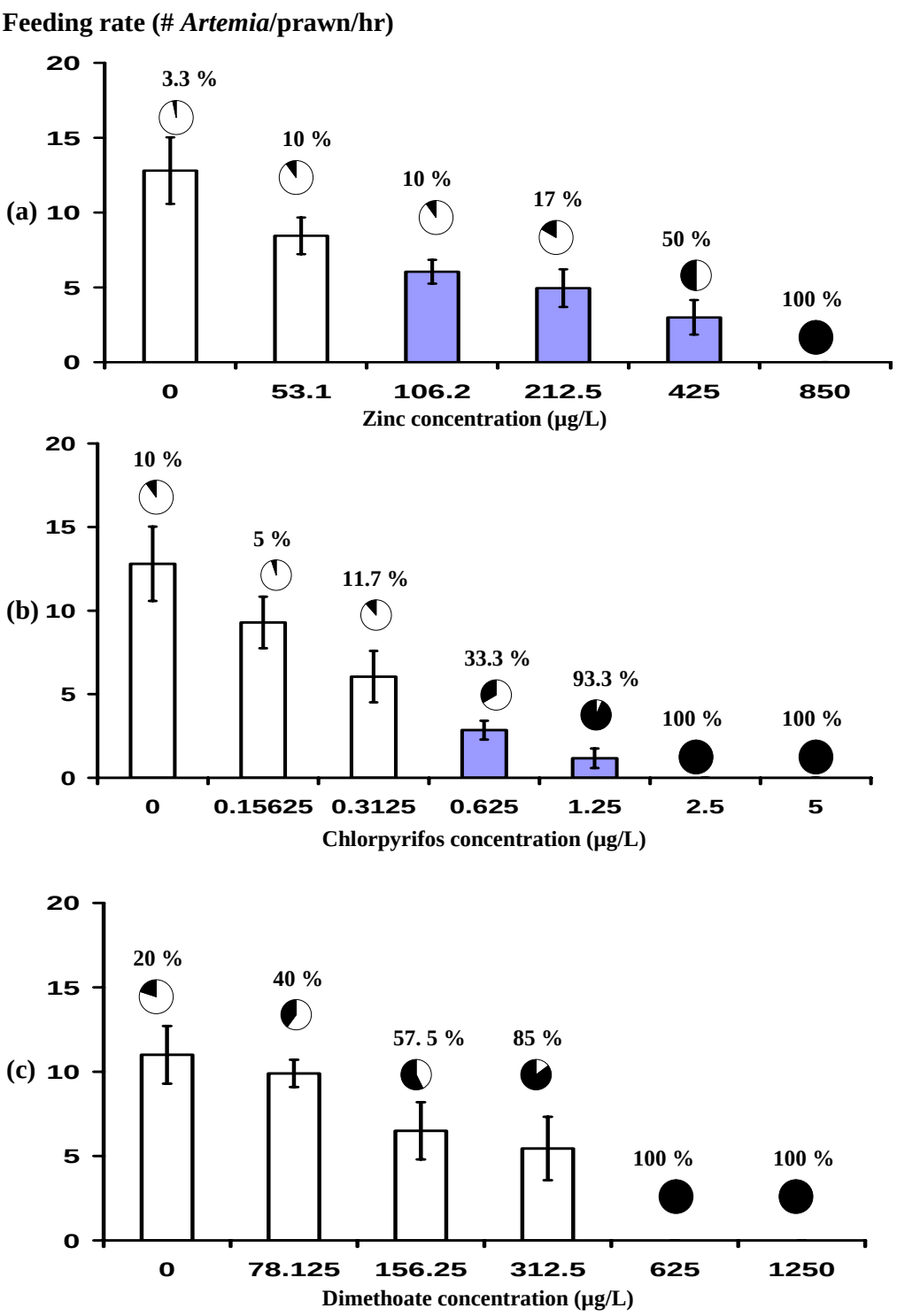


Figure 5.1 (a)-(c) Mean post-exposure feeding rates after 24 hours exposure to zinc, chlorpyrifos and dimethoate. Vertical bars indicate standard errors. Pie charts and percentages indicate average mortality of animals due to exposure for 24 hours. Shaded bars denote feeding rates which are significantly different from the control ($P < 0.05$).

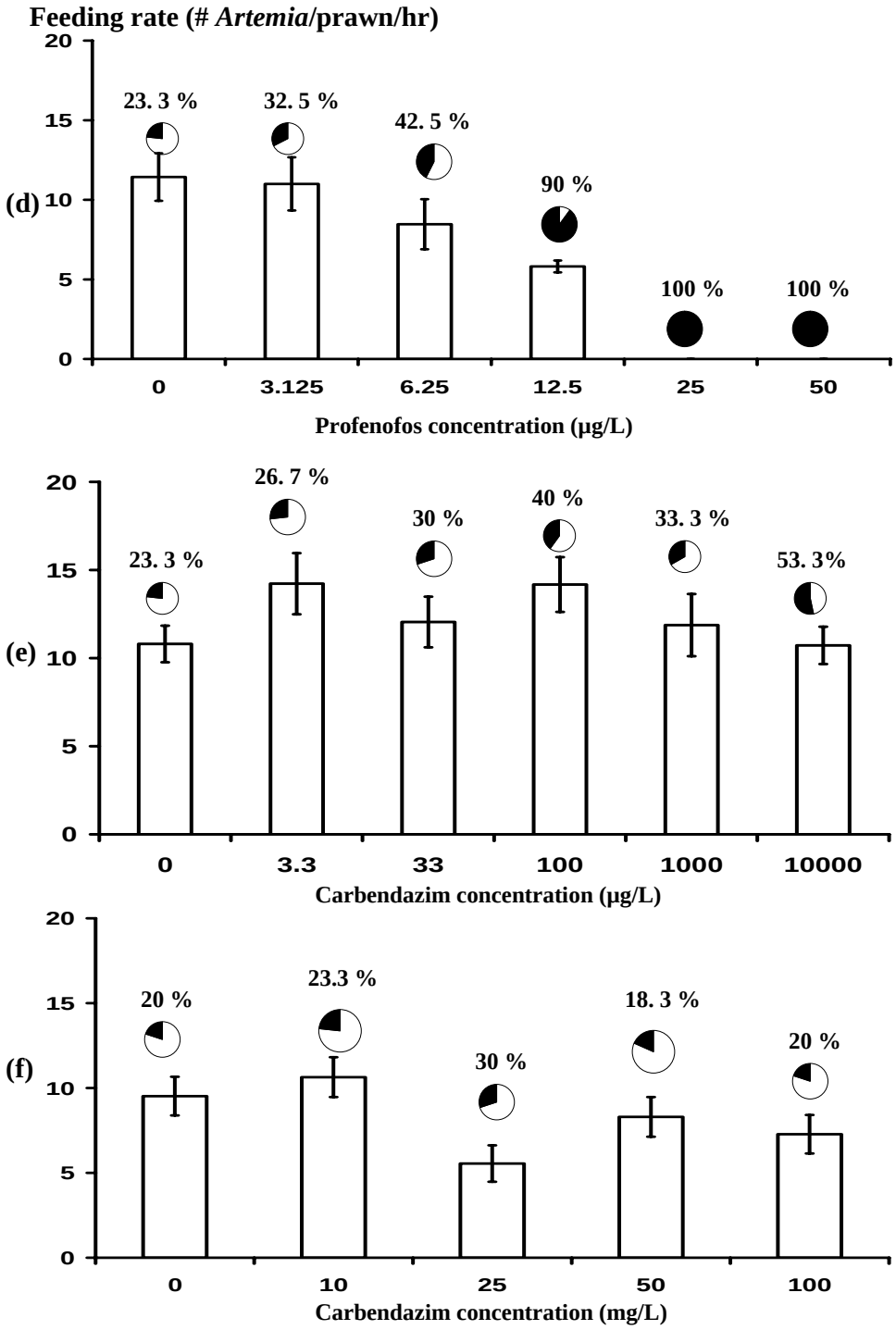


Figure 5.1 (d)-(f) Mean post-exposure feeding rates after 24 hours exposure to profenofos and carbendazim. Vertical bars indicate standard errors. Pie charts and percentages indicate average mortality of animals due to exposure for 24 hours. Shaded bars denote feeding rates which are significantly different from the control ($P < 0.05$).

Result of experiment on recovery from pesticide exposure :

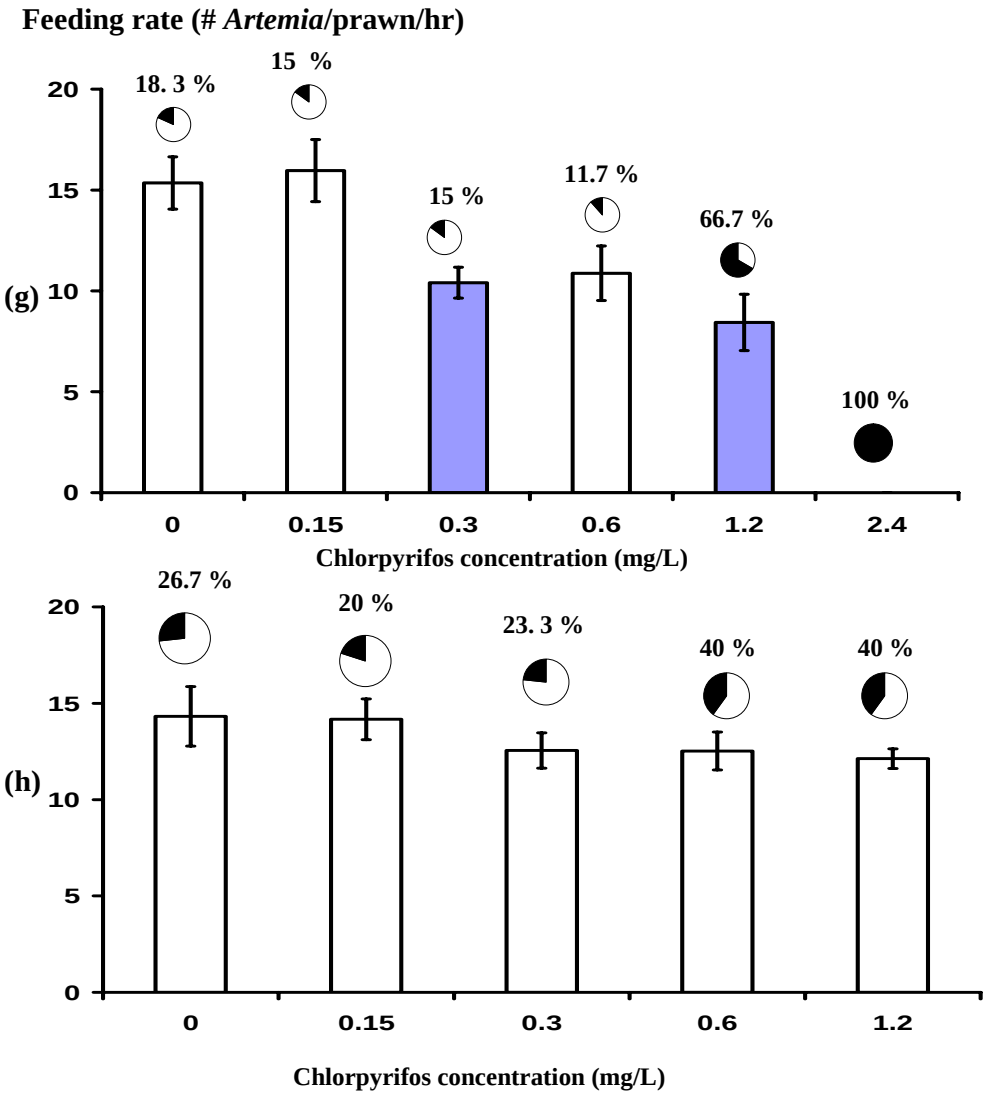


Figure 5.1 (g)-(h) Mean post-exposure feeding rates after 24 hours exposure to chlorpyrifos. Vertical bars indicate standard errors. Pie charts and percentages indicate average mortality of animals due to exposure for 24 hours. Shaded bars denote feeding rates which are significantly different from the control ($P < 0.05$).

The graphs in Figure 5.1 show post-exposure feeding rates of *M. rosenbergii* after exposure to test substances. Zinc and chlorpyrifos produced post-exposure feeding rates that were significantly different from the control ($F_{4,20}$ = 7.11, p = 0.001 for zinc and $F_{4,19}$ = 8.261, p < 0.001 for chlorpyrifos). Carbendazim, dimethoate and profenofos had some effect on feeding rate but no feeding rate at any concentration was significantly different from the control feeding rate (p > 0.05).

There was a 3 % mortality in the control treatment during exposure to zinc as seen in Figure 5.1(a). Mortalities in the 53.1, 106.2, 212.5, 425 and 850 µg/L zinc treatment groups were 10, 10, 17, 50 and 100 %, respectively. Zinc concentration of 106.2 µg/L showed a 52.7% decrease in post-exposure feeding rate, which was significantly different from the control ($F_{1,8}$ = 8.18; p = 0.021). A very pronounced decrease in feeding rate occurred when the percent difference from the control increased from 52.7 % at 106.2 µg/L zinc to 76.6% at 425 µg/L zinc. At 850 µg/L zinc, all prawns died.

During exposure of *M. rosenbergii* to chlorpyrifos, there was a 10 % mortality in the control group, as shown in Figure 5.1(b). Mortalities in the 0.16, 0.31, 0.62, 1.25, 2.5 and 5 µg/L chlorpyrifos were 5, 11.7, 33.3, 93.3, 100 and 100 %, respectively. The post-exposure feeding rate showed a significant decrease in 2 concentrations (0.625 and 1.25 µg/L) ($F_{2,9}$ = 13.23; p = 0.002). The percent differences from the control were 77.7 and 90.0 %, respectively. At the 2 highest levels of chlorpyrifos (2.5 and 5 µg/L), all prawns died.

Figure 5.1(c) shows the mortality of *M. rosenbergii* during exposure to dimethoate and post-exposure feeding rate of surviving *M. rosenbergii*. Mortality in the control group was 20 %. Mortality in the 78.12, 156.25, 312.5, 625 and 1,250 µg/L dimethoate treatment groups were 40, 57.5, 85, 100 and 100 %, respectively. During the post-exposure feeding tests, there were some decreases in post-exposure feeding at 78.125, 156.25 and 312.5 µg/L over the 24 hours period when *M. rosenbergii* was exposed to dimethoate, but these differences were not statistically significant. Percentage of decrease was 10.0, 40.9 and 50.4 %, respectively. All prawns died when exposed to the 2 highest dimethoate levels (625 and 1,250 µg/L).

During the post-exposure feeding test of *M. rosenbergii* exposed to profenofos, there was a 23 % mortality in the control group, as presented in Figure 5.1(d). Mortalities in the 3.125, 6.25, 12.5, 25 and 50 µg/L profenofos groups were 32.5, 42.5, 90, 100 and 100 %, respectively. During post-exposure there was a decrease in post-exposure feeding at 3.125, 6.25 and 12.5 µg/L over the 24 hour period when *M. rosenbergii* was exposed to profenofos, but these differences were not statistically significant. Percentages of decrease were 3.7, 25.9 and 49.1 % to the control (0 µg/L), respectively. All prawns died at the 2 highest profenofos concentrations (25 and 50 µg/L).

Figures 5.1(e) and (f) show post-exposure feeding rates (as % of control feeding rates) of *M. rosenbergii* exposed to carbendazim. Mortalities in control groups 1 (Figure 5.1e) and 2 (Figure 5.1f) were 23.3 % and 20 %, respectively. For group 2, there was a decrease in post-exposure feeding rate at 25, 50 and 100 mg/L over the 24 hours period when *M. rosenbergii* was exposed to carbendazim, but these differences were not statistically significant from the control. The percentages of decrease were 41.7, 12.9 and 23.6 % from the control (0 µg/L), respectively.

Carbendazim has a solubility of 8 mg/L (8000 µg/L) at pH 7 and 20°C (Tomlin, 2000). In this study, carbendazim concentrations used exceeded its solubility, where prawns were exposed up to 100 mg/L. The excess carbendazim that did not dissolve in water and the amount that dissolved reduced the post-exposure feeding rate of the prawns, but not significantly, as shown in Figure 5.1(f).

Figure 5.1(g) shows post-exposure feeding rate (as % of control feeding rates) of *M. rosenbergii* exposed to chlorpyrifos (from Experiment 2). Mortality in the control group was 18.3 % during this experiment. Mortalities in the 0.15, 0.3, 0.6 and 1.2 µg/L chlorpyrifos groups were 15, 15, 11.7 and 66.7 %, respectively. Post-exposure feeding rates significantly decreased with 2 concentrations (0.3 and 1.2 µg/L) ($F_{2,23} = 9.09$; $p = 0.001$). The percent differences from control were 32.2 and 45 % to the control (0 µg/L).

Figure 5.1(h) shows the evidence of recovery of prawns after being exposed to chlorpyrifos for 24 hours. Figure 5.1(g) shows that the post-exposure feeding rate of prawns decreased significantly due to chlorpyrifos exposure but during the recovery exposure, the feeding rate returned nearly to normal, as shown in Figure 5.1(h), with no significant difference from the control feeding rates ($F_{4,42} = 0.795$, $p = 0.535$).

5.5 Discussion

All the 24 and 48 hours LC_{50} values were within the ranges obtained from previous studies done by other researchers (Appendices 7 to 10). PAN pesticide database (2002) gave the ranges of toxicity for LC_{50} of chlorpyrifos as 0.18 - 623.3 µg/L, 0.8 - 3,000 µg/L for profenofos, 33 - 4,100 µg/L for dimethoate and 412.5-35,436 µg/L for zinc. All results from this study were in these toxicity ranges. However, LC_{50} values could not be compared directly because there were differences in methods, test media, size and species of test animals used.

According to the compiled list of toxicity tests done on *M. rosenbergii* (Table 1.2), none of these chemicals have ever been tested on the giant freshwater prawn post-larvae to determine their toxicity at this stage. A study by Dai *et al.* (2001) used zinc but only with juveniles of *M. rosenbergii.* The juveniles were cultured for 14 days in 850 µg/L of zinc and they were affected by toxicity, which resulted in decreased growth and survival rates. The 24 hour LC_{50} from Dai *et al.* (2001) at 850 µg/L was higher than that obtained from this study (439.7 µg/L) perhaps explained by the different age of the test animals. The test animals used in this study were post-larvae while Dai *et al.* (2001) used juveniles.

This was the first study done in the tropics for *M. rosenbergii* at the postlarval stage (9-10 mm) on lethal and sublethal effects of toxic substances and feed inhibition. Due to lack of literature and studies done on this specific area, the results of the LC_{50} and EC_{50} trials in this study could not be compared directly to any previous studies on the same species at the same stage. Nevertheless, the results of this study could certainly be used as reference material so further studies could be done especially in terms of refining methods and procedures.

M. rosenbergii post-larvae in this study were more sensitive to chlorpyrifos than *Palaemonetes pugio* (grass shrimp). Effective Concentration (EC_{50}) for 24 hours for *M. rosenbergii* post-larvae to chlorpyrifos was 0.27 µg/L (95 % C.I. = -0.40-0.92 µg/L). While for *P. pugio,* Lund *et al.* (2000) obtained the 24-h EC_{50} values for chlorpyrifos were 0.49 µg/L (95 % C.I. = 0.33-0.77 µg/L) and 0.36 µg/L (95 % C.I. = 0.33-0.38 µg/L) for Stages VI and VII embryos, respectively. EC_{50} using post-exposure feeding rate values from chlorpyrifos exposure were in the same range obtained by Van Wijngaarden *et al.* (1996) from their studies with macroinvertebrates and zooplankton. Their results for 48- and 96-h median effective concentrations (EC_{50}s) ranged from 0.1 to 3.4 µg/L.

Vijayram and Geraldine (1996) reported that the threshold dissolved concentration for zinc in *M. malcolmsonii* was 373 µg/L. In this study, the EC_{50} using post-exposure feeding rate value from zinc exposure was lower (132.4 µg/L). This confirmed the sensitivity of the post-exposure feeding rate response in *M. rosenbergii* since the response could be detected in a concentration that was lower than the threshold concentration. *M. rosenbergii* post-larvae were also more sensitive to zinc than *Daphnia magna*. EC_{50} 24 hours for *M. rosenbergii* post-larvae to zinc was 132.4 µg/L (95 % C.I. = -252.43-481.86 µg/L). But for *D. magna* the 48-h Effective Concentration (EC_{50}) values for zinc was 6,037 µg/L (95 % C.I. = 3,567-11,138 µg/L) without food (McWilliam and Baird, 2002a).

Overall, it could be seen that post-exposure feeding inhibition of *M. rosenbergii* was a sensitive endpoint to use with chlorpyrifos and zinc, where EC_{50} values obtained were lower than LC_{50} values.

Significant levels of feeding inhibition during post-exposure ($p < 0.05$) are presented in Figures 5.1(a) (zinc), 5.1 (b) (chlorpyrifos in Experiment I) and 5.1 (g) (chlorpyrifos in Experiment II). This indicated that the feeding inhibition which occurred during the exposures persisted throughout the post-exposure period (4 hours) at significant levels, allowing exposure effects to be detected during post-exposure within the same range of chlorpyrifos concentrations.

In the case of dimethoate and profenofos, the lethal effect concentrations were lower than the sub-lethal effect concentrations. Therefore, sub-lethal effects on post-exposure feeding in *M.* rosenbergii could not be used to detect dimethoate and profenofos toxicity.

For the post-exposure feeding rates for dimethoate (Figure 5.1(c)) and profenofos (Figure 5.1(d)), no significant difference from the control treatment was detected statistically for prawns exposed to varying levels of the chemicals. There are three possible points explanation for this. Firstly, the prawns exposed to these chemicals were able to recover and feed actively when placed in clean water, thus feeding rate was not statistically different from the control. Secondly, the 24 hours exposure was not enough for the chemicals (at the chosen concentrations) to take effect in the body of the animal. Thirdly, the negative effects of these chemicals did not immediately show any effect within the four hours that the feeding trials were conducted for those animals that survived. This demonstrates that *M. rosenbergii* was relatively more sensitive to chlorpyrifos and zinc than to dimethoate and profenofos as they needed only 24 hours exposure to be affected by the former.

The post-exposure feeding rates of the prawns with dimethoate and profenofos still appeared lower than the control feeding rate, showing that the chemicals might still have affected the prawns' biological and physiological processes, but could not be detected statistically due to low replicates/high variability in individual responses. In terms of the development of methods for these tests, the level of replication may need to be increased to reduce individual variability. The implication in real field situations is that any exposure to these chemicals could still lead to negative effects on the prawns' growth, survival and other bodily functions. Extoxnet (1996b) and the USEPA (2004c) mentioned that profenofos and dimethoate, respectively, were toxic to fish and aquatic organisms. Kumar and Chapman (2001) reported that profenofos affected the acetylcholinesterase (AChE) activity in wild fish in New South Wales, Australia, where residues were found in water (0.004-5.4 µg/L), sediments (4-670 µg/kg) and fish carcasses (0.13-10.7 mg/kg).

Figures 5.1(e) and 5.1(f) show the effect of carbendazim to post-exposure feeding rate and clearly suggests that under the current trial conditions, carbendazim

had no effect on post-exposure feeding, with feeding rates at all concentrations not significantly different from the control ($p > 0.05$). The reason might be the duration of exposure to carbendazim; Cuppen *et al.* (2000) suggested that the duration of exposure to carbendazim may have to be longer to get a significant effect. Van den Brink *et al.* (2000) also mentioned that carbendazim acted very slowly and that it required a period of 7 days, for the incipient LC_{50} of *D. magna* to be reached during the bioassay. They recommended the use of a exposure period of at least 96 hours for $L(E)C_{50}$ tests of fungicides.

In Figure 5.1(h), the feeding rates were higher than those in Figure 5.1(g). These feeding rates (after 24 hours recovery exposure to soft ASTM water without chlorpyrifos) were nearly the same as the normal feeding rate (control treatment). The lack of persistence of post-exposure feeding inhibition after exposure to chlorpyrifos suggests that if prawns were placed in clean water, they would recover.

This implies that 4 hours after exposure to chlorpyrifos is sufficient time to show the feeding inhibition effect of this chemical on prawns. The results from the recovery experiments clearly showed that *M. rosenbergii* would be able to recover if water was changed (in a setting where it is possible to change water in a culture system) and also in situations when it rained or some other type of water exchange occurred after contamination.

On the other hand, the feeding rates in Figure 5.1(h) are still lower than the control feeding rate which meant that the exposure to 0.3, 0.6 and 1.2 µg/L of chlorpyrifos was still able to reduce the feeding rates relative to the control during post-exposure by 12.4, 12.5 and 15.4%, respectively. However the differences are not statistically significant.

A concern will arise when prawn production systems are contaminated with these chemicals and their persistent levels might reduce feeding rate of prawns to levels which could make the operation less economically viable, i.e. prawns grow slowly or stop growing.

This study (as shown in Table 5.2) confirms the potential of *M. rosenbergii* as a test animal to detect the effects of different chemical contaminants in aquatic environments. Further investigation is required to observe whether post-exposure feeding rate effect of pesticides would occur with other more toxic organophosphates and pyrethroids. Feeding inhibition during exposure also needs to be investigated and methods for exposure times of more than 24 hours should be developed. This would need to consider that test animals would become stressed from lack of food over prolonged periods. Likewise, if post-exposure feeding times are lengthened practicality and cost need to be considered.

After the successful use of the *M. rosenbergii* bioassay for lethal and sublethal testing in the laboratory, techniques using *M. rosenbergii* were developed for microcosm (Chapter 6) and field *in situ* use (Chapter 7). Further experimental work was carried out to investigate the ecological relevance of the post-exposure feeding rate as the endpoint. However, it was foreseen that other factors (abiotic, environmental) might affect post-exposure feeding and survival in these outdoor systems.

CHAPTER 6

DEPLOYMENT OF THE *Macrobrachium rosenbergii* BIOASSAY IN MICROCOSM

6.1 Introduction

The use of microcosms provides a bridge between laboratory and the field, in terms of manageability and allowing replication (Calow, 1989; Daam and Van den Brink, 2003b), thus achieving a balance between an experimental set-up and real field conditions in terms of ecological processes and exposure to the chemical.

Microcosms are artificial multi-species test systems that simulate major characteristics of the natural environment for the purposes of ecotoxicological effects and risk assessment. Such aquatic systems may contain a limited amount and number of species of animals (vertebrates and invertebrates), microorganisms and plants (Leeuwen and Hermens, 1996), a model ecosystem contained in less than 15 m^3 of water (Crossland *et al.*, 1992)

Laboratory toxicity tests of single species may be able to show the effects of chemical substances directly but they fall short when it comes to showing the responses of test organisms to interactions and dynamics in ecosystems. These interactions can only be seen in cosm experiments (Rand *et al*, 2000 and Taub, 1997). In addition, single species tests do not represent the real conditions of test substances in the field (Brock, 1998). Because of these limitations of single species toxicity tests in the laboratory, it is therefore necessary to set-up cosm experiments. The end points which can be measured in cosm experiments include abundance of algae, zooplankton, micro and macroinvertebrates, nutrient dynamics and chemical fate (Leeuwen and Hermans, 1996).

6.2 Aims

The main aim of this chapter was to test the capacity of the post-exposure feeding rate bioassay of *M. rosenbergii* for use in the microcosm study. The hypothesis was that post-exposure feeding rate would decrease after exposure to different levels of the fungicide (carbendazim) when compared to the control treatment.

6.3 Materials and Methods

Due to the cost of settling up a microcosm experiment, *M. rosenbergii* microcosm experiment was conducted in cooperation with a carbendazim microcosm experiment being done by another researcher (Daam, in preparation).

Deployment was conducted on 28 February 2005. The experiment was performed in twelve outdoor microcosms at the hatchery of the Asian Institute of Technology (A.I.T.), located 42 km north of Bangkok (Thailand). Each microcosm consisted of a concrete tank (length 1 m, width 1 m, height 1.15 m, water volume 1,000 L) coated with epoxy paint. The tanks were filled with a 10-cm layer of sediment and 1-m water, taken from the canal surrounding A.I.T. The canal water was passed through a net (mesh size 0.1 mm) to prevent fish and prawn entering the systems. The microcosms modeled the community of irrigation drainage ditches in the immediate area.

In the preparatory phase of the experiment, zooplankton and macroinvertebrates were collected from the A.I.T. canal and introduced into the microcosms. Over an acclimation period of 5 weeks, a biocoenosis was allowed to develop in the microcosms. In this period, the water was circulated twice a week by collecting 100-L from each microcosm in a container and dividing it over the microcosms to achieve similarity between the communities in the systems. A nutrient addition of N (1.4 mg/L as urea) and P (0.18 mg/L as TSP) was applied twice a week during the entire experimental period (Daam, in preparation). Chambers used were similar to the ones used for field deployment (Figure 2.2).

During deployment, the chambers were placed on steel poles (Figure 6.1). The pole kept the chambers at 10 cm below water surface and 10 cm above the sediment. Two chambers were deployed in each tank.

The fungicide Bavistin FL (with carbendazim as the active ingredient) was applied once to 8 microcosms, in four duplicate doses (nominal levels: 3.3, 33, 100 and 1000 µg A.I../L) while four other untreated systems served as controls. Chambers were deployed 1 hour after carbendazim application. The time delay was to allow the pesticide to spread evenly throughout the tanks. It was necessary to stir and homogenize the pesticide in the tanks before deployment.

Figure 6.1 Steel poles for holding *M. rosenbergii* chambers in carbendazim microcosm tanks

6.3.1 Water quality parameters

Water samples from the microcosm tanks filling were collected by two (1 L) screwed-capped plastic sampling bottles with waters from each tank, ensuring no air bubbles were trapped the bottles were kept in Styrofoam boxes and were later analyzed for total hardness, ammonia, nitrite, chlorophyll a, total suspended solids and total volatile solids in the laboratory.

Duplicate 500 ml water samples were taken from each tank and put in brown glass bottles for carbendazim analysis. The bottles were rinsed using Acetonitrite 3 times and then with distilled water (5 times) before using to collect the samples. Carbendazim was analyzed directly from the water.

6.3.2 Post-exposure feeding rate

After the prawn exposure to treatment in microcosm, the number of dead and live prawns was counted. The surviving prawns from each treatment were mixed together in a wide-mouthed glass jar and randomly selected for the post-exposure feeding test. The following were the steps taken to measure the feeding rates :

1. 10 plastic jars were filled with 80 ml of soft ASTM fresh water. 100 *Artemia* nauplii were placed into each jar. Then prawns were added randomly at 1 prawn/jar to 7 of the jars. The 3 other jars were used as control (no prawns).
2. The prawns were left in the dark by black plastic for 4 hours.
3. After 4 hours, the number of dead and live prawns was counted and three drops of Lugol's solution were added to fix them.
4. The dead prawns were removed from the jar and set aside, while the remaining *Artemia* were concentrated by moving each jar clockwise.
5. The remaining *Artemia* was collected and placed into a 25 mL test tube/replicate, then they were set aside for 1 hour.
6. Using a glass dropper, all the *Artemia* were removed from the tube and placed in a Sedgwick-Rafter counting cell. They were then counted under the microscope. The number of *Artemia* was then recorded per replicate.

7. Prawn length was measured and recorded.
8. The feeding rate was calculated according to the method in Chapter 2 section 2.4.

6.4 Results

Carbendazim concentrations inside the microcosm from day 0 to day 1 increased in treatments 3.3, 33 and 100 µg/L and decreased in treatment 1000 µg/L by carbendazim pesticide analysis (Table 6.1 and carbendazim pesticide analysis in chapter 2). However, one way ANOVA revealed that there was no significant difference between concentrations on day 0 and day 1 at all treatment level ($F_{1,2}$ = 0.42; P=0.583, $F_{1,2}$ = 0.22; P=0.685, $F_{1,2}$ = 0.85; P=0.455 and $F_{1,2}$ = 1.19; P=0.388 for treatment 3.3, 33, 100 and 1000, respectively). Effectively concentrations did not change during the period of the test.

Mean mortality was 44 % in the control and less than 25 % in treatments 3.3 and 33. Mortalities in treatments 100 and 1000 µg/L were 52.5 % and 45.0 %, respectively (Figure 5.7) which are significantly different to the control treatments ($F_{2,24}$ = 5.95; P = 0.008). Table 6.2 presents the average mortality in each treatment, which were not significantly different in terms of mortality at different levels of the chambers in the tanks (10 cm below surface of water and 10 cm above the sediment).

Post-exposure feeding rates at all carbendazim treatments were compared to control post-exposure feeding rates using Bonferroni multiple comparisons test. There was a significant difference in treatments 100 and 1000 µg/L ($P < 0.05$). A significant difference was also found between post-exposure feeding rates using one way ANOVA test ($F_{3,32}$ = 4.62; P = 0.009) in treatments 33, 100 and 1000 µg/L compared with the control.

Table 6.1 Average carbendazim concentration (µg/L) within duplicate treatment level microcosm on day 0 and day 1 (24 hr)

Treatment (µg/L carbendazim)	Initial concentration (day 0) (µg/L ± SE)	Day 1 (24 hr) (µg/L ± SE)
3.3	2.46 ± 0.078	2.57 ± 0.156
33	25.15 ± 1.971	26.13 ± 0.672
100	81.54 ± 3.158	86.60 ± 3.079
1000	832.54 ± 47.919	777.36 ± 15.873

Table 6.2 Average mortality at below surface (10 cm) and above sediment (10 cm) of water in the microcosm tanks at 24 hours exposure; ANOVA results indicate significance of mortality in each treatment.

Treatments	Average mortality		F-value	p-value
	Below surface (10 cm)	Above sediment (10 cm)		
Control	35	52.5	$F_{(1,6)} = 0.54$	0.489
3.3 µg/L	35	10	$F_{(1,2)} = 0.51$	0.549
33 µg/L	15	30	$F_{(1,2)} = 9.00$	0.095
100 µg/L	55	50	$F_{(1,2)} = 0.04$	0.860
1000 µg/L	30	60	$F_{(1,2)} = 2.25$	0.272

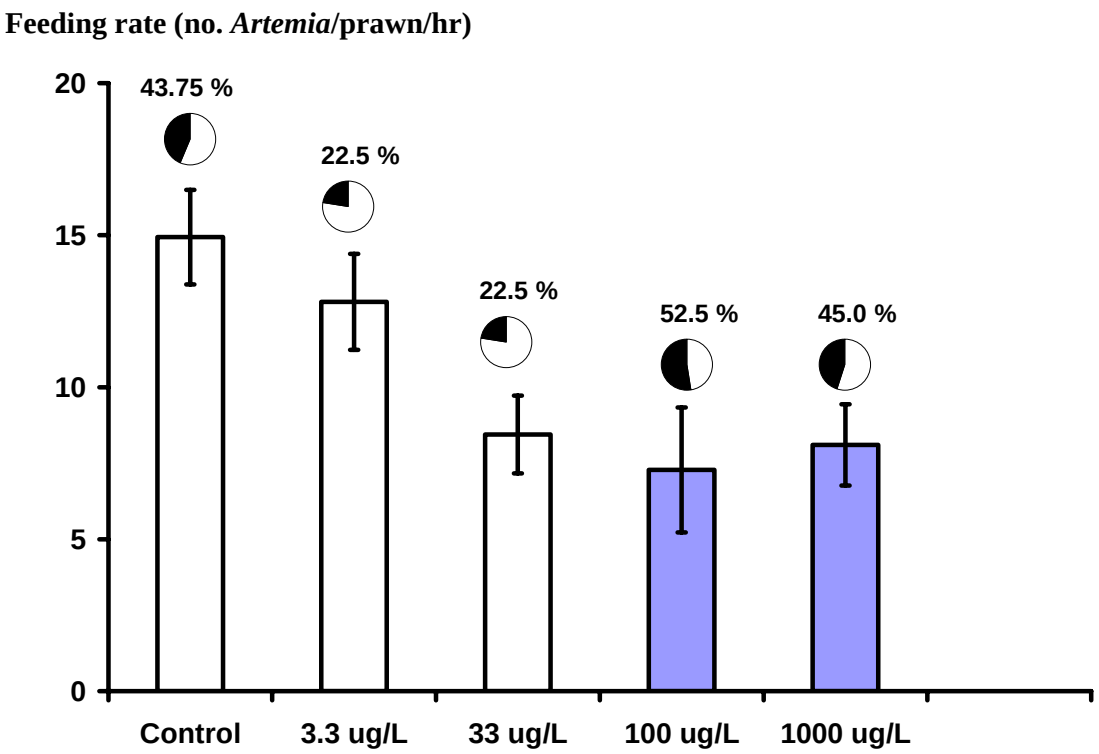


Figure 6.2 Post-exposure feeding rates of *M. rosenbergii* after the 24 hours exposure to carbendazim in microcosm and 4 hours of feeding. Shaded bars denote feeding rates which are significantly different from the control ($P < 0.05$). Vertical bars indicate standard errors. Pie charts and percentages present average mortality of animals during 24 hours exposure in microcosm.

6.5 Discussion

Previous experiments in Chapter 5 indicated that there was no significant difference in the post-exposure feeding rate of prawns exposed to carbendazim in the laboratory. But in the microcosm experiments, the post-exposure feeding rates in treatments 100 µg/L and 1000 µg/L decreased significantly ($P < 0.05$). There was also a significant decrease in treatment 33 µg/L but only when analysed differently (by ANOVA). In Cuppen *et al.* (2000) microcosm experiments, carbendazim at 33, 100, 330 and 1000 µg/L treatments affected the macroinvertebrate community with serious

noxious effects on certain organisms such as the Turbellarians (*Dugesia*), Oligochaetes (*Dero* and *Stylaria*), crustaceans (*Gammarus sp.*) and mollusks (*Bithynia*). At 100 µg/L or higher, the crustaceans *Gammarus* juvenile could not be found in the microcosms anymore, i.e. the abundance suddenly decreased within a week. At this level, the adults of *G. pulex* were also affected. Likewise, addition of copper in a microcosm study by Shawn and Manning (1996) also affected the benthic macroinvertebrate community mainly through changes in species composition. In a mesocosm study using cypermethrin, changes in the community structure and biodiversity of organisms occurred either by increasing or decreasing certain species (Medina *et al.*, 2004). Changes in community structure and water chemistry could have occurred in this study's microcosm experiments with carbendazim and could also have affected the responses of prawns upon exposure and during post-exposure feeding tests.

In the previous laboratory tests (Chapter 5), post-exposure feeding rates did not significantly decrease from the control. The results in this microcosm study confirmed the advantage of microcosm to provide a more realistic exposure to the chemical than the laboratory. Post-exposure feeding rates decreased as carbendazim concentration increased. In the control treatment, the mortality (more than 20 %) indicated that there might be another stress factor other than carbendazim (Cuppen *et al.*, 2000), which could also have caused the mortality and affected post-exposure feeding rate in the treatments, thus the difference in mortalities between the laboratory and the microcosm set-up. Furthermore, macroinvertebrates present in the microcosm (Daam, in preparation) and small enough to enter the chambers, such as flatworms, might have attacked and predated on the prawns, explaining the high prawn mortality in the control, while at intermediate concentration, these predator organisms were killed by carbendazim thus there was less predation on prawns. At highest concentration, these predator organisms inside the chambers and some of the prawns were affected by carbendazim (Table 6.2 and Figure 6.2). High variation in post-exposure feeding rates could be due to the mixing of surviving animals from the chambers on the surface and bottom.

Water quality in the microcosm set-up was within the optimum range for prawn post-larvae. Specifically, the dissolved oxygen saturation was more than 60 %

(4.5 mg/L). Cuppen *et al.* (2000) also mentioned that the structure of aquatic ecosystems was indirectly affected by the addition of carbendazim, i.e. encouraging the growth of phytoplankton and macrophytes, yet water quality parameters remained unchanged. In addition to carbendazim, the availability of natural food in the microcosm tanks might have contributed to the depression of post-exposure feeding rate, which was similar to the observation of Taylor *et al.* (1998) with *Daphnia magna*. Their study showed that the reduction in post-exposure feeding rate in animals pre-exposed to contaminated food (cadmium-loaded algae) was significantly greater than that found in animals that had been starved. Correia *et al.* (2003) also found that availability of natural food in the system may decrease the consumption of formulated feed. However, a conclusion could not be made if this was the case with the microcosm experiments on *Macrobrachium* post-larvae. This is one aspect in this study which was not investigated, i.e. whether the prawns were feeding while in the microcosms.

Water quality parameters need to be measured at different levels in the water column (surface, middle and bottom) where the chambers are set up, over a 24 hour period or throughout the duration of the exposure. The additional information on water quality parameters can help in the analysis of whether mortality during exposure and the effect on post-exposure feeding rate were caused only by the pesticides present or also by the water quality conditions in the microcosm. In this study, water quality parameters (DO, pH, temperature, conductivity, ammonia, total hardness, nitrite, total suspended solids, total volative solids and chlorophyll a) were measured only from the water surface (10 cm below water surface) which was not sufficient to conclude the effect of water quality on the performance of prawns during exposure and post-exposure feeding tests.

This microcosm study was a first step in improving information collection towards ecological risk assessment.

CHAPTER 7

DEPLOYMENT OF THE *Macrobrachium rosenbergii* BIOASSAY IN THE FIELD AT REFERENCE AND PESTICIDE CONTAMINATED SITES

7.1 Introduction

The use of laboratory tests as a basis for ecological risk assessment might be questionable due to a poor reflection of field conditions (Daam and Van den Brink, 2003b). In Chapter 4, a bioassay with *Macrobrachium rosenbergii* was developed under laboratory conditions. A microcosm experiment then identified differences in response to the fungicide (carbendazim) of *M. rosenbergii* postlarvae compared to laboratory toxicity test (chapter 6). Microcosms allow exposure to more complex environmental factors and their interaction, but do not necessarily reflect the full reality of field conditions; ideally field testing is still required. As a man-made system, the type and population of species in microcosms are controlled to avoid complexity in data interpretation (Rand, 1995). Likewise, nutrients are added into microcosms in a controlled manner to prevent eutrophication but in real field situation, the amount of organic matter going into the system varies depending on location and season.

Exposure to field conditions will allow *M. rosenbergii* to be continually exposed to the surrounding aquatic environment and to detect any deleterious effects on post-exposure feeding rates, induced by pesticide components present. Deployment of a single species *in situ* bioassay involves transplanting collected or cultured animals and enclosing them in mesh adapted chambers under field conditions e.g. Chappie and Burton (1997), Greenberg *et al.* (2002), Maltby *et al.* (2002a) and Daam and Van den Brink (2003).

Initially, single species *in situ* bioassays were developed using lethality as an endpoint. More recently, tests have been developed using sublethal endpoints such as scope for growth (SFG) (Widdows, 1985; Naylor *et al.*, 1989), feeding inhibition

(Maltby *et al.*,2002a; McWilliam and Baird, 2002 and Castro *et al.*, 2004) and reproduction (Pereira *et al.*, 2000).

It was argued by Lytle and Lytle (2001) that plants are more sensitive than animals to chemical effects, yet concluded that plant and animal sensitivities are specific to the type of contaminated chemical and species. Though there have been many studies using plants for toxicity assessment in aquatic ecosystem, the focus of this study is on animals, specifically in *Macrobrachium rosenbergii*, an important species in the tropics.

There have been several studies using the *in situ* approach with animals as test organisms. Successful *in situ* testing has been achieved with *Hyalella azteca* and *Chironomus tentans* (Chappie and Burton, 1997; Greenberg et. al., 2002 and Burton *et al.*, 2005), *Gammarus pulex* (Maltby *et al.*, 2002a), *Daphnia magna* (McWilliam and Baird, 2002b; Burton *et al.*, 2005), *Ceriodaphnia dubia*, and *Lumbriculus variegatus* (Greenberg et. al., 2002), *Poecilia reticulata* (Castro *et al.*, 2004) and *Chironomus riparius* (Burton *et al.*,2005). Chappie and Burton (1997) concluded that incorporating *in situ* tests in the assessment of contaminated sites was more realistic and the results obtained were more accurate than laboratory bioassays. They added that *in situ* testing is useful and effective when proper handling techniques, appropriate mesh size of the test chambers and substrates are used.

Lethality is a commonly used endpoint, but it does not consider sub-lethal effects of toxicity which could occur at lower contaminant concentrations. These lower contaminant concentrations could affect, for example, post-exposure feeding inhibition, as already presented in Chapter 4 with zinc and chlorpyrifos. A combined endpoint of survival/mortality and feeding behaviour could be used as an indicator of pesticide contamination in tropical freshwaters. The survival/mortality could be affected by environmental factors such as low temperature (Chappie and Burton, 1997), low pH (Castro *et al.*, 2004), low dissolved oxygen levels (Burton *et al.*, 2005) and predation from other species that invade the cages (Maltby, 1999; van den Brink *et al.*, 2000).

Sub-lethal endpoints are often more sensitive than lethality, but they are also more sensitive to abiotic factors as mentioned earlier. This might lead to misinterpretation of results where it is difficult to separate the effects of contaminants and abiotic factors on endpoints (McWilliam, 2001). Selection of bioassay endpoints depends not only on what the experimenter wishes to investigate, but also, critically, upon factors of time and cost. Tools for risk assessment should therefore be low cost and require less time to perform; in principle *in-situ* tests also not require expensive capital equipment or complex maintenance. The development of an *in situ* bioassay using *M. rosenbergii* was tested in field conditions to investigate the possibility of the procedures of *in situ* bioassay of *M. rosenbergii* to detect pesticide toxicity.

7.2 Aims

The main aim of this chapter was to test the capacity of the post-exposure feeding rate bioassay of *M. rosenbergii* for use in field studies, especially in areas known to be highly contaminated by pesticides. This was achieved by determining whether or not the bioassay was suitable for use in the field in terms of robustness (if prawns could survive during exposure to field conditions) and sensitivity (if toxicity could be detected). Deployments were set up at un-contaminated reference sites to compare whether there were any reductions in feeding rate due to the effects of pesticide contamination. The hypothesis was that post-exposure feeding rate under pesticide contamination would be reduced when compared to post-exposure feeding rate at reference sites.

7.3 Materials and Methods

7.3.1 Animals and acclimation

All animals used were post-larvae (PLs) with sizes ranging from 9-10 mm obtained from a commercial hatchery. They were acclimated in test medium as in section 2.1 and 2.2 in Chapter 2 for 48 hours, after which time animals were ready to be used in the field.

7.3.2 Field deployment

The following were the steps taken to conduct the field deployment of prawns:

1. One day before putting animals into the chambers, chambers in racks were installed at the field site. Four chambers were installed per site.
2. On the day of deployment, 100 *M. rosenbergii* were transported to the field in an oxygenated plastic bag (0.3 X 0.4 m) containing soft ASTM freshwater (3 litres). Animals can be kept without any negative impact or stress in this container for 4-6 hours.
3. At the field site, the plastic bag was allowed to float in the water for 15-30 minutes to equalize the temperature inside the bag with that of the surround water. After this, ten prawns were placed into each chamber by dropper through the air tube. All chambers in racks had been installed the day before and were submerged in the water. Each site had four chambers, requiring a total of 40 prawns.
4. After 1 day, the racks containing the chambers with prawns were removed from the water and transferred to a 10-L bucket filled with site water. They were then brought to a shaded area.
5. Under the shade, the number of dead and live prawns was counted. The live prawns were placed in a wide-mouthed glass jar and randomly selected for the post-exposure feeding test in plastic jar individually (100 mL).

7.3.3 Measurement of feeding rates

The following were the steps taken to measure the feeding rates:

1. 10 plastic jars were filled with 80 ml of soft ASTM fresh water. 100 *Artemia* nauplii were placed into each jar. Then prawns were added randomly at 1 prawn/jar to 7 of the jars. The 3 other jars were used as control (no prawns).
2. The prawns were left in the dark covered by black plastic for 4 hours (near the farm canal : field condition).
3. After 4 hours, the number of dead and live prawns was counted and three drops of Lugol’s solution were added to fix them.

4. The dead prawns were removed from the jar and set aside, while the remaining *Artemia* were concentrated by moving each jar clockwise.
5. The remaining *Artemia* was collected and placed into a 25 mL test tube/replicate, then they were set aside for 1 hour.
6. Using a glass dropper, all the *Artemia* were removed from the tube and placed in a Sedgwick-Rafter counting cell. They were then counted under the microscope. The number of *Artemia* was then recorded per replicate.
7. Prawn length was measured and recorded.
8. The feeding rate was calculated according to the following equation:

$$F = (C_o - C_t) / T$$

Where : F = feeding rate of a single animal (number of *Artemia*/hr)

C_o = initial nauplii (number of *Artemia*) X correction factor (from control)

C_t = final nauplii (number of *Artemia*)

T = time animals were allowed to feed (hours)

7.3.4 Water quality parameters and pesticide analysis during field deployment

Water quality parameters (DO, pH, temperature, conductivity, total hardness, chlorophyll a, ammonia, nitrite, TSS and TVS) were recorded at the start of the bioassay (deployment day) at around 9:00 a.m. to obtain the general water quality profile in the study sites. Water quality parameters, equipment and methods were the same as in Chapter 2.

Water samples for pesticide analysis were collected on every deployment day at the same time as water quality samples. Water sample from farm canals were collected by using glass bottles and stored on ice during transportation to laboratory for further analysis. Pesticide residue concentrations were analyzed only for profenofos, dimethoate, chlorpyrifos residue concentrations. Details of pesticide analysis method were in chapter 2.

7.4 Details of field deployment (*in situ*)

Field deployment of *M. rosenbergii* was conducted from June 2004 to February 2005 at the study sites mentioned in chapter 2. There were four main deployments.

The first deployment (deployment I) was conducted during the wet season from June to August 2004. *In situ* bioassay were conducted at study site 1 (Chuchart, Surat, Chanchai farms), study site 2 (Mali and Tew farms) and study site 3 (organic farm). The second deployment (deployment II) was conducted during the dry season from November to December 2004. *In situ* bioassay were conducted at study site 1 (Chumpon and Somjit farms), study site 2 (Add farm) and study site 3 (organic farm). The details of each farm are given in chapter 2.

The first and second deployments included farm canal deployment, water supply canal deployment and the jars system with soft water ASTM.

The jar system was set up by putting ten test animals per jar from the same batch used in the field deployment. Three glass jars were used. These glass jars with test animals were left under the shade near the farm canal for the same period of field deployment (24 hours). The purpose of this three glass jar system was to ensure the strength of the test animals and validation of the field bioassay in terms of error from mortality which should not be more than 20 %. The number of surviving and dead animals after 24 hours was recorded. The surviving animals were further used for the post-exposure feeding tests.

Deployments I and II did not coincide with the day of application of pesticides in the farms. However, they were conducted not more than 3 days before or after pesticide application. Deployment on the pesticide application day was often difficult because of the uncertain schedule of the farmers, weather condition (rain constrain, farmers applying pesticide) and test animal preparation. The pesticide sample and analysis procedure were mentioned in chapter 2.

Deployment III was conducted at Chuchart farm (study site 1) which used only profenofos as insecticide for the crop. An analysis of profenofos showed a high concentration. Chuchart farm fully cooperated in the project and the *in situ* bioassay was set up on the pesticide application day.

Deployment IV was conducted at Samnieng farm (study site 1). This deployment was different from previous deployments. The farmer used different pesticides, mixing them into a pesticide cocktail (carbendazim, cypermethrin, methomyl and parathion). The deployment was made in a time series (0, 3 and 7 days). Pesticide analysis was only carried out for carbendazim. Table 7.1 provides all the details of the deployments in this research including the type of pesticides used in the farms.

Table 7.1 Details of the deployments and *in situ* bioassay conducted at field sites in central Thailand (June 04 – February 05)

	Season	Date	Farm name	Supply canal	Locations	Applied pesticide
Deployment I						
Bioassay 1	Wet	9 June 04	Chuchart (veg1)	yes	Site 1	Profenofos
Bioassay 1	Wet	9 June 04	Surat (veg2)	yes	Site 1	Dicrotophos, chlorfenapyr, abamectin
Bioassay 1	Wet	9 June 04	Chanchai (veg3)	Yes	Site 1	Diafenthiuron + fenoxycarb, dicrotophos, senep and chlorfenapyr
Bioassay 2	Wet	22 June 04	Organic farm	No	Site 3	No pesticide applied
Bioassay 3	Wet	26 Aug 04	Mali (Tan1)	Yes	Site 2	Did not apply > 3 months
Bioassay 3	Wet	26 Aug 04	Tew (Tan2)	Yes	Site 2	Did not apply > 3 months
Deployment II						
Bioassay 4	Dry	25 Nov 04	Chumpon (veg4)	Yes	Site 1	Profenofos
Bioassay 4	Dry	25 Nov 04	Somjit (veg5)	Yes	Site 1	Profenofos
Bioassay 5	Dry	2 Dec 04	Add (Tan3)	Yes	Site 2	Did not apply > 3 months
Bioassay 5	Dry	2 Dec 04	Organic farm	Yes	Site 3	No pesticide applied
Deployment III						
Bioassay 6	Dry	9 Feb 05	Chuchart (veg6)	no	Site 1	Profenofos
Bioassay 6	Dry	9 Feb 05	Reference AIT	no	Site 4	No pesticide applied
Deployment IV						
Bioassay 7	Dry	14 Feb 05	Samnieng (Fr1)	no	Site 1	Carbendazim, cypermethrin, methomyl and parathion
Bioassay 7	Dry	14 Feb 05	Reference AIT	no	Site 4	No pesticide applied
Bioassay 8	Dry	17 Feb 05	Samnieng (Fr1)	no	Site 1	Carbendazim, cypermethrin, methomyl and parathion
Bioassay 8	Dry	17 Feb 05	Reference AIT	no	Site 4	No pesticide applied
Bioassay 9	Dry	21 Feb 05	Samnieng (Fr1)	no	Site 1	Carbendazim, cypermethrin, methomyl and parathion
Bioassay 9	Dry	21 Feb 05	Reference AIT	no	Site 4	No pesticide applied

Notes:
1. yes-deployment was set up; no-deployment was not set up.
2. Veg = vegetable farm; Tan = Tangerine farm; Fr = Fruit farm
3. See Appendix 13 for information on environmental parameters such as pH, DO, conductivity.

7.5 Results of field deployment I

Bioassay 1:

1st deployment on 9 June 2004

The first deployment was carried out on 9 June 2004 in Chuchart, Surat and Chanchai farms (**Study site 1**). As shown in Figure 7.7 (a), mortality of prawn was high in the supply canal. All prawns died in the supply canals of Chuchart and Surat farm while 80 % died in Chanchai farm. Water quality monitoring showed that the % saturation of oxygen in the supply canals at Chuchart and Surat farms was very low ranging between 1 % (0.1 mg/L) and 25 % (1.7 mg/L) at around 9:00 a.m., respectively (Appendix 7.2). Nitrite concentration (2.21 mg/L) was also high at Surat farm's supply canal.

All post-exposure feeding rate monitoring activities were done under better water quality water (soft ASTM) than occurring in the field. Post-exposure feeding rates in the farm canals of Chuchart, Surat and Chanchai farms were lower than that in the supply canal of Chanchai farm. Post-exposure feeding rates observed in jars (soft ASTM exposure) at Chuchart and Chanchai farms were not significantly different ($P > 0.05$) and the low mortality also indicated less transport or handling stress. In Surat farm, the post-exposure feeding rate in the jar systems decreased significantly from Chuchart and Chanchai farms which could be due to stress from handling as it was the last farm where deployment was conducted, at nearly noon time.

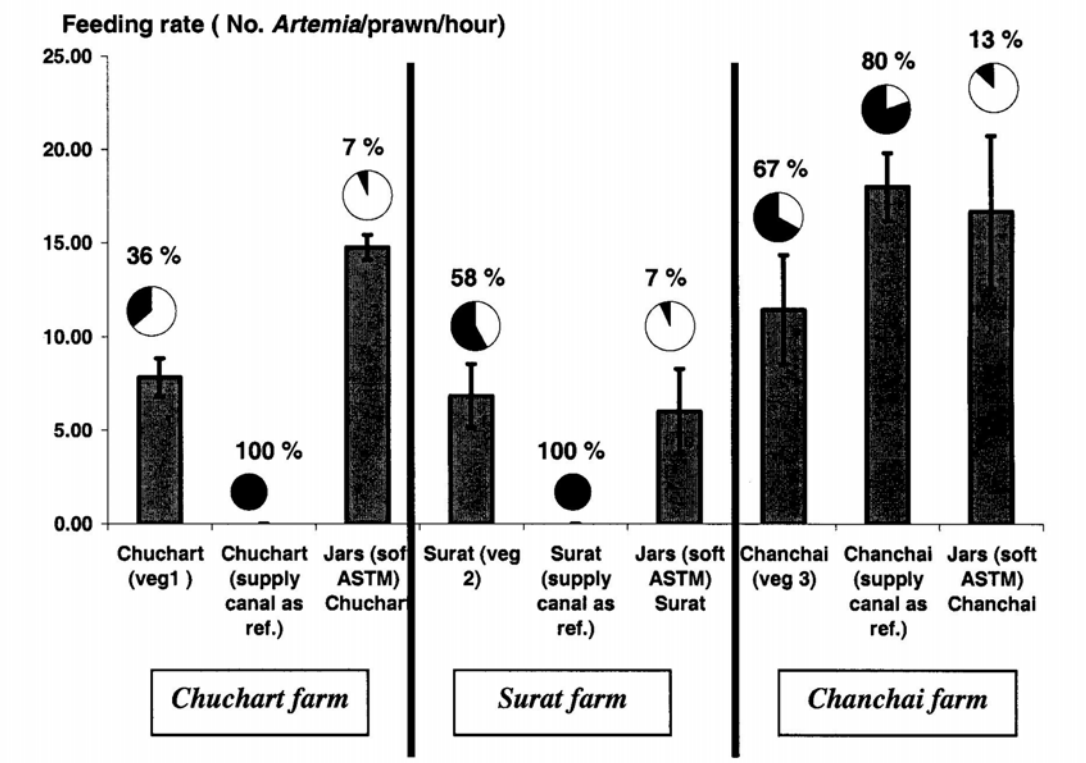


Figure 7.7 (a) ***Bioassay 1:*** Mean individual post-exposure feeding rate in 3 vegetable farms (Chuchart, Surat and Chanchai : **Study site 1**) for 24 hours. exposure period. Bars indicate mean post-exposure feeding rate in farm canal, supply canal and jar system. Vertical bars indicate standard errors. Pie charts and percentages present average mortality of animals during 24 hours exposure in farm canals, supply canals and jar systems

Bioassay 2:
2nd deployment on 22 June 2004

The second deployment was done on 22 June 2004 at the organic farm (**study site 3**). Figure 7.7(b) shows the mean individual post-exposure feeding rate in this organic farm and in the jar system near the farm canal. The average mortality of prawns located in farm canals was 55 %. Water quality conditions in the farm canal were within optimal ranges. DO was 4.5 mg/L at around 9:00 a.m. (61 % oxygen saturation). Nitrite and unionized ammonia levels were lower than 0.001 mg/L. Test animals were not stressed from transportation thus the low mortality in the jar system (9 %).

Post-exposure feeding rate from canal (field site) was not different from that of the jar systems ($P > 0.05$). The post-exposure feeding rate from farm canal was 15.8 ± 1.7 (number of *Artemia* per prawn per hour ± SE).

In comparing the post-exposure feeding from the organic farm with that from the first deployment in farm canals of Chuchart, Surat and Chanchai farms, there was a significant difference, especially compared to Chuchart & Surat farms ($F_{2,16} = 11.73$; $P = 0.001$). The post-exposure feeding rate in the farm canal of Chanchai farm was not different from that of the organic farm ($F_{1,10} = 1.98$; $P = 0.19$).

From this second deployment and after comparing the post-exposure feeding rate with those obtained from the first deployment, significant differences were observed. The post-exposure feeding rate was significantly lower in the first deployment (farms using pesticides) than that of the farm in the second deployment (organic farm, no pesticide used).

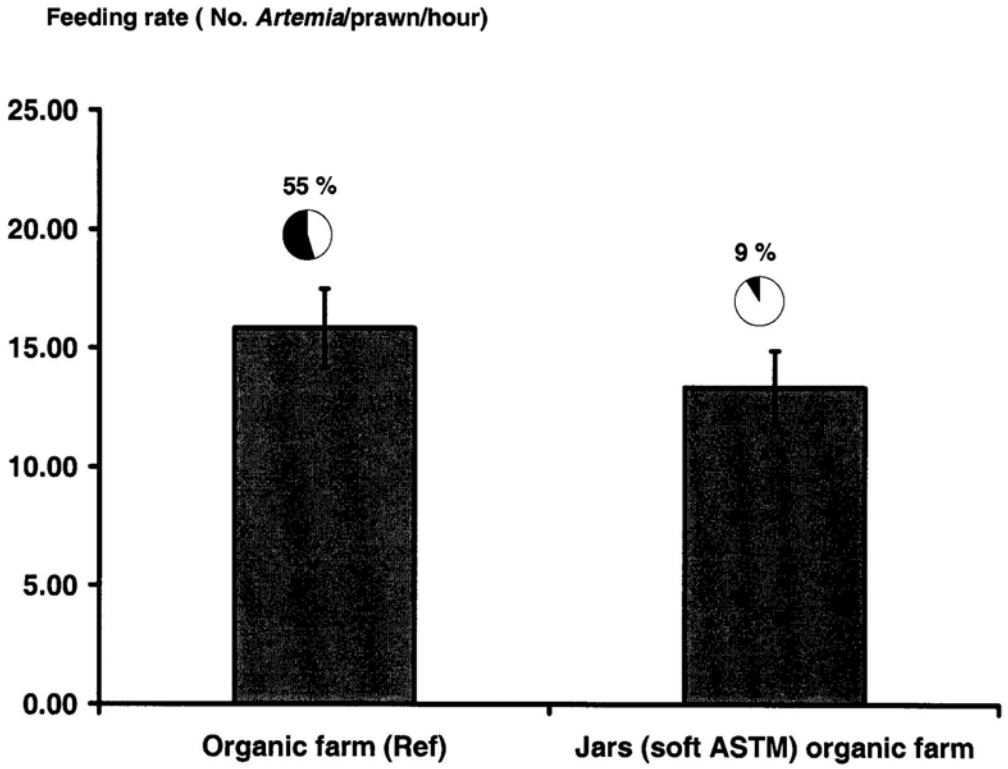


Figure 7.7 (b) ***Bioassay 2:*** Mean individual post-exposure feeding rate in organic farm (**Study site 3**) for 24 hours. exposure period. Bars indicate mean post-exposure feeding rate in farm canal and jar system.
Bars indicate mean post-exposure feeding rate in farm canal and jar system. Vertical bars indicate standard errors. Pie charts and percentages present average mortality of animals during 24 hours exposure in farm canals and jar systems.

Bioassay 3:
3rd deployment on 26 August 2004

The third deployment was done on 26 August 2004 at Mali and Tew farms (**study site 2**). Figure 7.7(c) presents the mean individual post-exposure feeding rates in Mali and Tew farms. Mortality of prawns in the supply canals of both farms was greater than 90 %. DO levels were 1.8 and 0.5 mg/L, respectively.

The surviving prawns were used for the post-exposure feeding test. The results shown in Figure 5.9(c) indicate that there were no differences in the post-exposure feeding rates obtained from the canal of Mali and Tew farms ($F_{1,10} = 2.18$; $P = 0.17$). However, these rates were lower than that of the jar systems in both farms. This indicates that exposure had an effect on later feeding.

The post-exposure feeding rate in the jar systems showed that there was no stress from traveling as there was no mortality. The prawns were healthy and hungry. All *Artemia* in the jars were consumed before the end of the feeding period (4 hours).

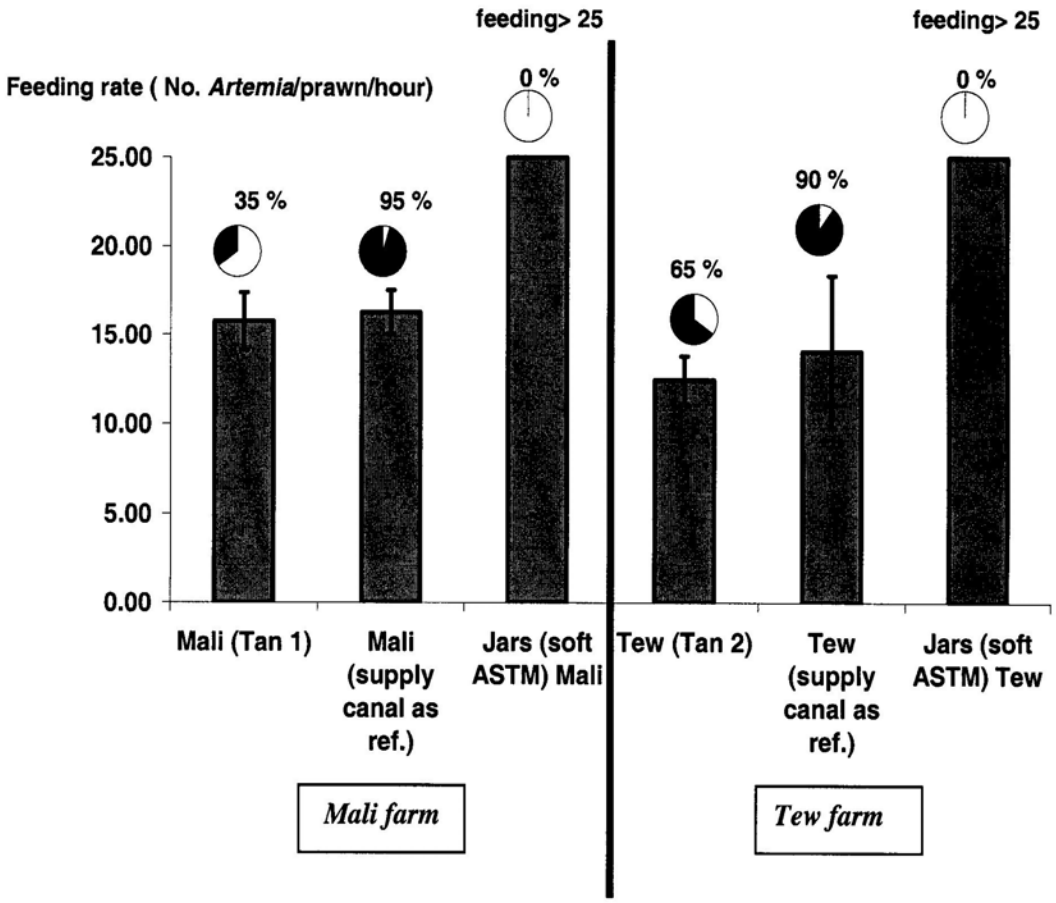


Figure7.7 (c) ***Bioassay 3:*** Mean individual post-exposure feeding rates in tangerine farm (Mali and Tew : study site 2) for 24 hours. exposure period.Bars indicate mean post-exposure feeding rate in farm canal, supply canal and jar system. Vertical bars indicate standard errors. Pie charts and percentages present average mortality of animals during 24 hours exposure in farm canals, supply canals and jar systems.

7.6 Result of field deployment II

Bioassay 4:

4th deployment on 25 November 2004

A fourth deployment was done on 25 November 2004. Figure 7.8 (a) shows the mean individual post-exposure feeding rates obtained in Chumpon and Somjit farms (**study site 1**). Mortality of prawns in the supply canals was high in Somjit farm (68 %), where DO was only 2.0 mg/L or 27 % oxygen saturation. In the jar system, the post-exposure feeding rate in Chumpon farm was very low for reasons which could not be explained. However, in the jar system in Somjit farm, feeding was normal and mortality was low (7 %). There was reduction in the feeding rate between Somjit farm canal, supply canal and jar systems but was not significantly different ($F_{2,18} = 1.09$; $P = 0.356$).

ANOVA showed that there was no difference in the feeding rates obtained in farm and supply canals of Chumpon and Somjit farms (Figure 5.10 (a)), ($F_{3,32} = 0.10$; $P = 0.958$). The jars system was not included in the ANOVA analysis. Comparing these post-exposure feeding rates with that obtained in the 2nd deployment (organic farm), there was also no difference in the feeding rate ($F_{4,38} = 0.24$; $P = 0.916$).

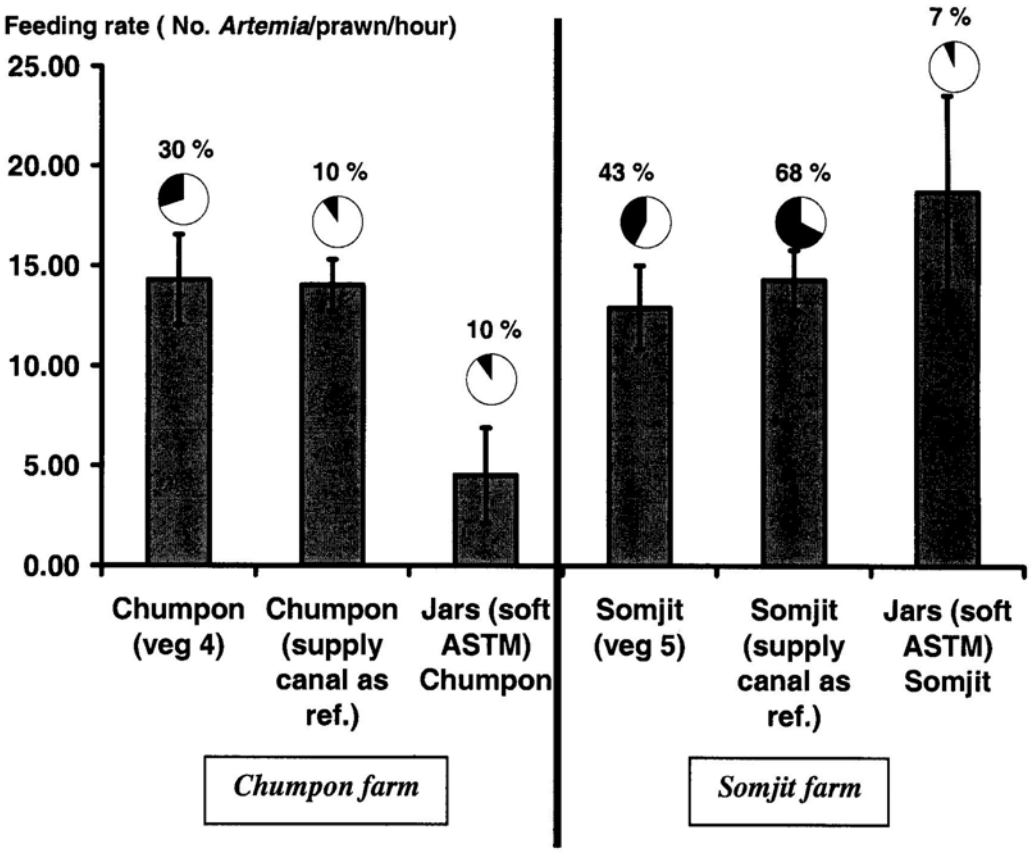


Figure 7.8 (a) ***Bioassay 4:*** Mean individual post-exposure feeding rates in 2 vegetable farms (Chumpon and Somjit farms : **Study site 1**) for 24 hours exposure period. Bars indicate mean post-exposure feeding rates in farm canal, supply canal and jar system. Vertical bars indicate standard errors. Pie charts and percentages present average mortality of animals during 24 hours exposure in farm canal, supply canal and in jar system.

Bioassay 5:

5th deployment on 2 December 2004

A fifth deployment was done on 2 December 2004. Figure 7.8 (b) presents the mean individual post-exposure feeding rate obtained from Add farm (**Study site 2**) and organic farms (**Study site 3**). Mortality of prawns was less than 30 %, with the exception of the organic farm where mortality during exposure was 74 %. Mortality in the organic farm could be attributed to low DO (0.7 mg/L) and the presence of profenofos (15.5 µg/L) in its farm canal.

The post-exposure feeding rates of animals deployed at Add farm and the organic farm (5th deployment) were not different from each other ($F_{1,18}$ = 3.87; P = 0.065). When the post-exposure feeding rate of Add farm was compared with the 2nd deployment results in organic farm, there was also no difference in the feeding rate ($F_{1,15}$ = 1.22; P = 0.287). However, the post-exposure feeding rate from Add farm (12.80±1.97 *Artemia* per prawn per hour ± SE) was lower than that obtained from the organic farm during the 2nd deployment (15.82±1.65 *Artemia* per prawn per hour ± SE).

The post-exposure feeding rate in the organic farm during this deployment (5th) was significantly different from that obtained in the same organic farm during the 2nd deployment ($F_{1,15}$ = 18.71; P = 0.001).

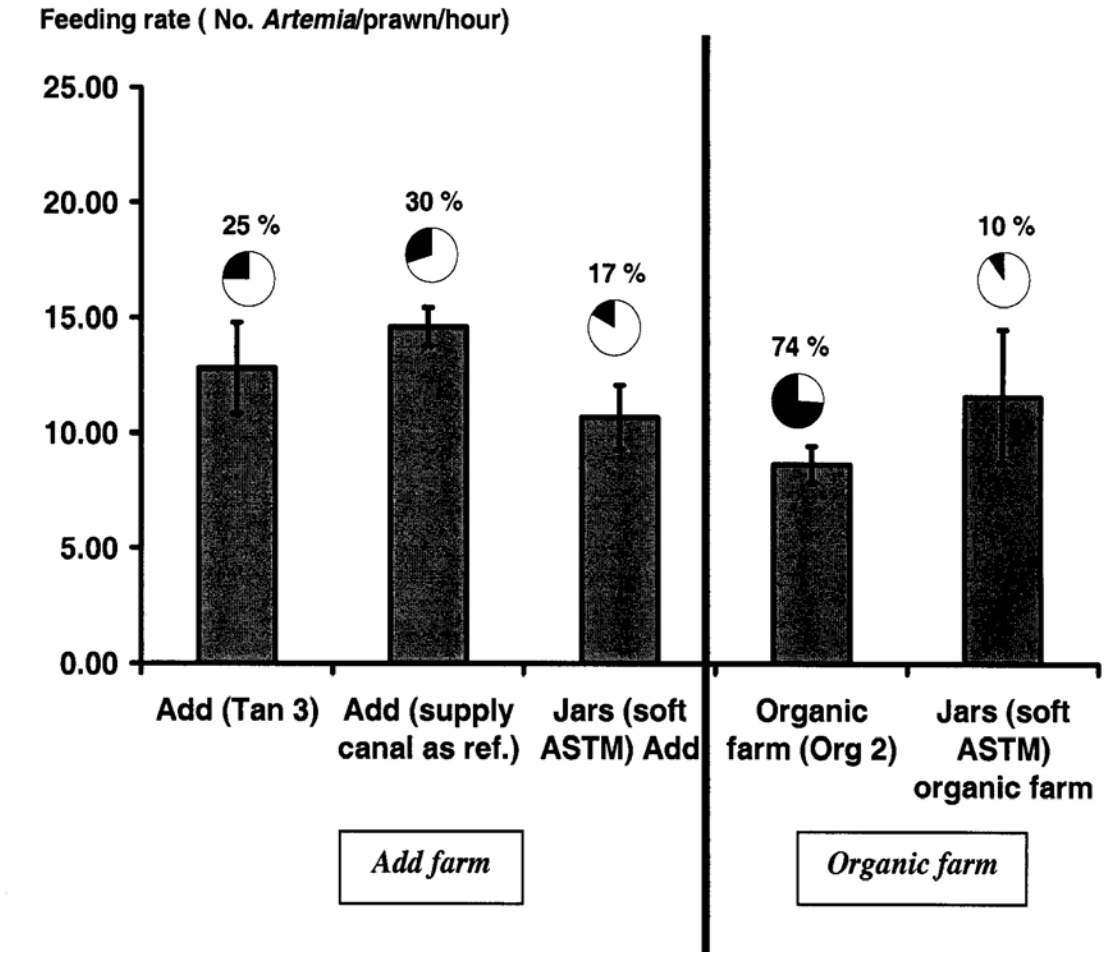


Figure 7.8(b) ***Bioassay 5:*** Mean individual post-exposure feeding rate in tangerine farm (Add farm : **Study site 2**) and organic farm (**Study site 3**) for 24 hours exposure period. Bars indicate mean post-exposure feeding rates in farm canal, supply canal and jar system. Vertical bars indicate standard errors. Pie charts and percentages present average mortality of animals during 24 hours exposure in farm canal, supply canal and in jar system.

7.7 Result of field deployment III

Bioassay 6:

6th deployment on 9 February 2005

A sixth deployment was done on 9 February 2005. Figure 7.9 presents the mean individual post-exposure feeding rates obtained in Chuchart farm(**Study site 1**) and the reference site (AIT canal : **Study site 4**). Mortality of prawns was high (80 %) at the reference site.

Mortality during exposure in the jar system was less than 30 %. Post-exposure feeding rates in jar systems at both AIT and Chuchart farm sites were not significantly different ($F_{1,15}$ = 0.43; P = 0.520). There was also no difference, when comparing post-exposure feeding rates of both jar systems with that obtained from the 2nd deployment in the organic farm ($F_{2,21}$ = 1.72; P = 0.203). However, the post-exposure feeding rate from the canal in Chuchart farm was significantly different from that of the 2nd deployment in the organic farm ($F_{1,15}$ = 18.49; P = 0.001). DO was high (9.9 mg/L) and % oxygen saturation was 140. Profenofos residue concentration in Chuchart farm canal was 29 µg/L (Figure 7.10).

Deployment III :

6th deployment on 9 February 2005

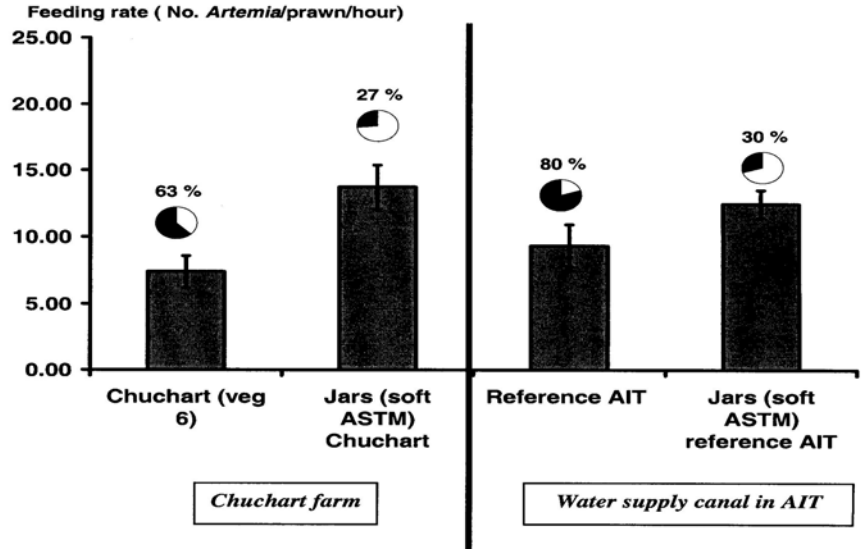


Figure 7.9 Mean individual post-exposure feeding rates in vegetable farm (Chuchart : Study site 1) and reference canal at AIT (Study site 4) for 24 hours. exposure period. Bars indicate mean post-exposure feeding rates in farm canal and jar system. Vertical bars indicate standard errors. Pie charts and percentages present average mortality of animals during 24 hours exposure in farm canal and jar system.

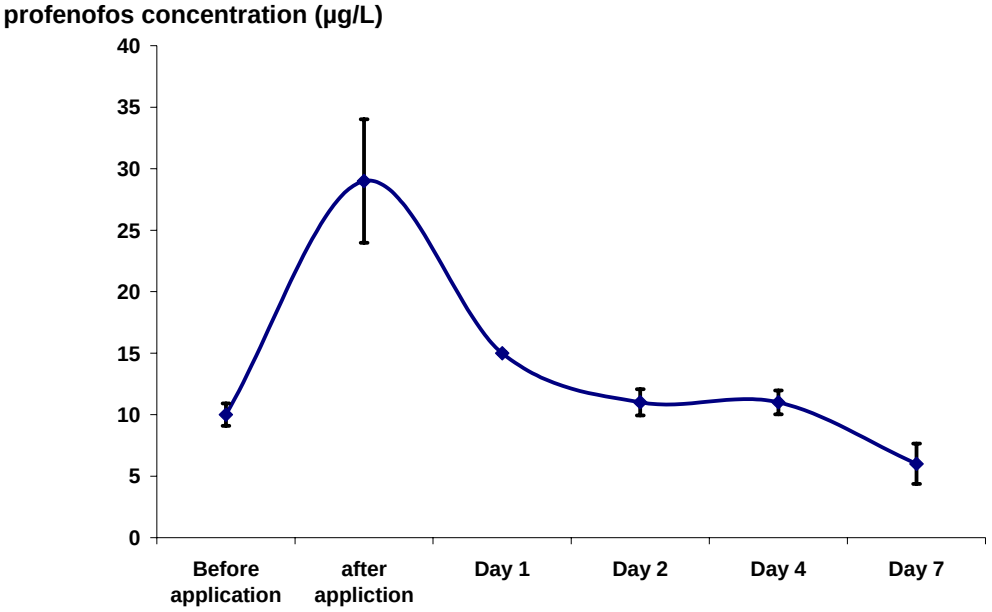


Figure 7.10 Profenofos concentration (µg/L) in the vegetable farm (Chuchart farm) before pesticide application and the day after application. Vertical bars indicate standard errors. (n = 12)

7.8 Result of field deployment IV

Bioassay 7:

7th deployment on 14 February 2005

A seventh deployment was done on 14 February 2005. Figure 7.11 (a) presents the mean individual post-exposure feeding rates in Samnieng farm (**Study site 1**) and the reference site (AIT canal : **Study site 4**). All prawns died in the canal at Samnieng farm while in the AIT reference canal 70% died. But mortality in the jar systems at both sites were lower than 20 %.

Post-exposure feeding rate in the jar systems at both AIT and Samnieng farm were not significantly different ($F_{1,14}$ = 1.44; P = 0.250). There was also no difference when compared with that of the 2nd deployment in organic farm ($F_{2,24}$ = 4.28; P = 0.028). There was also no difference between the post-exposure feeding rate of the AIT reference canal (Ref 1) and the 2nd deployment in organic farm ($F_{1,11}$ = 0.65 ; P = 0.436). As all test animals died in Samnieng farm, post-exposure feeding rates could not be measured. Carbendazim was diluted in the canal at 8.9 µg/L. The farmer at Samnieng farm also used other pesticides such as cypermethrin, parathion and methomyl which could also be present in the water at that time and caused the death of the test animals. Cypermethrin, parathion and methomyl were not analysed because they were not part of this study so no further conclusions could be drawn.

Bioassay 8:

8th deployment on 17 February 2005

The eighth deployment was done on 17 February 2005. Figure 7.11 (b) presents the mean individual post-exposure feeding rates in Samnieng (day 3) and at the reference site (AIT canal). The mortality of animals during exposure in Samnieng farm canal was 100 %. Carbendazim was detected at a low concentration (0.42 µg/L). Mortality of prawn at the reference site (AIT canal) was also high at 65 %. However, mortality in the jar systems at both sites were less, 3 % and 20 % in Samnieng farm and AIT laboratory at room temperature, respectively.

Post-exposure feeding in the jar system at Samnieng farm could not be calculated because all *Artemia* were consumed (before end of feeding period). No

difference was found between the post-exposure feeding rate in jar systems at AIT with that of the 2nd deployment in organic farm ($F_{1,7}$ = 2.17; P = 0.185). Likewise there was also no difference between the post-exposure feeding rate at AIT (H) (Ref 2) and that of the 2nd deployment in organic farm ($F_{1,11}$ = 0.58 ; P = 0.464).

The post-exposure feeding rates at the AIT reference canal on 14 and 17 February 2005 were not different from each other ($F_{1,10}$ = 0.07; P = 0.790). Also there was no difference when compared with that of the 2nd deployment in organic farm ($F_{2,16}$ = 0.49 ; P = 0.620). Post-exposure feeding rates in non-contaminated chemical sites (AIT) were consistent.

Bioassay 9:

9th deployment on 21 February 2005

A ninth deployment was carried out on 21 February 2005. This deployment was day 7 in Samnieng farm where the seventh deployment was conducted. Figure 7.11 (c) presents the mean individual post-exposure feeding rates in Samnieng farm (day 7) and at the reference site (AIT canal : **Study site 4**). Mortality of animals during exposure in Samnieng farm canal was 30 %. Carbendazim could not be detected in the canal water. However, at the reference site at the AIT canal, the mortality rate of prawns was 68 %. Mortality during exposure in the jar systems in both sites were 20 % and 17 % at Samnieng farm and AIT lab, respectively.

Post-exposure feeding rates in the reference site (AIT canal) and jar system in AIT but were not stignificantly different ($F_{2,17}$ = 5.09; P = 0.019) from that of the 2nd deployment in the organic farm. However, the feeding rates were significantly different ($F_{3,21}$ = 9.26; P < 0.001) when compared with that of the reference site (AIT canal) obtained on 14 and 17 February 2005. DO was low (1.8 mg/L) in the AIT canal, resulting in test animals becoming stressed leading to a mortality of 68 %.

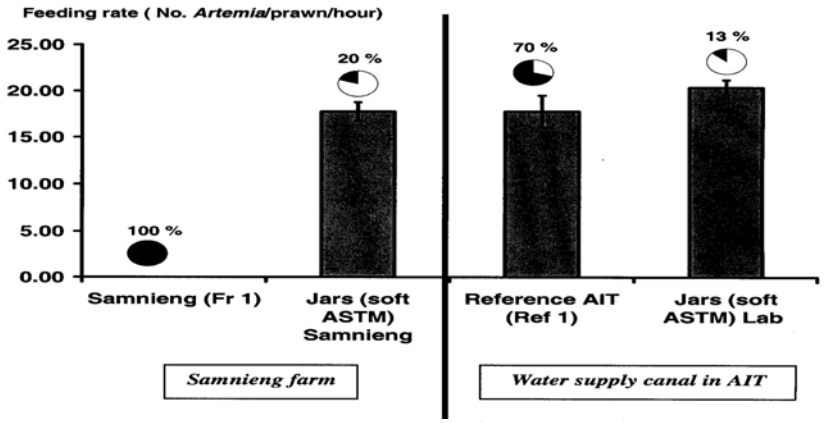
Post-exposure feeding rate in Samnieng canal was significantly different ($F_{2,17}$ = 7.0; P = 0.006) from that of the reference site (AIT canal) on 14 and 17 February 2005. When comparing post-exposure feeding rate in Samnieng canal in this deployment with that of the 2nd deployment in organic farm, there was no difference ($F_{1,13}$ = 5.52; P = 0.035). However, post-exposure feeding rate decreased which

confirmed that the farm canal still had some pesticide residue. Carbendazim concentration was not detected (Figure 7.12) but other pesticides (cypermethrin, parathion and methomyl) might still be present in the farm canal based on the observation and pesticide application from farm owner.

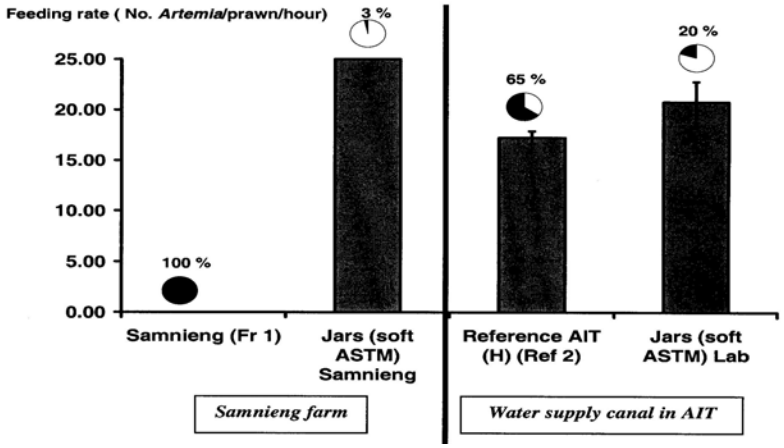
Carbendazim fungicide concentration was measured in the farm canal before and after application, as well as 1, 2, 3, 4, 5, 8 and 10 days after application. Figure 7.12 presents the changes in carbendazim concentration over time. Carbendazim was not detected in the farm canal before pesticide application. The farmer applied a mixture of carbendazim, cypermethrin, parathion and methonmyl to maximize time to work. After the mixed pesticide application, carbendazim was detected at 8.9 µg/L, decreasing to 3.65, 0.74 and 0.42 µg/L on days 1, 2 and 4, respectively. After day 4, no carbendazim was detected when analysis was done on days 5, 8 and 10 after application.

This deployment indicated that there was deterioration of pesticide in the water over time. It appeared that some test animals survived exposure because of this decline in pesticide levels. Remaining live animals from deployment in the Samnieng farm canal exhibited a significantly lower post-exposure feeding rate ($P < 0.05$).

7.11 (a)



7.11 (b)



7.11 (c)

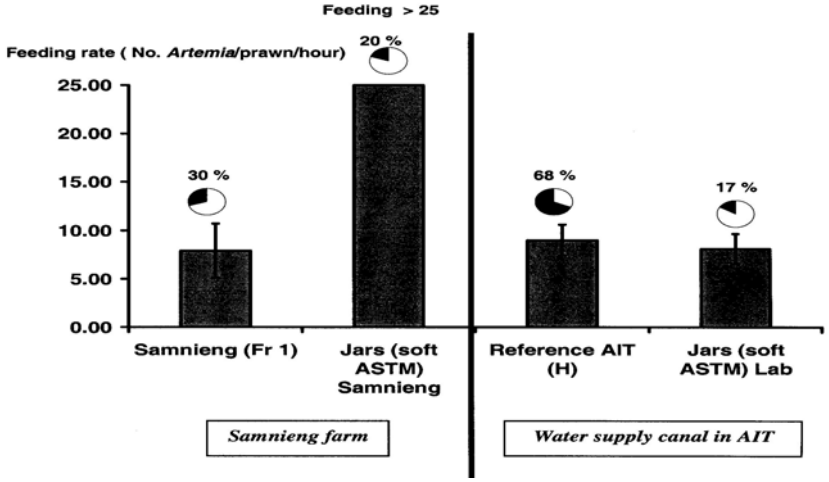


Figure 7.11 (a) ***Bioassay 7:*** Mean individual post-exposure feeding rate in fruit (guava) farm-day 1 (Samnieng farm : **Study site 1**) and reference canal in AIT (**Study site 4**) for 24 h. exposure period.

7.11 (b) ***Bioassay 8:*** Mean individual post-exposure feeding rate in fruit (guava) farm-day 4 (Samnieng farm : **Study site 1**) and reference canal in AIT (**Study site 4**) for 24 h. exposure period.

7.11 (c) ***Bioassay 9:*** Mean individual post-exposure feeding rate in fruit (guava) farm-day 7 (Samnieng farm : **Study site 1**) and reference canal in AIT (**Study site 4**) for 24 h. exposure period.

Bars indicate mean post-exposure feeding rate in farm canal and jar system.Vertical bars indicate standard errors. Percentages present average mortality of animals during 24 h exposure in farm canal and in jar system. (Note : Fr = Fruit farm)

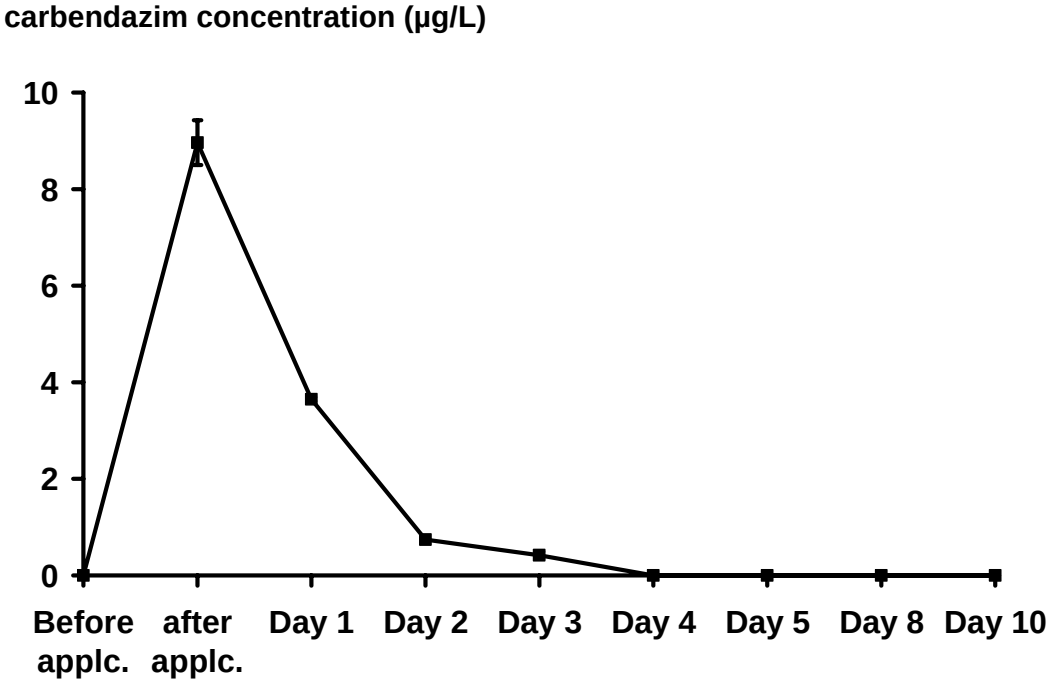


Figure 7.12 Carbendazim concentration (µg/L) in the Samnieng farm before and after application (n = 18). Vertical bars indicate standard error.

7.9 Result of jar systems as the reference in field deployment

At every deployment (there were 18 deployment sites), three glass jars (jar systems) were also set up to expose animals to soft ASTM for 24 hours for the same exposure time as in the field. Post-exposure feeding tests were then also conducted on prawns at the same time as prawns for the *in situ* bioassay.

The average post-exposure feeding rate from the glass jars was 13.52 ± 1.36 (*Artemia* per prawn per hour ± SE) (Figure 7.13). This feeding rate was not different from that of the 2nd field deployment in organic farm and 7th and 8th deployments in AIT canal ($P > 0.05$). Four sites were not included in the figure because *Artemia* were completely consumed before the end of the feeding time.

Mortality in the glass jars generally happened during deployment and 20 % mortality was considered an acceptable level. Only 2 samples (AIT 1 and Chuchart 2) failed this standard with mortalities of 30 and 26.7 %, respectively.

No significant correlation was observed between the mortality and the post-exposure feeding rate of *M. rosenbergii* deployed in jar systems (r = 0.158, P-value = 0.588, n = 14).

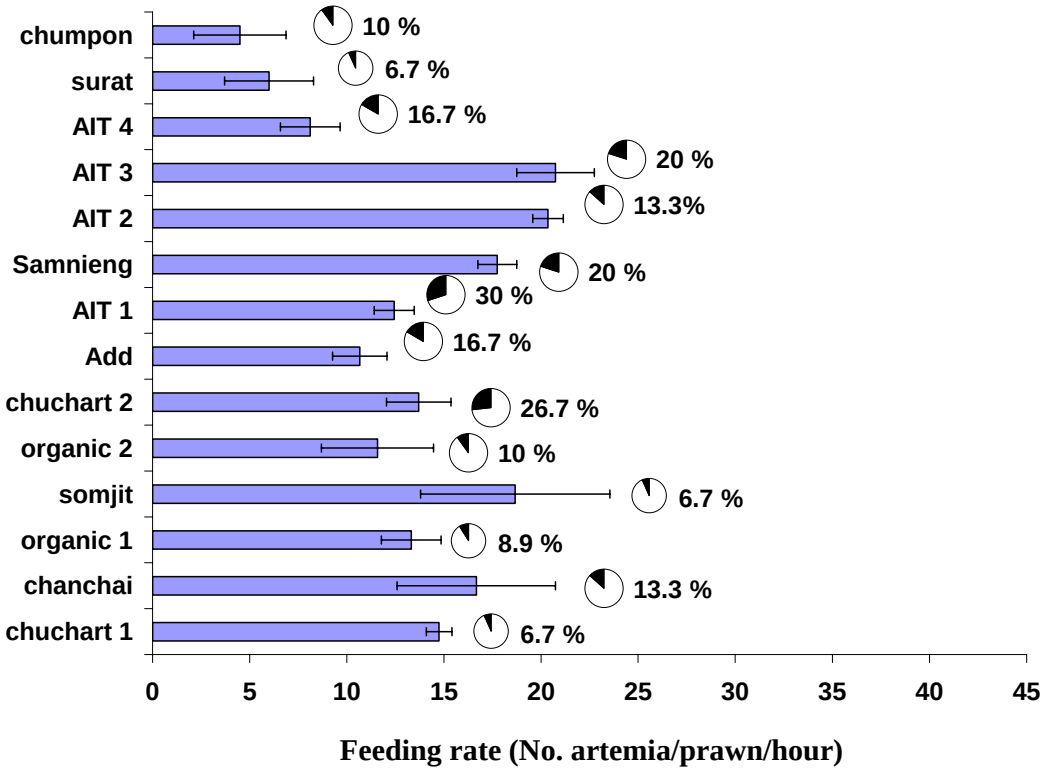


Figure 7.13 Mean individual post-exposure feeding rates in glass jars set near farm canals. Horizontal bars indicate standard errors. Pie charts and percentages present average mortality of animals during 24 hours exposure in soft ASTM jar systems.

7.10 Summary of results from all field deployments

The organic farm in bioassay 2 (deployment I) was used as a reference site as the farm had not used pesticides. The post-exposure feeding rate of prawns in the organic farm ranged from 14.17 to 17.47 *Artemia*/prawn/hour. This range was then used as a standard reference to compare and validate the post-exposure feeding performance in other sites. In addition of the AIT site, bioassays 7 and 8 was also used as reference as the area did not use any pesticide as well and pesticide analysis shown no residue pesticide (profenofos, dimethoate and chlorpyrifos). The post-exposure feeding rate obtained at the AIT site was 17.75 ± 1.72 and 17.25 ± 0.63 *Artemia*/prawn/hour which was not significantly different from that of the organic farm.

Figure 7.14 presents the post-exposure feeding rate from vegetable farms during the wet season (May to October). There was a significant difference between Chuchart (veg1) and Surat (veg 2) farms ($F_{2,16} = 11.73$; $P = 0.001$). The post-exposure feeding rate in the farm canal at Chanchai farm (veg3) was not different from that of the organic farm (ref) ($F_{1,10} = 1.98$; $P = 0.19$).

Figure 7.15 presents the post-exposure feeding rate from vegetable farms during the dry season (November to April). Chuchart (veg6) and Samnieng (Fr1) farms showed a significant difference compared to the reference ($F_{2,21} = 23.66$; $P < 0.001$). Profenofos (29 µg/L) was found in Chuchart farm canal. Samnieng farm had other insecticides (cypermethrin, methomyl and parathion) which could not be analysed in this study. This deployment was conducted in these farms during the same day that they applied pesticides.

Figures 7.16 and 7.17 present post-exposure feeding rates in the farm canals of tangerine farms during wet and dry seasons. No significant difference from the reference was found in both seasons ($F_{2.18} = 1.26$; $P = 0.310$ at wet season and $F_{2,21} = 2.57$; $P = 0.103$ at dry season).

There was no significant correlation between the post-exposure feeding rate of *M. rosenbergii* and the mortality deployed in the supply canals (r = 0.466, P-value = 0.351, n = 6).

There was also no significant negative correlation between post-exposure feeding rate of *M. rosenbergii* and mortality during exposure in organic farms and AIT canals (r = -0.593, P-value = 0.215, n = 6).

Finally there was no significant negative correlation between post-exposure feeding rate of *M. rosenbergii* and mortality during exposure in farm canals (r = -0.227, P-value = 0.527, n = 10).

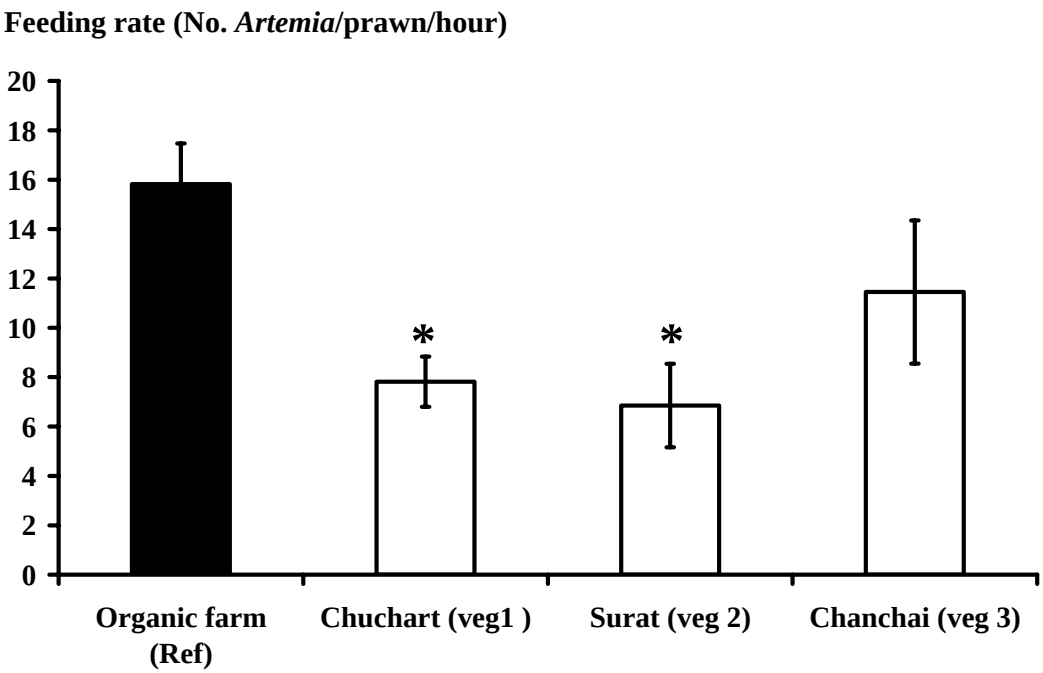


Figure 7.14 Post-exposure feeding rate of prawn after exposure in vegetable farm canals during wet season (May-October). Vertical bars indicate standard errors. Asterisks indicate post-exposure feeding rates that are significantly different from the reference ($P < 0.05$).

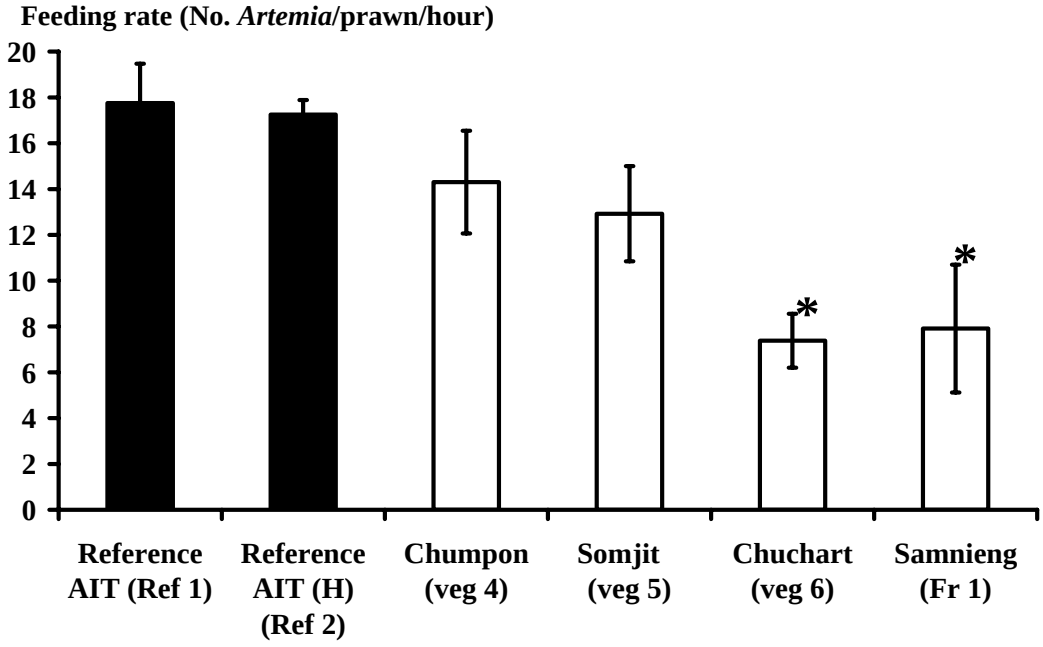


Figure 7.15 Post-exposure feeding rate of prawn after exposure in the vegetable farm canals during dry season (Nov-April). Vertical bars indicate standard errors. Asterisks indicate post-exposure feeding rates that are significantly different from the reference ($P < 0.05$).

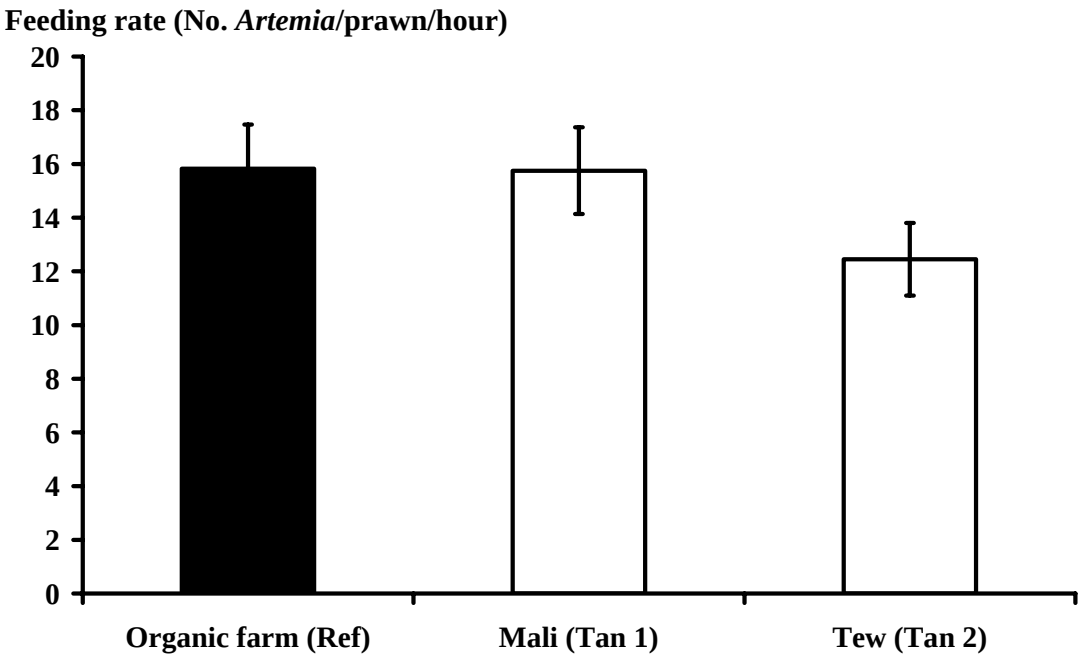


Figure 7.16 Post-exposure feeding rate of prawn after exposure in tangerine farm canals during wet season (May-October). Vertical bars indicate standard errors.

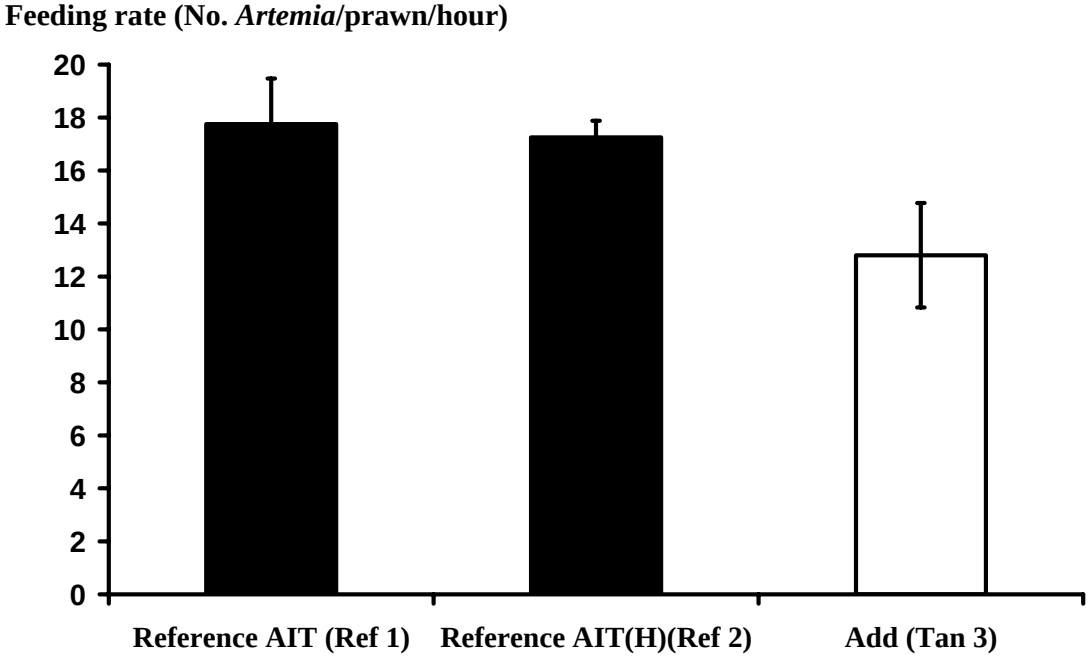


Figure 7.17 Post-exposure feeding rate of prawn after exposure in tangerine farm canals during dry season (Nov-April). Vertical bars indicate standard errors.

7.11 Discussion

The recovery and survival of the animals were important to determine the ability of the bioassay that can be conducted in the field. The size of post-larvae *M. rosenbergii* (9-10 mm.) was advantageous as they could be recovered and were easy to identify after the exposure period. It was not necessary to have more animals to compensate for loss of animals during the exposure period as is required for the *Daphnia magna* bioassay (McWilliam and Baird, 2002b) or other crustaceans which have a very small size. The size of the post-larvae also made their recovery in the chamber relatively easy, compared to smaller animals. If the animals are too small, it is very difficult to recover after exposure period.

The choice of a reference site was problematic. Results from a reference site are used to compare the inhibition in post-exposure feeding rate of organisms from exposed sites with those from uncontaminated sites. The water supply canals located near each farm was the first option considered for the reference sites. However, only two out of the eight sites had mortality less than 50 %. This suggested that such canals located near to tested farms are unsuitable as references sites. Dissolved oxygen tended to be very low in such canals and they are easily contaminated with pesticides from the surrounding farms as they receive effluent water.

An organic farm and AIT canals were considered as alternative reference sites and also had high mortality of animals after the exposure period. However, prawns remained to allow completion of the bioassay. Such high mortalities during the exposure might be expected to also affect the post-exposure feeding rate but the organic farm and AIT canals used were the best references identified within the limitations of the study. A justification for their validity as control was the results from the post-exposure feeding rate from organic farm and AIT canals being not significantly different from treatment controls in microcosm (Chapter 6) ($P < 0.05$). Microcosms guaranteed pesticide-free condition in control treatments. The post-exposure feeding rate from the control treatment in microcosms can therefore be used for validating the post-exposure feeding rate from reference sites.

The results of the field deployments revealed considerable variation in the mortality of test animals after exposure. While the presence of pesticides in the water resulted in mortality, it could not be discounted that environmental factors such as low DO could also have interacted to effect a higher mortality of test animals. But according to the correlation test, there were no significant correlations between mortality during exposure and post-exposure feeding rate. In contrast, mortality was low (9-20 %) in the jar systems (with clear soft ASTM) in the same sites. In the post-exposure feeding tests, clean and good quality water (soft ASTM) was used. The effect of environmental factors on the prawns may or may not persist or continue during post-exposure feeding tests. This warrants further study.

Post-exposure feeding rate proved to be effective in detecting effects of the pesticide at some sites. At Chuchart farm (Figure 7.9), the post-exposure feeding rate at the contaminated farm canal was significantly lower than that of the organic farm in bioassay 2/deployment I (Figure 7.7(b)) and AIT reference canal on bioassays 7 and 8 ($P < 0.05$). The contamination at Chuchart farm resulted from farmer pesticide application of profenofos. This was confirmed through observation and pesticide analysis. Profenofos concentration was detected at 29 µg/L during bioassay 6 in Chuchart farm canal, a level exceeding the EC_{50} and LC_{50} (19.8 µg/L and 11.6 µg/L, respectively) as reported in Chapter 5 (Table 5.2). The mortality levels during bioassay 6 at Chuchart farm canal was 63 % (Figure 7.9). The post-exposure feeding rate reduction of more than 40 % at Chuchart farm (veg 6) when compared to bioassay 2 in the organic farm, was also most likely explained by this measured exposure to profenofos.

The reduced post-exposure feeding rates measured were likely to result from the combination of the effects of pesticides and other factors (environmental) on the test animals. In real field situations, poor water quality conditions and contamination from substances such as pesticides could work together to stress the animals or even cause mortality. In this study, tangerine farms (Mali, Tew and Add) which had not used pesticides for many months showed lower post-exposure rates (12 *Artemia*/prawn/hour) than the reference, which could be attributed to other factors (environmental) (Figure 7.16 and 7.17). In vegetable farms such as Chuchart (veg1 and veg6) and Surat (veg2) which were using pesticides intensively, post-exposure

feeding rates decreased by more than 50 % compared to the reference (Figure 7.14 and 7.15). In McWilliam and Baird (2002b), low post-exposure feeding rates were caused by a combination of toxic effluents, high flow rates and suspended solids in a river where a wool processing mill and a sewage treatment plant emptied their wastes. Metals and acidity were also observed to cause feeding impairment and other behavioural change with small fish (Castro, *et al*, 2004).

In this study, in addition to pesticides, low dissolved oxygen (DO) contributed to the decrease in post-exposure feeding rate. Dissolved oxygen levels in most of the farm and supply canals were below the optimum level of 3 mg/L. The DO measurement was taken at 9 am and based on diurnal cycles, DO at these levels mean that night and pre-dawn DO levels will be lower (Boyd, 1990; 1998). As the prawns were left in the system for 24 hours, the low DO levels could also have affected their survival (in addition to the effect of pesticides). Field canals are shallow and when the water becomes eutrophic during the day, the phytoplankton at night and dawn will consume large amounts of oxygen, resulting in very low DO in the system (Boyd, 1990; 1998).

In general, low DO levels in the supply and farm canals may also be caused by the pumping of water high in total suspended solids and organic matter from the farms as well as into the farm canals. Water from the farms is pumped into supply canals to maintain water levels in the farm. Likewise, water from the outside irrigation canal is pumped into the farm canals when water levels are low, which occurs nearly everyday in the dry season (Van den Brink *et al*., 2003a). The canals are thick with mud and during pumping of water into these canals, the sediment is disturbed causing turbidity as well as lower DO levels especially when anaerobic layers of the sediment are disturbed (Boyd, 1990; 1998).

pH levels in some of the farms were not optimal for prawns (pH 7.0 – 8.0 as reported in Chapter 4), which ranged from 8.2 to 9.0 (Appendix 13). This could also have affected the post-exposure feeding rate of prawns in the field.

The *in situ M. rosenbergii* bioassay in this research could be considered as a preliminary study (laboratory methods) but needs more evaluation of the field

methods. The number of samples for the field study was small and insufficient to show the significance of major factors. An *in situ* evaluation study by Maltby *et. al.* (2002b) with *Gammarus pulex* involved setting up thirty-five deployments at 24 reference sites and 22 deployments at 15 contaminated sites over the time period. They also considered the seasonal effects on the *in situ* bioassay.

In the present work, the sensitivity of the post-exposure feeding response in *M. rosenbergii* postlarvae was demonstrated. But the *in situ M. rosenbergii* bioassay required access to an undisturbed/uncontaminated area to provide the baseline information. In an area of interconnected irrigation canals supplying intensive, commercial agriculture, such sites are difficult to identify. Seasonal differences were also likely to exacerbate such problems. Profenofos was detected in the farm canal (average 15.5 µg/L) of the reference sites (organic farm) during the dry season in this study.

The quality of the test organism may also have varied seasonally. In some deployments (bioassays 3, 8 and 9) *Artemia* were finished before the end of the post-exposure feeding test, suggesting stronger and more tolerant test animals at this time (Figure 7.13). Others have observed significant variations in the *in situ* feeding rate of *G. pulex* by season and over the time period (Maltby *et. al.*, 2002b).

Biological monitoring will still require chemical/physical monitoring (Cairns, 2005) to underpin interpretation of results. Furthermore, the concentration of any chemical in the environment and the toxicological responses at these concentrations provide the basis for an efficient and orderly process of hazard assessment. The limited frequency of monitoring in this study (9:00 a.m.) for environmental measurement may not have been enough to build up an understanding of the relationship between the effect of pesticides and environmental concentrations. Significant diurnal variation in key environmental variables occurs in tropical freshwater systems. Moreira, *et.al.* (2005) found the influence of temperature and salinity on the post-exposure feeding rate of *Hediste diversicolor*. Pereira, *et al.* (1999) mentioned that variables such as pH, conductivity, dissolved oxygen, particle size and organic matter content are important factors in toxicity effects measured in any environment. Further study is needed to point out specific factors that could affect

responses because isolating pesticide effects from other stressors could be difficult (Shaw and Manning, 1996).

Pesticide cocktails were another complication in these *in situ* trials. The common practice among the farmers in the area is to combine at least three compounds and use them in one pesticide application. In this study, cypermethrin and parathion were applied at the same time with carbendazim. Combination of pesticides can cause toxicological interaction and the effect of pesticides might be different from the response to each of the pesticide alone (Rand, 1995).

Time series in *in situ* bioassays (same site over time) is necessary in tropical bioassays. In this study, the presence of pesticide (applied on first day only) continued to affect prawns resulting in their mortality even four days after pesticide application (bioassays 7, 8 and 9). Results from time series studies in *in situ* bioassay could show the effect of pesticides over time (at the same site) and could be useful in analyzing the extent of pesticide effects on post-exposure feeding as well as in identifying the effects of environmental factors on prawn performance. This should also take into consideration the fluctuations in environmental conditions, water use and varied agricultural activities near the farms studied.

CHAPTER 8

GENERAL DISCUSSION AND IMPLICATIONS OF FINDINGS

8.1 Summary and implications of research findings

The purpose of this research was to develop and standardize a novel feeding bioassay with *Macrobrachium rosenbergii* for use in the laboratory and allowing it to be easily deployed under field conditions. Standardization of the test aimed to minimize feeding rate variation under clean (non-toxic) conditions and to ensure that subsequent statistical analyses had sufficient power to consistently detect changes in feeding rates. These were accomplished through the development of a post-exposure feeding toxicity test under laboratory (Chapters 4 and 5), microcosm (Chapter 6) and *in situ* or field (Chapter 7) conditions.

The tiered approach used in the preliminary risk assessment of pesticide using TOXSWA (Chapter 3) was capable of screening the risk level of pesticide in the study area, with the assumption that only a single pesticide was used in each area. As a result, profenofos and dimethoate were chosen as test chemicals for the lethal and sub-lethal experiments in Chapter 5. This model was beneficial in the preliminary assessment of the risk of pesticide use in the tropics. It was not necessary to set up laboratory work as this method can provide preliminary data to support the environmental planner and the decision/policy maker. This is an alternative way to develop a cost efficient model to inform and warn of the risk of pesticide use. However, the challenge for this kind of computer modeling is the “pesticide cocktail” or mixed pesticide prevalent among farmers especially in Thailand. The complexity of chemical reaction from a mixture of pesticides makes it difficult to study residues in the water.

In Chapter 4, methodological development of the bioassay and its optimization were described. All experiments were set up to determine variability inherent in parameters associated with the development of a *M. rosenbergii* bioassay. The

bioassay test procedure proved to be repeatable under laboratory conditions as all the experiments resulted in no significant differences from their controls. Test animals were readily available in terms of quantity and uniformity in sizes. Material effects (different batches of PLs from different brooders and spawnings) and seasonal effects might explain variability observed in post-exposure feeding rates in the controls at different times.

Chapter 4 also reported on the assessment of the effects of pH, temperature and hardness on control post-exposure feeding rates of *M. rosenbergii*. These assessments indicated that *M. rosenbergii* was very sensitive to acidic and basic conditions. This would cause difficulty when deploying the bioassay in areas with acidic and basic conditions as the effect on post-exposure feeding rates could not be attributed to pesticide effects alone.

The results in Chapter 5 demonstrated the use of post-exposure feeding inhibition as the endpoint under laboratory conditions. Carbendazim, chlorpyrifos, dimethoate, profenofos and zinc were the test chemicals used. The study included the recovery of *M. rosenbergii* after exposure to pesticide contamination using chlorpyrifos. The bioassay clearly showed the sensitivity of *M. rosenbergii* to pesticides and heavy metals. Post-exposure feeding rate inhibition could be used as a sublethal endpoint as the EC_{50} values obtained for chlorpyrifos and zinc were lower than their lethal levels. Mortality of prawn was also another endpoint used to define the toxicity of pesticides such as carbendazim, in which mortality occurred during exposure but post-exposure feeding rate of the surviving animals did not decrease.

The results obtained from the carbendazim microcosm experiment in Chapter 6 proved that this method was able to link the laboratory toxicity tests and the effects observed in the field. Microcosm studies are different from laboratory toxicity tests, where tests are conducted in synthetic water under conditions different from the field situation. Microcosm studies provide another dimension to studies looking at pesticide effects on aquatic systems. In this research, carbendazim affected feeding and survival rates in the microcosm set-up but in the laboratory only mortality showed a significant difference ($P < 0.05$). Some treatments in the microcosm showed mortality more than two times that of the control (Figure 5.1 (e)).

In situ bioassays were able to show the effects of pesticides on post-exposure feeding rates using the methods developed (Chapter 7). Post-exposure feeding rates were significantly lower than control in farms using pesticides while in uncontaminated sites (pesticide-free), the post-exposure feeding rates did not decrease. However, mortality was observed even in the uncontaminated sites which could be attributed to other factors such as low dissolved oxygen and presence of some other chemical substances. Therefore, the degree of mortality and the effect on feeding rates depends not only on the type and concentration of the known pesticide but also on water quality parameters. Since the water analysis concentrated only on specific pesticides, the presence of other chemicals or factors were not identified which made it difficult to validate causes of effects on post-exposure feeding rates. This implies the importance of thorough analysis of the sites but due to limited resources and analytical procedures, this could not be done during the research period. This is one of the challenges of *in situ* bioassays in contrast to laboratory bioassays where the degree of control of conditions is higher.

In terms of the selection of test species, based on the criteria by Rand et al. (1995) mentioned in Chapter 1, *M. rosenbergii* is a reliable species to use for bioassays and can be a representative species for many inland tropical areas. They are easily available and well distributed in the tropics. Their response during the tests conducted showed that they are sensitive to several factors such as pH, hardness, organophosphates and heavy metals. Since there has been other research conducted on prawn sensitivity to factors such as temperature, dissolved oxygen, ammonia, nitrite (Sarver et al., 1982; Hummel, 1986; Rogers and Fast, 1988; Strauss et al., 1991; Diaz-Barbosa, 1995; Chen and Kou, 1996; Nelson et al., 1997; New and Valenti, 2000), these were not studied in detail in this research. This species has been studied since 1965 resulting in a strong knowledge base and a well-developed production culture system, thus there is no problem with its availability and quality (New and Valenti, 2000).

Reliability of the post-exposure feeding bioassay was also addressed, where the influencing factors through the standardization of test conditions were investigated. The standardization of testing conditions for the laboratory-based test produced post-exposure feeding rates with coefficient of variation (CV) of 17.79 %

for freshwater prawn (*M. rosenbergii*) of sizes 9-10 mm, much lower than the 24 % and 23 % obtained by McWilliam (2001) and Crichton (2003) for *D. magna* and *Lymnaea peregra* (freshwater snail), respectively. Therefore, the *M. rosenbergii* post-exposure feeding bioassay could be a reliable test as it had less variability. However, if the aim is to decrease the CV further, then more work on the sources of variability in baseline rates needs to be conducted. As discussed in detail in Chapter 4, increasing the number of replicates could reduce the CV further and reach the 0.8 power level, thereby achieving a 20% difference between means of the post-exposure feeding rate.

Time required to complete the *in situ* bioassay was only 4 days, but required intensive work during this period. Preparation and collection of results of the bioassay required 7 days to complete in both the laboratory and *in situ*. Expertise and high knowledge of chemistry are not required to follow the test either in the laboratory or in the field except for the chemical solution preparation, provided that procedures and materials are available and clearly understood.

The basic methods developed for *in situ* bioassay from this research is a simple, easy and fast way to determine the effect of pesticides because the results can be seen in the field compared to the traditional and costly way of analysing pesticide, i.e. taking water samples from the field and bringing them to the laboratory for analysis.

This research study can be considered a preliminary study in tropical ecotoxicology. The procedures developed and results obtained from this study can be used as a basis for further toxicity studies on *M. rosenbergii* and other potential tropical species in the laboratory.

8.2 Recommendations for further study

During the research period, a number of issues for further study arose.

- Investigation into the effect of pesticides on feeding activity of prawns during exposure period. This would provide a better analysis of post-exposure feeding performance. Two possibilities may occur to cause an effect on post-exposure feeding rate as suggested by Taylor *et al*. (1998) i.e. animals have weakened due to starvation or the animals may reject the food.
- The number of replicates for post-exposure feeding may need to be increased to 16 to reduce variation in individual response (as explained in section 4.4.3). Crane *et al.* (2000) used 30 individual measurement for *Gammarus pulex*.
- Alternative test food for *M. rosenbergii* post-larvae (9-10 mm), i.e. instead of *Artemia.* Ease in calculating the quantity of feed before and after feeding should be the main criterion.
- Further studies should be done on the combined effects of pesticides and environmental factors such as dissolved oxygen, pH, temperature, water flow and suspended solids on the survival during exposure and post-exposure feeding rates of *M. rosenbergii*. This should include developing methods in areas with poor water quality conditions.
- A wider range of chemicals specifically pesticides should be studied on their lethal and sublethal effects on *M. rosenbergii*
- *M. rosenbergii* should be assessed together with other species for bioassays by deploying simultaneously with longer exposure times. It will give more accuracy in assessing species-specific effects of pesticides at different trophic levels.
- Perform bioassays under more controlled conditions using water collected from the field and filtered to remove items that would affect feeding behaviour of test organisms. Results should be compared with *in situ* bioassays.
- Study fate processes of pesticides in the tropics including the validation of the predicted exposure concentration by TOXSWA with field observations and analysis.

8.3 Critique of the methodology

Post-exposure feeding tests done using individual instead of groups of prawns was a good method due to the problem of cannibalism and aggressive behaviour. Counting of *Artemia* before and after feeding, although labour-intensive, was easier in the long-run when compared with powdered feed which would involve a more complex process of calculating remaining feed (extracting from the water by filtration, drying and weighing).

Methods of pesticide analysis are complex and difficult especially for organophosphates. The lack of sample preparation procedures for pesticide analysis made it difficult for a thorough analysis of water samples. Pesticide analysis also requires expensive equipment and chemicals which is a constraint in countries with poor facilities. Oftentimes research institutions may have the equipment but it is used for other analysis such as for food properties and will not allow pesticide analysis to be done on the same equipment for fear of contamination.

Lack of trained staff to handle equipment for pesticide analysis (if available) hinders analytical work when problems with machines come up as they could not be resolved quickly.

Most of the analysis being done in Thailand is on organochlorine which is more persistent and are easily detected by machines but less persistent chemicals in the water such as organophosphates are difficult to detect and the machines available have detection limits of 0.1 µg/L.

A more detailed water quality analysis before and during deployments should have been conducted at all sites to provide a better analysis of the field situation in *in situ* studies. However this was not done during this research because of time constraints.

Pesticide use is a sensitive topic among farmers and the researcher needs to establish good rapport with them to gain their trust to be able to discuss pesticide

issues and be given permission to use their farms as trial sites. Communication between researcher and farmers is important. The researcher has to be flexible to changes in schedules and activities in the farm study sites even though this could also affect the research schedule and material preparation especially when an experiment has to be set-up.

8.4 Contribution of the thesis

Many organic chemicals in tropical areas still need to be studied so the information obtained can create a database to improve the regulation of pesticide use in tropical systems. The pesticide regulations currently used in tropical countries are still based on regulations developed by temperate countries and according to their requirements (especially for importation of products from the tropics). In a review by Lacher and Goldstein (1997), it was mentioned that ecotoxicology studies are focused mostly on temperate ecosystems and the tropical ecosystems which contain 75 % of global diversity have been largely ignored.

The results of this research using *M. rosenbergii* is a step towards meeting the need for more studies on tropical species working towards the development of policy and regulations on pesticide. It is expected that published outputs from this thesis research and the MAMAS project will be able to reach sectors of the government and institutions to campaign for more awareness about agrochemical use in the tropics. The aim is to give a choice to farmers to either reduce pesticide use in farms to safe levels or to stop using them altogether. Incentives for those following these policies could be given by the government in the form of market or health incentives and certifications (such as healthy product approval).

A closer collaboration among agencies in each tropical country will enable a concerted effort when lobbying the government to give more attention to the effects of pesticides on the population and the environment and specifically on aquatic systems. These countries, through regional collaborations, can share experiences and expertise on existing methods to build towards more cost-effective methods of monitoring pesticide use and effects.

Multidisciplinary research is something new among researchers and other professionals in many countries, and especially in Thailand. During the course of this work, the researcher had to contact different agencies and individuals from many disciplines. There is potential for cooperation among all these sectors to be able to integrate the results, avoid overlap and double spending that will not waste effort and time and lastly more relevant results can cover many aspects and disciplines.

Most farmers in developing countries such as Thailand believe that their survival comes first and they do not have any concern for the environment as they need to produce more for the lowest cost (Farmer Chuchart S., personal communication). However, there are signs of growing awareness among farmers and other stakeholders regarding issues related to pesticide use and safety, although information is difficult to obtain as not enough research is being done due to lack of funding and low prioritization (MAMAS, 2003).

It is hoped that through this research study, a breakthrough in laboratory and field research as well as in policy making and pesticide regulation will happen not only in Thailand but also in the whole region.

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Appendix 1

Satapornvanit, K., Baird D.J., Little D.C., Milwain, G.K., Van den Brink, P.J., Beltman, W.H.J., Nogueira, A.J.A., Daam, M.A., Domingues, I., Kodithuwakku, S.S., Perera, M.W.P, Yakupitiyage, A., Sureshkumar, S.N. and Taylor, G.J. (2004). Risk of pesticide use in aquatic ecosystems adjacent to mixed vegetable and monocrop fruit growing areas in Thailand. Australasian Journal of Ecotoxicology, 10:85-95.

RISKS OF PESTICIDE USE IN AQUATIC ECOSYSTEMS ADJACENT TO MIXED VEGETABLE AND MONOCROP FRUIT GROWING AREAS IN THAILAND

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ABSTRACT

A preliminary ecological risk assessment approach was used to determine the potential ecological consequences of pesticide use for aquatic systems in Thailand. Using a two-tiered approach, data from participatory community appraisals and household surveys were employed to assess exposure risk. Two sites representing monocrop (tangerine) and mixed-crop (vegetables and fruit) systems were studied. In the first tier the Predicted Environmental Concentration (PEC) is based on an 'extreme worst case' loading scenario. The PECs were compared with No Effect Concentrations (NECs) calculated from results of laboratory tests performed with standard test species. In the second tier, the TOXSWA model (TOXic substances in Surface WAters) was used to simulate pesticide fate for estimation of the PEC. The PEC:NEC ratios indicated that there is an extremely high risk that pesticide use will adversely affect the environment. The PECs of nearly all pesticides were higher than their corresponding NEC, some by several orders of magnitude. The highest PEC:NEC ratios were calculated for the insecticides Mevinphos and Carbaryl (mixed-crop and monocrop areas, respectively). The highest risks were associated with the rose apple and tangerine crops. The results of this study should provide useful information for stakeholders and policy-makers to increase awareness of the potential seriousness of this problem, and to prioritise action to avoid serious problems in the future.

Key words: ecological risk assessment, pesticides, environment, developing countries, tropics.

INTRODUCTION

Nearly two-thirds of the world's population live in Asia, and many countries in that region are currently undergoing very rapid industrial, agricultural and economic development. Changes induced by these developments, such as increases in water-use and pollution loads, are reducing the availability of high quality fresh water, which is an essential resource sustaining human activity and is critical in the maintenance of biodiversity (Lebel 1996).

An important reason for the increase in pesticide use in Asian countries has been the shift of farmers from traditional subsistence farming towards market-oriented intensive-crop farming. This increase has been driven by an increasing demand for vegetables and other crops, caused by urbanisation and economic development (Midmore and Janssen 2003). Capable of growing two or three crops a year, these countries are becoming the future "breadbaskets" for the world, exporting agricultural produce to regions having colder climates and shorter growing seasons (Ecobichon 2001). Given the increased rate of cropping, environmental side-effects of pesticide use are likely to be more severe in developing countries than in the developed world, even where regulatory guidelines are enforced. In addition, the general knowledge and public awareness of the scale and importance of such problems is limited (Yudelman *et al.* 1998), and as a consequence, environmental regulations are 'imported' from the developed world, to function in systems and environments which differ significantly from those where the guidelines were derived.

Thailand is no exception to the above, and in recent years has seen pesticide use increase significantly. All pesticide products are imported into Thailand since no pesticide factories exist in the country. These pesticides are diluted, reformulated or re-packed for the retail markets. The quantity of pesticides imported during the first quarter of 2003 (11,268 tons) was nearly the same amount that was imported for the whole year in 1985 (12,832 tons) (Anon. 2003).

The economic downturn from 1997 to 2000 compelled farmers to use cheaper but more hazardous pesticides, instead of using more costly but less toxic and hazardous products. In addition, the current authorized registration system for pesticides is oversimplified, and does not encourage full engagement with the risk assessment process (Ecobichon 2001; Thapinta and Hudak

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2000). This has encouraged more entrepreneurs to engage in the agrochemical business. These conditions have resulted in an increased use of more hazardous pesticides, leading to an increase in the number of people reportedly affected by pesticide poisoning (Thapinta and Hudak 2000).

Another consequence of increased pesticide use, particularly in the rural areas, has been environmental degradation such as pollution and contamination of the soil, water and air (Thapinta and Hudak 2000). Besides having detrimental effects on the biodiversity and ecological stability of aquatic ecosystems, the residues in water and other environmental compartments may cause illnesses or disabilities, forcing the public and the government to bear increased health care costs. The extent of the problem is, however, difficult to assess.

Ideally, when assessing the ecological risks of a new pesticide, one investigates its fate and effects under realistic field conditions, taking into account good agricultural practices and the spatial and temporal variability of the ecosystems potentially under stress. The time, costs and logistics necessary for this approach, however, makes it impossible to evaluate all active ingredients and formulated products in this way. Thus, a tiered approach has been adopted, as has been employed previously in Europe (EU 1991, 1997).

Pesticides may enter surface waters via spray drift, runoff, drainage or atmospheric deposition. In this preliminary risk assessment only entry via spray drift is taken into account. For the other entry routes additional data on soils and field practices are needed, data which are not yet available. Furthermore, spray drift is considered to be one of the main entry routes causing peak concentrations in surface waters. Point sources, e.g. via spills, are not considered in the risk assessment because it is assumed that good agricultural practice is followed. Nevertheless in reality, point sources are a major risk to be considered.

The first tier of aquatic risk assessment is based on the estimation of a PEC/NEC (Predicted Environmental Concentration/No Effect Concentration) ratio. In this ratio, the calculated concentration of the pesticide in surface water (PEC) is compared to the expected "safe" concentration (NEC). If the PEC does not exceed the NEC, the pesticide is not expected to have an effect on the aquatic ecosystem. The first tier PEC is calculated with the help of a simplified standard scenario for a freshwater system on the basis of the recommended dose used for pest control and the expected drift percentage (see e.g. FOCUS 2001). The NEC is based on concentration-effect relationships studied in the laboratory with a limited number of "standard" species, viz., an alga, macrophytes, waterflea (*Daphnia*) and fish. These test procedures have been adapted as standard protocols and are well described in, for instance, OECD (Organisation for Economic Co-operation and Development) guidelines (OECD 1993). To protect sensitive indigenous aquatic populations, the NEC is usually calculated by multiplying the toxicity value of the most sensitive standard test species by an assessment factor (EU 1997).

In the first tier, the PEC is based on an 'extreme worst case loading' scenario. If, based on this PEC compared with the NEC, the use is considered safe, no further risk assessment is required. If, however, the result indicates that use is not safe, it is necessary to do a second tier assessment, which accounts for the application pattern and for the dissipation processes in the water body.

This paper is a result of a project assessing the risks of pesticide use to aquatic life and the human health via dietary exposure for Thailand and Sri Lanka. This preliminary study used a tiered approach in assessing the risks of pesticide applications to the aquatic environment. The risk assessment calculations used a realistic worst-case approach, which can ultimately be followed by more detailed assessments where risks are indicated. The preliminary ecological risk assessment performed within this project was presented in report form by Van den Brink *et al.* (2003), the results of the participatory community appraisals and household interviews are summarised in Little *et al.* (2003). This paper aims to present the risk assessment performed for Thailand in greater detail and to make the findings of the study available to the wider scientific community.

MATERIALS AND METHODS

Study Sites

Based on the diversity of agricultural systems in Central Thailand, two sites were selected, namely, a monocrop (tangerine) site in Salakru sub-district, Nong Sua District, Pathumthani province, and a more diverse multi-crop vegetable and fruit-growing site in Kokprajedee sub-district, Nakornchaisri district, Nakhon Pathom province. Three villages from each study site were selected for both participatory community appraisals and household surveys. Twenty households in each village were interviewed to obtain more information, i.e. 60 households in each study site.

In the monocrop study site, village selection was based on the villages' high production levels of tangerines within the district, whereas in the mixed crop study site, village selection was based on the intensive production of a wide variety of fruit and vegetables in the area and the use of canals and ponds as a source of food fish through fishing and aquaculture.

In the recent past, rice was the main crop in both areas. In Salakru, commercial tangerine farming only became established in the last two decades but now forms the vast majority of highly intensive agricultural production in this area. Recently, however, an unidentified disease has been placing significant constraints on tangerine production. Fish production, as culture in both ponds and cage-in-canal systems has appeared in recent years as a significant activity for some, and fishing in farm canals, sub-canals and main canals remains important among most households for meeting consumption needs (Little *et al.* 2003). Some other aquatic plants and animals are also collected from local canals for home consumption (Van den Brink *et al.* 2003). In both study sites, the water systems adjacent to the crop production areas are used intensively for food production, indicating the possibility that ecological effects due to pesticide use may be present. To ensure the ecological integrity of these surface waters, and thus maintain fish and macrophyte production of these waters, we performed an ecological risk assessment of pesticide use in these two study sites in terms of potential impacts on the adjacent aquatic environment.

First tier PEC calculation

This involved a preliminary estimation of the risks posed by each pesticide to the aquatic environment. The total quantity of each pesticide applied on each crop during the crop cycle was used to calculate a first tier PEC, which allowed the identification of pesticide-crop combinations that could produce the highest risks. It is calculated using the following equation:

$$PEC\ (\mu g/L) = \frac{\text{Dose x Number of applications x (Drift percentage/100) x 0.1}}{\text{Volume of water body}}$$

Where,

Dose (g a.i./ha): The loading of active ingredients (a.i.) for one application is calculated from the applied dose per hectare of the formulated product accounting for the percentage of active ingredient of the pesticide product.

Number of applications: The highest number of applications in a crop during its crop cycle was gathered from the household surveys. A crop cycle of 45 days was assumed for the vegetable crops and for fruit in mixed-crop site, while for the monocrop site, a crop cycle of 28 to 182 days was used depending on crop type.

Drift percentage (%): This is the percentage of the pesticide dose (as active ingredient) applied to 1 m^2 of soil which deposits on 1 m^2 of surface water. The assumptions used to estimate this percentage depended on whether the pesticide was applied from a boat or by knapsack spray. Applications by boat are carried out from the boats used for irrigation of the crops. These boats sail through the irrigation ditches spraying water behind them in a fan. The pesticide is diluted with the irrigation water, and the resulting spray pattern falls equally on both the water and the crops on the adjacent strips of land. Based on these observations a drift percentage of 100% is assumed for the boat applications.

***Table 1.** Parameter values of the water body defined to calculate 2nd tier PECs with TOXSWA.*

Parameter	Dimension	Value
Water body		
Dimensions	m	1 x 1
Slope	-	0
Water depth	m	0.50
Concentration of suspended solids	g/m^3	50
Mass ratio organic matter suspended solids	-	0.5
Macrophytes	g D.W./m^2	0
Flow velocity	m/d	0
Sediment		
Thickness	m	0.05
Bulk density	kg/m^3	800
Porosity	-	0.5
Tortuosity*	-	0.5
Mass ratio organic matter	-	0.085
Temperature (water and sediment)	°C	33

* factor that corrects diffusion in the sediment for increased path length through the pores in the system.

The crops are grown directly next to the watercourse, so when the pesticide is sprayed with a knapsack it is expected that water directly next to soil does receive the same mass of pesticide as the soil. The width of the watercourse is between 1 and 2 m. Assuming that both sides of the watercourse are sprayed it is estimated that the average pesticide deposition via spraydrift is 30% with the knapsack method. The percentage is divided by 100 to obtain a fraction.

0.1: This factor is added to convert from g/ha to mg/m^2.

Volume of water body (m^3): This is the volume of water which the pesticide enters, based on a realistic worst-case value of 0.5 m depth of a ditch in the ditch-dike system in the dry season, and waterbody dimensions of 1 m by 1 m the volume is 0.5 m^3. This water volume represents the water volume adjacent to 1 m edge of cropped soil.

Second tier PEC calculation

If the first-tier PEC/NEC calculations indicated a risk for a certain pesticide-crop combination, a second-tier PEC was calculated to obtain a more realistic estimate of the concentrations of this pesticide in the surface water adjacent to the defined crop. The first tier PEC was calculated assuming all loadings entered instantaneously into the water body, causing a peak concentration which was the PEC. In calculating the second-tier PEC, a more realistic application scheme was considered and the processes that determine the fate of the pesticide in surface water were taken into account. The difference between the first-tier PEC and the second-tier PEC was due to the dissipation processes between applications, resulting in lower PECs. The second tier PEC was the concentration present after the last application, including residues left from earlier applications. The TOXSWA model (Adriaanse *et al.* 2003; Beltman and Adriaanse 1999) was used to calculate the second tier PEC.

Second-tier PEC calculations for risk assessment involved the input of data on pesticide use, pesticide properties and selected environmental parameters to the TOXSWA model. For each pesticide-crop combinations the combination revealing the highest PEC values from the first-tier assessment were selected for the second-tier PEC calculation.

For this calculation, the dry season case was chosen to represent the worst-case scenario, since at that time pesticide concentrations are likely to be highest because the canal water is shallow and replenishment is minimal. The scenario parameters used in TOXSWA are given in Table 1. The chemical parameter values of the pesticides used for the calculations are given in Table 2. The drift deposition on the water surface in mg/m^2 for each application is the dose (g a.i./ha) multiplied by the drift percentage and the factor 1/10 to convert from g/ha to mg/m^2.

The number of applications and the time interval between applications were extracted from the household surveys (see Tables 3 and 4). A period of 45 days for vegetable and fruit in the mixed-crop site and a maximum period of 180 days in the dry season for the monocrops were simulated. These are the periods during which the pesticides are applied.

Table 2. Pesticides used in the study areas with their type of use and their physico-chemical properties. The temperatures (T) at which the saturated vapour pressure, solubility and transformation half-lives (DT50-water, DT50-sediment) were measured are needed to correct to the temperature in the scenario. The physico-chemical properties were used to calculate the second tier PECs (Predicted Environmental Concentrations).

Active ingredient	Pesticide type	Molecular mass	Saturated vapour pressure (mPa)	T saturated vapour pressure (°C)	Solubility (g/L)	T solubility (°C)	DT50-water (days)	DT50-sediment (days)	K_{om}* (L/kg)
Abamectin	Insecticide. acaricide	873.1	$2{\cdot}1{\cdot}10^{-7}$	22.5	0.005	20	56	d.v.	2860
Captan	Fungicide	300.61	$1.1{\cdot}10^{-5}$	25	0.0051	22.5	1	1	75
Carbaryl	Insecticide, plant growth regulator	201.23	$1.6{\cdot}10^{-4}$	24	0.12	30	14	14	34
Carbendazim	Fungicide	191.19	$6.5{\cdot}10^{-8}$	20	0.008	20	90	d.v.	76
Carbofuran	Insecticide, nematocide	221.25	$8.0{\cdot}10^{-5}$	22.5	0.351	25	50	50	13
Carbosulfan	Insecticide	380.5	$4.1{\cdot}10^{-5}$	25	$3{\cdot}10^{-4}$	25	7.6	d.v.	d.v.
Chlorfenapyr	Insecticide, acaricide	407.6	d.v.	20	1.0	20	d.v.	d.v.	d.v.
Chlorfluazuron	Insecticide	540.7	$1.0{\cdot}10^{-8}$	20	$1{\cdot}10^{-5}$	20	42	d.v.	d.v.
Cypermethrin	Insecticide	416.3	$1.9{\cdot}10^{-7}$	20	$4{\cdot}10^{-6}$	20	14	d.v.	2137
Dicrotophos	Insecticide, acaricide	237.2	$9.3{\cdot}10^{-3}$	20	1.0	20	20	20	d.v.
Difenoconazole	Fungicide	406.3	$3.3{\cdot}10^{-8}$	25	0.015	25	145	d.v.	d.v.
Diflubenzuron	Insecticide	310.69	$1.2{\cdot}10^{-7}$	25	$8{\cdot}10^{-5}$	20	10	d.v.	104
Dimethoate	Insecticide, acaricide	229.2	$1.1{\cdot}10^{-3}$	25	23.8	20	21	d.v.	17
EPN	Insecticide, acaricide	323.3	$4.1{\cdot}10^{-5}$	23	$9.2{\cdot}10^{-7}$	24	15	d.v.	96 700
Fipronil	Insecticide	437.2	$3.7{\cdot}10^{-7}$	25	0.0019	25	28	d.v.	d.v.
Glyphosate-isopropylammonium	Herbicide	169.1	1.0	25	12	25	30	d.v.	3200
Malathion	Insecticide, acaricide	330.3	$5.3{\cdot}10^{-3}$	30	0.145	25	1	d.v.	d.v.
Mancozeb	Fungicide	330	1.0	25	0.006	25	70	d.v.	1143
Metalaxyl	Fungicide	279.3	$7.5{\cdot}10^{-4}$	25	8.4	22	56	d.v.	27
Methamidophos	Insecticide, acaricide	141.1	$2.3{\cdot}10^{-3}$	20	200	20	23.5	d.v.	5
Methomyl	Insecticide, acaricide	162.2	$6.7{\cdot}10^{-3}$	25	58	25	30	d.v.	12
Mevinphos	Insecticide, acaricide	224.15	$1.7{\cdot}10^{-2}$	20	600	22.5	20.5	d.v.	17
Profenofos	Insecticide, acaricide	373.6	$1.24{\cdot}10^{-4}$	25	0.028	25	8	d.v.	13 965
Propineb	Fungicide	289.8	0.0001	22.5	0.01	20	1	d.v.	d.v.
Prothiofos	Insecticide	345.2	$6.0{\cdot}10^{-4}$	22.5	$7{\cdot}10^{-5}$	20	280	d.v.	d.v.
Tetradifon	Acaricide	356	$3.2{\cdot}10^{-8}$	20	$7.8{\cdot}10^{-5}$	20	52	d.v.	455
Zineb	Fungicide	275.8	$1.0{\cdot}10^{-5}$	20	0.01	22.5	37	d.v.	571

d.v.: default value used because data are not available; ie. Psat = $1.0{\cdot}10^{-15}$ mPa, DT50-sediment = 10 000 d, K_{om} = 1000 L/kg

* K_{om}, sorption coefficient for organic matter, can be used for sorption to suspended solids and for sorption to sediment.

Table 3. *Pesticides used in the mixed crop site, with their application patterns assessed from household surveys, and also the recommended dosages obtained from labels and references The 2nd tier PECs (Predicted Environmental Concentrations) have been calculated with TOXSWA using the recommended dosages, the scenario from Table 1, and the pesticide properties from Table 2. The recommended dose and the maximal dose from the household survey are both shown for comparison. The NEC (No Effect Concentration) is derived via the method described in paper. The PEC/NEC ratio is calculated from the PEC calculated with the recommended dose (rec) and the NEC given in the table.*

Pesticide	Crop	Recommended dose (g a.i./ha).	Max. dose from household survey (g a.i./ha)	Application interval (days)	Number of applications	Spray drift (%)	PEC (rec) (µg/L)	NEC (µg/L)	PEC/NEC (rec)
Mevinphos	Drumstick moringa	60	533	15	3	100	18.6	0.0019	9763
Cypermethrin	Rose apple	14	100	7	6	100	3.5	0.0012	2926
Profenofos	Lettuce	94	375	3	15	100	26.9	0.011	2444
Malathion	Guava	156	5390	7	6	100	30.4	0.018	1688
Chlorfluazuron	For all crops	13	17	5	9	100	8.8	0.0091	964
Dimethoate	Rose apple	50	2261	7	6	100	21.6	0.026	831
Chlorfenapyr	Amaranth	25	60	5	9	100	35.6	0.061	583
Methomyl	Chinese kale	88	579	5	9	100	36.4	0.080	455
Abamectin	Lettuce	2.3	14	3	15	100	1.4	0.0034	402
Carbosulfan	Rose apple	25	101	7	6	100	5.9	0.015	393
Carbendazim	Lettuce	188	281	3	15	100	124.7	0.56	223
Mancozeb	Rose apple	250	101	7	6	100	48.6	0.22	221
Methamidophos	Rose apple	150	565	7	6	100	69.9	0.33	212
Prothiofos	Guava	94	893	10	5	100	18.3	0.14	131
Difenoconazole	For all crops	31	88	7	6	100	28.1	0.50	56
Diflubenzuron	Rose apple	70	100	10	5	100	16.8	0.33	51
Metalaxyl	For all crops	3125	1097	7	6	100	2391.0	120	20
Glyphosate-isopropylammonium	For all crops	750	2892	7	6	30	327.0	32	10
Fipronil	Amaranth	6.3	30	5	9	100	3.9	0.46	9
Dicrotophos	Chinese kale	62	1432	5	9	100	23.0	3.4	7
Propineb	Drumstick moringa	263	136	15	3	100	51.2	19	3

Note : Glyphosate-isopropylammonium was applied by knapsack spraying.

Table 4. *Pesticides used in the monocrop site, with their application patterns assessed from household surveys, and also the recommended dosages obtained from labels and references. The 2nd tier PECs (Predicted Environmental Concentrations) have been calculated with TOXSWA using the recommended dosages, the scenario from Table 1, and the pesticide properties from Table 2. The recommended dose and the maximal dose from the household survey are both shown for comparison. The NEC (No Effect Concentration) is derived via the method described in paper. The PEC/NEC ratio is calculated from the PEC calculated with the recommended dose (rec) and the NEC given in the table*

Pesticide	Crop	Recommended dose (g a.i./ha.)	Max. dose, from household survey (g a.i./ha)	Application interval (days)	Number of applications	Spray drift (%)	PEC (rec) (µg/L)	NEC (µg/L)	PEC/NEC (rec)
Carbaryl	Tangerine	159	27	15	4	100	36.9	0.0042	8793
EPN	Longbean	84	260	7	9	100	4.9	0.0010	4955
Cypermethrin	Tangerine	7.7	188	7	4	100	2.2	0.0012	1869
Profenofos	Tangerine	94	52	7	13	100	14.5	0.011	1317
Dimethoate	Tangerine	50	2674	7	26	100	13.4	0.026	517
Methomyl	Tangerine	88	347	10	6	100	37.8	0.080	472
Abamectin	Tangerine	2.3	9.3	7	26	100	1.4	0.0034	402
Carbendazim	Tangerine	188	5639	7	4	100	189.0	0.56	338
Methamidophos	Tangerine	150	622	7	17	100	86.8	0.33	263
Mancozeb	Tangerine	250	600	7	9	100	48.6	0.22	221
Zineb	Tangerine	400	3889	10	18	100	198.6	2.0	99
Captan	Tangerine	156	97	7	26	100	32.5	1.3	24
Dicrotophos	Tangerine	62	165	7	4	100	22.4	3.4	7
Tetradifon	Tangerine	19	147	10	12	100	10.7	9.0	1

The DT50 values used to quantify transformation in the simulations with TOXSWA model were those given in Tomlin (1997, 2000) and Linders *et al.* (1994). When available, the DT50 of a water-sediment study was used, but if unavailable, the hydrolysis rate or degradation rate in water at pH 7 were used. As a last choice the DT50 of soil was used. If still unavailable, the DT50 value for water and for sediment was set at 10 000 days, which means that transformation was assumed not to occur. Using the molar enthalpy for transformation, the transformation rate was corrected for the temperature considered in the scenario. An activation energy for transformation of 55 000 J/mole was used for all pesticides (Beltman and Adriaanse 1999).

The sorption coefficient K_{om} for suspended solids and for sediment were both taken from the sorption coefficient for soil according to Tomlin (1997, 2000) and Linders *et al.* (1994). If not available the K_{om} was calculated from the K_{oc} via $K_{om} = 1.724 / K_{oc}$ (FOCUS 2001).

If neither K_{om} nor K_{oc} were available, a default value of 1,000 L/kg was used. The Freundlich exponent for the nonlinear sorption isotherm was set at the default value of 0.9 (Calvet *et al.* 1980).

The molar mass, saturated gas pressure and solubility are needed to calculate the volatilisation of the pesticide from the water compartment. These properties were obtained from Tomlin (1997, 2000) and Linders *et al.* (1994). If the references did not have a value for saturated gas pressure, the value was set at $1 \cdot 10^{-15}$ mPa. The temperatures at which the saturated gas pressure and solubility were measured were also required. With these temperatures and the molar enthalpies, the saturated gas pressure and the solubility could be calculated for the temperature in the scenario. For the molar enthalpy of vaporization the default value of 95,000 J/mole was used (Beltman and Adriaanse 1999). For the molar enthalpy of solution, the default value of 27,000 J/mole was used (Beltman and Adriaanse 1999).

All calculations were carried out for all combinations of crops and pesticides recorded in the household surveys, using the number of applications and the time interval between applications, but only for the combinations that resulted in the highest PEC, because all PEC:NEC ratios were higher than 1. The recommended dose was used for calculation of the PEC instead of the actual dose that was obtained from the household surveys. By doing so the results of the ecological risk assessment evaluates the risks of normal pesticide use for all crop-pesticide combinations. If using actual doses from the household interviews, risks are biased to specific locations, farmers and/or specific times, such as when certain pests were locally abundant in the year that the households were interviewed.

No Effect Concentration (NEC)

The preliminary ecological risk assessment is based on the estimation of a PEC/NEC ratio. For the calculation of the ratio, the second tier PECs were calculated according to the procedures described above.

The calculation of the NEC was based on existing laboratory toxicity data (LC50/EC50/NOECs) gathered for a limited number of 'standard species': including algae, macrophytes, *Daphnia* and fish. These species were chosen because of their ease in handling

Table 5. Relevant endpoints employed in laboratory toxicity tests and their duration.

Species group	Toxicity measure	Relevant endpoints	Relevant duration of test (days)
Daphnia / fish	EC50	Mortality Behaviour	1 — 4
	NOEC	see EC50 + Reproduction	> 4
Macrophytes	EC50	Growth Population	2 — 14
	NOEC	see EC50	
Algae	EC50	Growth Population	1 — 4
	NOEC	see EC50	1 — 4

Table 6. Assessment factors for extrapolation of toxicity values in standard test species to give the No Effect Concentration (NEC) (EU 1997)

Short term exposure
0.01 x acute EC50 fish, *Daphnia*
0.1 x acute EC50 algae and macrophyte
Long-term exposure
0.1 x chronic NOEC fish, *Daphnia*, algae and macrophyte

and rearing in the laboratory. A list of standard test species was derived from OECD (1993); this list was used to search the AQUIRE data base (US-EPA 2002) for relevant toxicity values. Only toxicity data from laboratory tests were taken into account. Furthermore, values obtained from laboratory tests with non-reported endpoints or endpoints that were not considered relevant were excluded, including toxicity values from laboratory tests with a duration that was considered out of range. Table 5 lists relevant endpoints and duration for laboratory toxicity tests.

When more than one EC50 or NOEC value was found for the same species, the geometric mean was calculated. If no EC50 or NOEC data were available for any of the three species groups, the database of the RIVM was used (De Zwart 2002). If toxicity values were still missing for the most relevant species groups for a certain pesticide (i.e. insecticide and acaricide: *Daphnia*, fish; herbicide and plant growth regulator: algae and macrophytes; fungicide: *Daphnia*, fish, algae and macrophytes), the Pesticide Manual (Tomlin 2000) was checked. For all pesticides, a NOEC or EC50 value was obtained from at least one species within the most relevant species group(s). Because of the uncertainties associated with the extrapolation from one species to another and to protect sensitive indigenous aquatic populations, the NEC was calculated by multiplying the toxicity value by an assessment factor (Table 6). After application of the assessment factors, the lowest NEC for each pesticide was taken.

RESULTS AND DISCUSSION

The first tier PECs calculated with the recommended doses combined with the NECs showed that for all 241 pesticide-crop combinations the PEC/NEC ratio exceeded 1 (from 1.8 to 25575). For each pesticide-crop combination, the one that yielded the highest PECs in the first-tier calculation was subjected to the second-tier PEC calculation. Combined with the NECs the 36 pesticide-crop combinations that resulted in PEC/NEC ratios exceeding 1, are presented in Table 3 and 4. The tables also show the maximal dosages applied by farmers from the household surveys.

The environmental preliminary risk assessment indicates that significant effects of pesticide exposure could be expected on aquatic life. The predicted risks are so large that it is expected that the species inhabiting the local aquatic ecosystem present in the farm channels are highly resilient to high pesticide exposure or have a high potential to reinvade the systems rapidly.

The top ten pesticides in the mixed crop site all belonged to the insecticide group. These chemicals not only affect insect pests (the 'target organisms') but also kill non-target insects and crustaceans present in the water system, e.g. low concentrations of Mevinphos (1.46 µg/L) can kill *Daphnia magna* (FAO 1998). The largest exceedence of the NEC is calculated for the use of Mevinphos in the drumstick moringa crop. To evaluate the potential consequences of the predicted exposure level to the indigenous ecosystem more realistically, the PERPEST model was employed (Van den Brink *et al.* 2002). The PERPEST model predicts the ecological risks posed by herbicides and insecticides in freshwater ecosystems by using relevant (toxicity) characteristics of the compound and the results of all microcosm and mesocosm experiments published in the open literature (see Brock *et al.* 2000a,b for a review). PERPEST searches for analogous situations in the database allowing the model to predict effects of pesticides for which no effects on a semi-field scale have been published (Van den Brink *et al.* 2002). Using PERPEST, for the drumstick moringa crop, the probability of observing a clear effect of Mevinphos on insects and crustaceans was calculated to be between 96 and 100%, on other invertebrates and fish between 50 and 74% and 45% for primary producers. The ten pesticides showing highest risk in the mixed crop site were all insecticides. The low NEC for these pesticides is a consequence of their high toxicity to their target group - insects - and other

arthropods, especially *Daphnia*. The fungicides exhibit higher NEC values because their target group of organisms (fungi) do not have a sensitive counterpart in the risk assessment "ecosystem" consisting of algae, macrophyte, *Daphnia* and fish (Van den Brink *et al.* 2000). The rose apple crop was associated with the highest risks, although for lettuce, guava, amaranth, Chinese kale and drumstick moringa high risks were also indicated.

Of the ten pesticides showing highest risk in the monocrop site, eight were insecticides and two were fungicides. Again, using the PERPEST model to evaluate the insecticide Carbaryl which showed the highest PEC/NEC ratio, its PEC was predicted to result in a 72 to 90% probability of clear effects on insects and crustaceans, a 33 to 37% probability for other invertebrates and fish and a 62% probability of clear effects on primary producers. From the data it was clear that tangerine farming as currently practised within the Salakru area poses significantly high risks to aquatic life in surrounding habitats, especially when multi-substance exposure is considered.

In this risk analysis, the dose recommended by the company or extension service or used for the registration of the pesticide in the local market, was used. Through this approach, the variations between years and between farmers did not influence the results of the risk assessment of pesticide. The PEC/NEC results based on the recommended dose which were compared with the doses given by respondents in the household surveys to evaluate the degree of overuse. Tables 3 and 4 show that the recommended doses were invariably lower than actual doses used by farmers (from household surveys) except for Mancozeb, Metalaxyl and Propineb in the mixed crop site and Carbaryl, Profenofos and Captan in the monocrop site. The quantity of pesticides used by farmers greatly exceeded the recommended amount, thereby leading to a situation of pesticide overdose. On average, the maximum dose was 8.2 times the recommended dose for the mixed crop sites and 9.8 times for the monocrop site. Overuse is a known problem for Thailand; Jungbluth (2000) found indications for pesticide overuse in citrus production areas in central Thailand. Similarly, Jungbluth (1996) reported that about half of the farmers apply higher than recommended concentrations and do not observe recommended intervals between spraying and harvest. Small animals in the food chain such as fish may already have been affected by the overuse of pesticides in agriculture (Girard 1994). Since the relationship between the dosages and the PEC/NEC ratios was approximately linear, the exceedence of the NECs as indicated in the last columns of Table 3 and 4 was in most cases a gross underestimation of risk when compared to that posed from actual dosages applied at the two study sites. The results of the tiered PRA approach indicated that several pesticides had the potential to have an impact on non-target species within the aquatic ecosystem. Pookpakdi (1995) stated that there is a growing concern in Thailand regarding the undesirable effect of chemical pesticides on non-target species along with the gradual development of resistant pest species and the adverse effect of chemicals on human beings and the ecosystem.

In this preliminary ecological risk assessment only the spray drift entry route was considered, yet runoff and drainage entries will also contribute to pesticide loadings in surface waters. Those entry routes would likely further elevate the PECs obtained for the water bodies studied here. Spray drift percentages in the study areas are often over 10 times higher than in European situations (FOCUS 2001). These high drift percentages are due to the more intensive use of farm land, with no use of buffer strips. The crops are grown as close as possible to the ditch, which is also close to the water surface for the purpose of watering. Thus, mitigation measures that are more appropriate for local circumstances should be explored to reduce spray drift entries into the surface water.

The scenario used here was a realistic worst case. It assumed that in the dry season the water in the ditches was not replenished during the whole crop cycle, leading to the accumulation of pesticides in the system. Furthermore, the organic matter content of suspended solids sediments within the system may have been underestimated. Although the organic matter content does not affect the concentrations in water strongly (Westein *et al.* 1998), for pesticides with high sorption coefficients it would lower the exposure concentrations somewhat. Therefore it is it necessary to collect data to further refine the scenario to local conditions.

Another major source of uncertainty was the fact that pesticide properties used for this assessment were obtained from databases originating from Europe and North-America. It is questionable whether these are representative for tropical conditions. It is possible that the breakdown of pesticides may be different under warmer and more eutrophic conditions compared to the water-sediment system used to establish the degradation rates for registration purposes in Europe. The same can be argued regarding the toxicity of the chemicals towards tropical species although Maltby *et al.* (2004) could not demonstrate differences in sensitivity between temperate and tropical species for a few pesticides (chlorpyrifos, fenitrothion and carbofuran). Brock *et al.* (2000a,b) also found that there were no systematic differences in threshold levels derived from semi-field experiments conducted in temperate and warmer conditions although temperature may considerably influence the extent and types of secondary effects if safe threshold levels were exceeded (Van Wijngaarden *et al.* 2003). Therefore it is important that more studies be done to determine the differences in the fate and effects of pesticides under temperate and tropical conditions.

The NEC calculation in the first tier of the risk assessment procedure is often considered conservative because of the limited number of species tested and the lack of ecological realism (Brock *et al.* 2000a,b). Therefore, if the first tier indicates potential risks, European guidelines offer the possibility to include more ecologically relevant data in a higher tier risk assessment procedure. Possible concepts and tools that can be used to calculate second tier NECs are the Species Sensitivity Distribution (SSD) concept (Posthuma *et al.* 2002), effect models like PERPEST (Van den Brink *et al.* 2002) or results from semi-field experiments (Brock *et al.* 2000a,b). The SSD concept is based on the assumption that the sensitivities of a set of species can be described in a statistical distribution (Posthuma *et al.* 2002). The available ecotoxicological data for all species tested are considered as a sample from this distribution and are used to estimate the parameters of the SSD. The variance in sensitivity

among the test species and the mean are used to calculate a concentration expected to protect most species (eg. 95%). This concentration (HC5, Hazardous Concentration 5%) is supposed to prevent effects on ecosystems and is validated for a limited number of compounds (Maltby *et al.* accepted 2004). For future assessments it is recommended to use the SSD concept and the PERPEST model in order to obtain more realistic NECs for each pesticide. It must be noted that the second tier NEC is not always found to be higher compared to the first tier one, and that only an increase up to two orders of magnitude can maximally be expected (Van den Brink *et al.* 2003) which would not eliminate the risks indicated by this assessment.

High risks of pesticide use are indicated for the aquatic environment adjacent to crops at both study sites. It is, however, essential for a true estimation of risks, that the results from this preliminary ecological risk assessment are validated using chemical measurements, bioassays and biomonitoring (the TRIAD approach, Chapman 2000). For instance, the predicted effects are so large that it is to be expected that the species inhabiting the local aquatic ecosystem present in the farm channels and tanks are highly resilient to high pesticide exposure or have a high potential to reinvade the systems rapidly. This could be verified by biomonitoring, which should be carried out under the same circumstances as the defined scenarios, .g. under zero water flow. By monitoring, it should therefore be possible to gain insight into realistic worst case conditions, eg. what is a realistic low value for replenishment of the water bodies? In other words, are the two scenarios defined for the two study sites truly representative of the Thai situation? By performing chemical analysis, a degree of validation of the PECs as calculated with TOXSWA using these scenarios can be obtained. In this way a more refined risk assessment can be carried out after the monitoring programme. On the other hand, possible effects indicated by the risk assessment can be validated using bioassays and biomonitoring.

The risks of pesticides to the biodiversity of fresh water as indicated by this study may lead to a reduced resilience to cope with other stressors linked to urbanisation and other consequences of global change. Pesticides will contribute to species loss (Van Wijngaarden *et al.* 2004), changes in food webs (Baird *et al.* 2001; Traas *et al.* 2004) and, as a result, to an increase of algal biomass (Van den Brink *et al.* 1997). In Asia, including Thailand, surface water is often used as drinking water for livestock, as process water for industries, as a resource for fishing and also for human and household consumption. Thus a decline in water quality has significant negative consequences for aquatic life in surface waters, and also on the working and living conditions of poor rural and peri-urban people. Poor people are disproportionately affected by any deterioration in the environment (Maxwell 1999), and it is therefore important for ensuring the future availability of clean water in Asia to predict the effects of intensified agriculture on the biodiversity and quality of fresh water. This should be done using an interdisciplinary approach by assessing the use of pesticides and its effects on aquatic ecosystems, and linking the results obtained to sound environmental policies aimed at reducing the use and effects of pesticides. Social science approaches must be used to understand farmers' intentions and motivations for the use of pesticides in their systems, to assess the societal and economic functions of water, and to understand and evaluate the effectiveness of existing and designed policy regimes and programmes on the system.

For a more integrated risk assessment of pesticides, the risks of pesticide use towards other environmental compartments like the terrestrial environment and groundwater and towards humans via dietary exposure should also be considered (Van den Bosch *et al.* 2004). The first can be done by including models such as PEARL (Leistra *et al.* 2001), which describe the fate of pesticides in soil, into risk assessment schemes. This will permit the calculation of the concentrations of pesticides in topsoil and subsequent leaching to groundwater. Moreover, it will also allow the risks to non-target invertebrates in the topsoil and the risks associated with using the groundwater as drinking water to be properly assessed. Van den Brink *et al.* (2003) present a first set-up to calculate the risks associated with drinking surface water adjacent to the crops and eating fish and macrophytes raised in these waters. This was done by multiplying the concentrations calculated for these food items with a typical Asian diet as set by the WHO (2003). By comparing the resulting estimated daily intakes with the acute reference dose and acceptable daily intakes (Van Raaij 2001) as set by the JMPR reports as published on their web-site (FAO 2002) the risk assessment was performed analogous to the PEC/NEC approach. This approach could be improved by using the more realistic concentration in the groundwater as input for exposure due to drinking water and including residues on crops for estimating exposure due to vegetable consumption

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Appendix 2: Second tier PEC / NEC risk quotients for crop-pesticide combinations in Thailand (recommended dosages)

mixed crop

Pesticide name	Crop name	PEC/1st tier NEC
Mevinphos	Drumstick Moringa (Ma rum)	9763
Cypermethrin	Roseapple	2926
Profenofos	Lettuce (Pak kad horm)	2444
Malathion	Guava	1688
Chlorfluazuron	For all crops	964
Dimethoate	Roseapple	831
Chlorfenapyr	Amaranth (Pak Khom)	583
Methomyl	Chinese kale (Ka na)	455
Abamectin	Lettuce (Pak kad horm)	402
Carbosulfan	Roseapple	393
Cabendazim	Lettuce (Pak kad horm)	223
Mancozeb	Roseapple	221
Methamidophos	Roseapple	212
Prothiofos	Guava	131
Difenoconazole	For all crops	56
Diflubenzuron	Roseapple	51
Metalaxyl	For all crops	20
Glyphosate-isophopylammonium	For all crops	10
Fipronil	Amaranth (Pak Khom)	9
Dicrotophos	Chinese kale (Ka na)	7
Propineb	Drumstick Moringa (Ma rum)	3

mono-crop

Pesticide name	Crop name	PEC/1st tier NEC
Carbaryl	Tangerine	8793
EPN	Longbean	4955
Cypermethrin	Tangerine	1869
Carbofuran	Coconut	1556
Profenofos	Tangerine	1317
Dimethoate	Tangerine	517
Methomyl	Tangerine	472
Abamectin	Tangerine	402
Carbendazim	Tangerine	338
Methamidophos	Tangerine	263
Mancozeb	Tangerine	221
Zineb	Tangerine	99
Captan	Tangerine	24
Dicrotophos	Tangerine	7
Tetradifon	Tangerine	1

Appendix 3: First and second tier PEC's for crop-pesticide combinations (application dosages)

PEC's of crop-pesticide combinations are based on dosages collected in household surveys in Thailand. The second tier PEC is only calculated for pesticide-crop combination yielding the highest first-tier PEC.

mixed crop site : application dosages

Pesticide name	Crop name	Load g a.i./ha.	Application Interval days	Number of applications	Spray drift -	1st tier PEC µg/L	2nd tier PEC µg/L
Abamectin	Chinese leek(Kui chai)	9.1	7	6	1	11	
Abamectin	Chinese leek(Kui chai)	12.5	7	6	1	15	
Abamectin	For all crops	12.7	7	6	1	15	
Abamectin	Amaranth (Pak Khom)	10.9	5	9	1	20	
Abamectin	Chinese cabbage (Kwang tung)	9.0	3	15	1	27	
Abamectin	Lettuce (Pak kad horm)	13.5	3	15	1	41	
Abamectin	Chinese kale (Ka na)	18.0	3	15	1	54	
Abamectin	For all crops	78.9	7	6	1	95	
Abamectin	Chinese kale (Ka na)	208.3	5	9	1	375	195
Carbendazim	Holy basil	68.5	14	3	1	41	
Carbendazim	Chinese cabbage (Kwang tung)	187.5	3	15	1	563	
Carbendazim	Roseapple	500.0	7	6	1	600	
Carbendazim	Lettuce (Pak kad horm)	281.3	3	15	1	844	
Carbendazim	Roseapple	753.3	7	6	1	904	
Carbendazim	Chinese kale (Ka na)	375.0	3	15	1	1125	734
Carbendazim	Guava	992.1	10	5	1	992	
Carbendazim	Guava	1250.0	10	5	1	1250	841
Carbendazim	Roseapple	95.5	7	6	1	115	
Carbosulfan	Roseapple	100.8	7	6	1	121	24.0
Chlorfenapyr	For all crops	48.4	7	6	0.3	17	
Chlorfenapyr	For all crops	70.7	7	6	1	85	
Chlorfenapyr	Amaranth (Pak Khom)	60.4	5	9	1	109	99
Chlorfluazuron	Lettuce (Pak kad horm)	10.6	24	2	1	4.2	
Chlorfluazuron	For all crops	16.7	5	9	1	30	14
Cypermethrin	Roseapple	19.1	7	6	1	23	
Cypermethrin	Roseapple	100.0	10	5	1	100	26
Dicrotophos	For all crops	233.4	7	6	1	280	
Dicrotophos	For all crops	1157.9	7	6	1	1389	
Dicrotophos	Chinese kale (Ka na)	1432.3	5	9	1	2578	740
Difenoconazole	For all crops	88.4	7	6	1	106	80
Diflubenzuron	Roseapple	100.0	10	5	1	100	24
Dimethoate	Roseapple	2260.8	7	6	1	2713	984
Fipronil	Amaranth (Pak Khom)	30.2	5	9	1	54	20
Glyphosate	For all crops	2891.6	7	6	0.3	1041	428
Glyphosate	Grass	393.4	183	1	0.3	24	
Glyphosate	Grass	761.9	365	1	0.3	46	
Glyphosate	Grass	1326.7	243	1	1	265	
Lannate (methomyl)	Drumstick Moringa (Ma rum)	133.3	15	3	1	80	
Lannate (methomyl)	Chinese kale (Ka na)	578.7	5	9	1	1042	
Mancozeb	Lettuce (Pak kad horm)	50.8	24	2	1	20	
Mancozeb	Chinese leek(Kui chai)	60.9	7	6	1	73	

Pesticide name	Crop name	Load g a.i./ha.	Application Interval days	Number of applications	Spray drift -	1st tier PEC µg/L	2nd tier PEC µg/L
Mancozeb	Roseapple	100.8	7	6	1	121	61
Mancozeb	Amaranth (Pak Khom)	72.4	5	9	1	130	60
Malathion	Guava	5389.6	7	6	1	6468	1067
Metalaxyl	For all crops	1096.5	7	6	1	1316	788
Methamidophos	Holy basil	205.5	14	3	1	123	
Methamidophos	angled gourd	480.0	7	6	0.3	173	
Methamidophos	Roseapple	229.2	7	6	1	275	
Methamidophos	Roseapple	565.2	7	6	1	678	
Methamidophos	Roseapple	800.0	10	5	1	800	
Methamidophos	Guava	3600.0	10	5	1	3600	
Methamidophos	Guava	3896.1	7	6	1	4675	
Methamidophos	Roseapple	24107.1	7	6	1	28929	11040
Methomyl	For all crops	116.3	7	6	0.1	14	
Methomyl	Roseapple	20.2	7	6	1	24	
Methomyl	Guava	82.8	10	5	1	83	
Methomyl	Roseapple	160.0	10	5	1	160	
Methomyl	Guava	396.8	10	5	1	397	
Methomyl	Roseapple	400.0	7	6	1	480	
Methomyl	Guava	500.0	10	5	1	500	
Mevinphos	Drumstick Moringa (Ma rum)	533.3	15	3	1	320	142
Phosphorus acid	For all crops	1754.4	7	6	1	2105	
Profenofos	For all crops	242.2	7	6	0.3	87	
Profenofos	For all crops	166.7	5	9	1	300	
Profenofos	For all crops	333.3	7	6	1	400	
Profenofos	For all crops	1204.8	7	6	0.3	434	
Profenofos	Chinese cabbage (Kwang tung)	250.0	3	15	1	750	
Profenofos	Lettuce (Pak kad horm)	375.0	3	15	1	1125	
Profenofos	Chinese kale (Ka na)	500.0	3	15	1	1500	166
Propineb	Drumstick Moringa (Ma rum)	136.1	15	3	1	82	
Propineb	For all crops	233.3	7	6	1	280	
Propineb	Roseapple	933.3	10	5	1	933	184
Prothiofos	Guava	892.9	10	5	1	893	710

mono-crop site : application dosages

Pesticide name	Crop name	Load g a.i./ha.	Application Interval days	Number of applications	Spray drift -	1st tier PEC µg/L	2nd tier PEC µg/L
Abamectin	Longan	22.50	15	2	1	9.0	6.7
Abamectin	Tangerine	0.77	5	12	1	1.8	
Abamectin	Tangerine	6.79	10	3	1	4.1	
Abamectin	Tangerine	14.63	15	2	1	5.9	
Abamectin	Tangerine	8.12	15	4	1	6.5	
Abamectin	Tangerine	6.75	7	9	1	12	
Abamectin	Tangerine	9.33	7	26	1	48	8.0
Captan	Tangerine	8.01	7	4	1	6.4	
Captan	Tangerine	8.01	15	4	1	6.4	
Captan	Tangerine	97.15	7	26	1	505	19
Carbaryl	Tangerine	26.98	15	4	1	22	6.3
Carbendazim	Longan	312.50	15	2	1	125	107
Carbendazim	Longbean	195.31	5	6	1	234	
Carbendazim	Longbean	195.31	7	9	1	352	205
Carbendazim	Tangerine	173.61	30	2	1	69	
Carbendazim	Tangerine	125.00	7	9	1	225	
Carbendazim	Tangerine	757.27	15	2	1	303	
Carbendazim	Tangerine	757.27	10	3	1	454	
Carbendazim	Tangerine	260.42	7	9	1	469	
Carbendazim	Tangerine	260.42	7	9	1	469	
Carbendazim	Tangerine	1437.77	10	3	1	863	
Carbendazim	Tangerine	773.99	7	9	1	1393	
Carbendazim	Tangerine	1174.78	5	6	1	1410	
Carbendazim	Tangerine	5639.10	7	4	1	4511	3603
Carbendazim	Tangerine	5639.10	15	4	1	4511	
Carbofuran	Coconut	245.45	30	2	0.3	29	20
Carbofuran	Coconut	245.45	30	2	0.3	29	
Cypermethrin	Tangerine	46.40	15	4	1	37	
Cypermethrin	Tangerine	46.40	7	4	1	37	
Cypermethrin	Tangerine	187.50	7	4	1	150	58
Cypermethrin	Tangerine	187.50	15	4	1	150	
Dicrotophos	Tangerine	165.07	30	2	1	66	
Dicrotophos	Tangerine	165.07	7	4	1	132	62
Dimethoate	Longan	1041.67	15	2	1	417	267
Dimethoate	Tangerine	83.33	30	2	1	33	
Dimethoate	Tangerine	83.33	7	9	1	150	
Dimethoate	Tangerine	378.49	15	2	1	151	
Dimethoate	Tangerine	204.08	7	4	1	163	
Dimethoate	Tangerine	367.65	20	3	1	221	
Dimethoate	Tangerine	555.56	30	2	1	222	
Dimethoate	Tangerine	200.00	10	6	1	240	
Dimethoate	Tangerine	204.08	10	6	1	245	
Dimethoate	Tangerine	378.49	7	4	1	303	
Dimethoate	Tangerine	200.00	7	9	1	360	
Dimethoate	Tangerine	555.56	7	4	1	444	
Dimethoate	Tangerine	600.00	7	4	1	480	
Dimethoate	Tangerine	600.00	15	4	1	480	
Dimethoate	Tangerine	1238.39	15	2	1	495	
Dimethoate	Tangerine	694.44	15	4	1	556	

Pesticide name	Crop name	Load g a.i./ha.	Application Interval days	Number of applications	Spray drift -	1st tier PEC µg/L	2nd tier PEC µg/L
Dimethoate	Tangerine	694.44	7	4	1	556	
Dimethoate	Tangerine	312.50	7	9	1	563	
Dimethoate	Tangerine	312.50	7	9	1	563	
Dimethoate	Tangerine	1009.70	10	3	1	606	
Dimethoate	Tangerine	854.70	7	4	1	684	
Dimethoate	Tangerine	1258.97	10	3	1	755	
Dimethoate	Tangerine	1009.70	15	4	1	808	
Dimethoate	Tangerine	1142.86	7	4	1	914	
Dimethoate	Tangerine	367.65	7	13	1	956	
Dimethoate	Tangerine	1653.44	10	3	1	992	
Dimethoate	Tangerine	1666.67	10	3	1	1000	
Dimethoate	Tangerine	1258.97	15	4	1	1007	
Dimethoate	Tangerine	1258.97	7	4	1	1007	
Dimethoate	Tangerine	848.74	15	6	1	1018	
Dimethoate	Tangerine	848.74	5	6	1	1018	
Dimethoate	Tangerine	1428.57	7	4	1	1143	
Dimethoate	Tangerine	1428.57	15	4	1	1143	
Dimethoate	Tangerine	2083.33	10	3	1	1250	
Dimethoate	Tangerine	500.00	7	13	1	1300	
Dimethoate	Tangerine	1653.44	15	4	1	1323	
Dimethoate	Tangerine	1653.44	7	4	1	1323	
Dimethoate	Tangerine	1666.67	7	4	1	1333	
Dimethoate	Tangerine	2401.96	10	3	1	1441	
Dimethoate	Tangerine	1804.51	7	4	1	1444	
Dimethoate	Tangerine	1804.51	15	4	1	1444	
Dimethoate	Tangerine	848.74	7	9	1	1528	
Dimethoate	Tangerine	4000.00	15	2	1	1600	
Dimethoate	Tangerine	2203.70	7	4	1	1763	
Dimethoate	Tangerine	2401.96	15	4	1	1922	
Dimethoate	Tangerine	1666.67	5	6	1	2000	
Dimethoate	Tangerine	1142.86	10	9	1	2057	
Dimethoate	Tangerine	1238.39	7	9	1	2229	
Dimethoate	Tangerine	641.03	10	18	1	2308	
Dimethoate	Tangerine	4000.00	20	3	1	2400	
Dimethoate	Tangerine	2083.33	5	6	1	2500	
Dimethoate	Tangerine	6033.18	10	3	1	3620	
Dimethoate	Tangerine	6033.18	30	3	1	3620	
Dimethoate	Tangerine	2083.33	7	9	1	3750	
Dimethoate	Tangerine	1666.67	15	12	1	4000	
Dimethoate	Tangerine	2203.70	10	12	1	5289	
Dimethoate	Tangerine	1487.32	7	26	1	7734	
Dimethoate	Tangerine	2673.80	7	26	1	13904	
Dimethoate	Tangerine	7407.41	15	12	1	17778	2066
EPN	Longbean	260.42	5	6	1	313	
EPN	Longbean	260.42	7	9	1	469	45
Mancozeb	Tangerine	408.16	7	4	1	327	
Mancozeb	Tangerine	1135.48	15	2	1	454	
Mancozeb	Tangerine	408.16	10	6	1	490	
Mancozeb	Tangerine	600.00	10	6	1	720	

Pesticide name	Crop name	Load g a.i./ha.	Application Interval days	Number of applications	Spray drift -	1st tier PEC µg/L	2nd tier PEC µg/L
Mancozeb	Tangerine	1135.48	7	4	1	908	
Mancozeb	Tangerine	600.00	7	9	1	1080	
Mancozeb	Tangerine	5600.00	15	2	1	2240	
Mancozeb	Tangerine	5600.00	20	3	1	3360	1705
Methamidophos	For all crop	200.00	7	4	1	160	
Methamidophos	For all crop	200.00	15	4	1	160	
Methamidophos	For all crop	200.00	5	6	1	240	112
Methamidophos	Guava	218.18	30	2	1	87	48
Methamidophos	Guava	218.18	30	2	1	87	
Methamidophos	Tangerine	62.82	10	3	1	38	
Methamidophos	Tangerine	62.82	7	13	1	163	
Methamidophos	Tangerine	454.55	7	4	1	364	
Methamidophos	Tangerine	454.55	15	4	1	364	
Methamidophos	Tangerine	1406.25	10	3	1	844	
Methamidophos	Tangerine	2819.48	15	2	1	1128	
Methamidophos	Tangerine	2819.48	15	2	1	1128	
Methamidophos	Tangerine	3450.66	15	2	1	1380	
Methamidophos	Tangerine	3656.25	15	2	1	1463	
Methamidophos	Tangerine	1406.25	5	6	1	1688	
Methamidophos	Tangerine	2819.48	10	3	1	1692	
Methamidophos	Tangerine	2819.48	10	3	1	1692	
Methamidophos	Tangerine	750.00	7	13	1	1950	
Methamidophos	Tangerine	3450.66	10	3	1	2070	
Methamidophos	Tangerine	621.76	7	17	1	2114	
Methamidophos	Tangerine	3656.25	10	3	1	2194	
Methamidophos	Tangerine	1406.25	7	9	1	2531	
Methamidophos	Tangerine	3656.25	7	4	1	2925	
Methamidophos	Tangerine	2819.48	5	6	1	3383	
Methamidophos	Tangerine	2819.48	5	6	1	3383	
Methamidophos	Tangerine	3450.66	5	6	1	4141	1929
Methomyl	Tangerine	347.22	7	4	1	278	
Methomyl	Tangerine	347.22	10	6	1	417	148
Profenofos	Tangerine	52.35	10	3	1	31	
Profenofos	Tangerine	43.40	15	4	1	35	
Profenofos	Tangerine	43.40	7	4	1	35	
Profenofos	Tangerine	55.84	7	4	1	45	
Profenofos	Tangerine	55.84	5	6	1	67	
Profenofos	Tangerine	206.68	10	3	1	124	
Profenofos	Tangerine	52.35	7	13	1	136	
Profenofos	Tangerine	260.42	10	3	1	156	
Profenofos	Tangerine	206.68	15	4	1	165	
Profenofos	Tangerine	206.68	7	4	1	165	
Profenofos	Tangerine	260.42	7	4	1	208	
Profenofos	Tangerine	260.42	5	6	1	313	
Profenofos	Tangerine	651.04	10	3	1	391	
Profenofos	Tangerine	260.42	15	12	1	625	
Profenofos	Tangerine	390.63	7	9	1	703	
Profenofos	Tangerine	390.63	7	9	1	703	
Profenofos	Tangerine	651.04	5	6	1	781	
Profenofos	Tangerine	651.04	7	9	1	1172	

Pesticide name	Crop name	Load g a.i./ha.	Application Interval days	Number of applications	Spray drift -	1st tier PEC µg/L	2nd tier PEC µg/L
Profenofos	Tangerine	2467.11	7	4	1	1974	
Profenofos	Tangerine	2467.11	15	6	1	2961	378
Tetradifon	Tangerine	65.28	7	4	1	52	
Tetradifon	Tangerine	65.28	10	6	1	78	
Tetradifon	Tangerine	201.43	7	4	1	161	
Tetradifon	Tangerine	147.45	10	12	1	354	93
Zineb	Tangerine	26.67	7	13	1	69	
Zineb	Tangerine	1135.48	15	2	1	454	
Zineb	Tangerine	833.33	10	3	1	500	
Zineb	Tangerine	833.33	7	4	1	667	
Zineb	Tangerine	833.33	15	4	1	667	
Zineb	Tangerine	1135.48	7	4	1	908	
Zineb	Tangerine	833.33	5	6	1	1000	
Zineb	Tangerine	2742.86	7	4	1	2194	
Zineb	Tangerine	5035.88	10	3	1	3022	
Zineb	Tangerine	3888.89	7	4	1	3111	
Zineb	Tangerine	5035.88	15	4	1	4029	
Zineb	Tangerine	5035.88	7	4	1	4029	
Zineb	Tangerine	2742.86	10	9	1	4937	
Zineb	Tangerine	3888.89	10	18	1	14000	1947

Appendix 4: Results of NEC calculations for all pesticides evaluated

Active ingredient name	Cass-number	Pesticide type	NEC 1st tier (µg/L)
Abamectin	71751-41-2	insecticide, acaricide	0.0034
Alachlor	15972-60-8	herbicide	0.46
Bispyribac-sodium	125401-75-4	herbicide	63
Captan	133-06-2	fungicide	1.3
Carbaryl	63-25-2	insecticide, plant growth regulator	0.0042
Carbendazim	10605-21-7	fungicide	0.56
Carbofuran	1563-66-2	insecticide, nematocide	0.40
Carbosulfan	55285-14-8	insecticde	0.015
Chlorfenapyr	122453-73-0	insecticide, acaricide	0.061
Chlorfluazuron	71422-67-8	insecticide	0.0091
Chlorpyrifos	2921-88-2	insecticide	0.0038
Cypermethrin	52315-07-8	insecticide	0.0012
Diafenthiuron	80060-09-9	insecticide, acaricide	0.0070
Dicrotophos	141-66-2	insecticide, acaricide	3.4
Difenoconazole	119446-68-3	fungicide	0.50
Diflubenzuron	35367-38-5	insecticide	0.33
Dimethoate	60-51-5	insecticide, acaricide	0.026
EPN	2104-64-5	insecticide, acaricide	0.0010
Fenobucarb	3766-81-2	insecticide	1.0
Fenoxycarb	72490-01-8	insecticide	0.00016
Fipronil	120068-37-3	insecticide	0.46
Gibberellic acid	77-06-5	plant growth regulator	1430
Glyphosate	1071-83-6	herbicide	100
Glyphosate-isopropylammonium	38641-94-0	herbicide	32
Glyphosate-trimesium	81591-81-3	herbicide	107
Malathion	121-75-5	insecticide, acaricide	0.018
Mancozeb	8018-01-7	fungicide	0.22
MCPA	94-74-6	herbicide	4.3
Metalaxyl	57837-19-1	fungicide	120
Methamidophos	10265-92-6	insecticide, acaricide	0.33
Methomyl	16752-77-5	insecticide, acaricide	0.080
Mevinphos	26718-65-0 and 7786347	insecticide, acaricide	0.0019
Omethoate	1113-02-6	insecticide, acaricide	0.21
Oxadiazon	19666-30-9	herbicide	0.56
Phosalone	2310-17-0	insecticide, acaricide	0.0069
Profenofos	41198-08-7	insecticide, acaricide	0.011
Propanil	709-98-8	herbicide	0.50
Propargite	2312-35-8	acaricide	0.72
Propineb	12071-83-9	Fungicide	19
Prothiofos	34643-46-4	Insecticide	0.14
Tetradifon	116-29-0	Acaricide	9.0
Zineb	12122-67-7	Fungicide	2.0

Appendix 5: Second tier PEC/NEC risk quotients for crop-pesticide combinations in Thailand (application dosages)

mixed crop site

Pesticide name	Crop name	PEC/1st tier NEC
Mevinphos	Drumstick Moringa (Ma rum)	74737
Malathion	Guava	59278
Abamectin	Chinese kale (Ka na)	57353
Dimethoate	Roseapple	37846
Methamidophos	Roseapple	33455
Cypermethrin	Roseapple	21667
Profenofos	Chinese kale (Ka na)	15091
Prothiofos	Guava	5069
Chlorfenapyr	Amaranth (Pak Khom)	1616
Carbosulfan	Roseapple	1600
Chlorfluazuron	For all crops	1516
Carbendazim	Guava	1502
Carbendazim	Chinese kale (Ka na)	1311
Mancozeb	Roseapple	276
Mancozeb	Amaranth (Pak Khom)	271
Dicrotophos	Chinese kale (Ka na)	218
Difenoconazole	For all crops	159
Diflubenzuron	Roseapple	72
Fipronil	Amaranth (Pak Khom)	42
Propineb	Roseapple	10
Metalaxyl	For all crops	6.6
Glyphosate	For all crops	4.3

Thailand (mono-crop site)

Pesticide name	Crop name	PEC/1st tier NEC
Dimethoate	Tangerine	79462
Cypermethrin	Tangerine	48583
EPN	Longbean	44700
Profenofos	Tangerine	34364
Dimethoate	Longan	10269
Mancozeb	Tangerine	7750
Carbendazim	Tangerine	6434
Methamidophos	Tangerine	5845
Abamectin	Tangerine	2344
Abamectin	Longan	1976
Methomyl	Tangerine	1850
Carbaryl	Tangerine	1490
Zineb	Tangerine	974
Carbendazim	Longbean	365
Methamidophos	For all crop	339
Carbendazim	Longan	190
Methamidophos	Guava	145
Carbofuran	Coconut	49
Dicrotophos	Tangerine	18
Captan	Tangerine	15
Tetradifon	Tangerine	10

Appendix 6: First and second tier PEC's for crop-pesticide combinations in Thailand

PEC's of crop-pesticide combinations were based on **recommended dosages**. The second tier PEC was only calculated for pesticide-crop combination yielding the highest first-tier PEC.

mixed crop site : recommended dosages

Pesticide name	Crop name	Load g a.i./ha.	Application Interval days	Number of applications	Spray drift -	1st tier PEC µg/L	2nd tier PEC µg/L
Abamectin	For all crops	2.25	7	6	1	3	
Abamectin	Chinese leek(Kui chai)	2.25	7	6	1	3	
Abamectin	Chinese leek(Kui chai)	2.25	7	6	1	3	
Abamectin	For all crops	2.25	7	6	1	3	
Abamectin	Amaranth (Pak Khom)	2.25	5	9	1	4	
Abamectin	Chinese kale (Ka na)	2.25	5	9	1	4	
Abamectin	Chinese cabbage (Kwang tung)	2.25	3	15	1	7	
Abamectin	Chinese kale (Ka na)	2.25	3	15	1	7	
Abamectin	Lettuce (Pak kad horm)	2.25	3	15	1	7	1
Cabendazim	Holy basil	187.5	14	3	1	120	
Carbendazim	Guava	187.5	10	5	1	169	
Carbendazim	Guava	187.5	10	5	1	169	
Cabendazim	Roseapple	187.5	7	6	1	240	
Cabendazim	Roseapple	187.5	7	6	1	240	
Carbendazim	Roseapple	187.5	7	6	1	240	
Cabendazim	Chinese cabbage (Kwang tung)	187.5	3	15	1	563	
Cabendazim	Chinese kale (Ka na)	187.5	3	15	1	563	
Cabendazim	Lettuce (Pak kad horm)	187.5	3	15	1	563	125
Carbosulfan	Roseapple	25	7	6	1	32	6
Chlorfenapyr	For all crops	25	7	6	0.3	10	
Chlorfenapyr	For all crops	25	7	6	1	32	
Chlorfenapyr	Amaranth (Pak Khom)	25	5	9	1	45	36
Chlorrfluazuron	For all crops	12.5	5	9	1	23	9
Cypermethrin	Roseapple	14.0625	10	5	1	13	
Cypermethrin	Roseapple	14.0625	7	6	1	18	4
Dicrotophos	For all crops	61.875	7	6	1	79	
Dicrotophos	For all crops	61.875	7	6	1	79	
Dicrotophos	Chinese kale (Ka na)	61.875	5	9	1	111	23
Difenoconazole	For all crops	31.25	7	6	1	40	28
Diflubenzuron	Roseapple	70.3125	10	5	1	63	17
Dimethoate	Roseapple	50	7	6	1	64	22
Fipronil	Amaranth (Pak Khom)	6.25	5	9	1	11	4
Glyphosate-isopropylammonium	Grass	750	365	0	0.3	6	
Glyphosate-isopropylammonium	Grass	750	183	0	0.3	11	
Glyphosate-isopropylammonium	Grass	750	243	0	1	28	
Glyphosate-isophopylammonium	For all crops	750	7	6	0.3	288	327

Pesticide name	Crop name	Load g a.i./ha.	Application Interval days	Number of applications	Spray drift -	1st tier PEC µg/L	2nd tier PEC µg/L
Methomyl	Drumstick Moringa (Marum)	87.5	15	3	1	52	
Methomyl	Chinese kale (Ka na)	87.5	5	9	1	158	36
Mancozeb	Lettuce (Pak kad horm)	37.5	24	2	1	14	
Mancozeb	Chinese leek(Kui chai)	37.5	7	6	1	48	
Mancozeb	Amaranth (Pak Khom)	37.5	5	9	1	68	
Mancozeb	Roseapple	250	7	6	1	321	49
Melathion	Guava	155.625	7	6	1	31	30
Metalaxyl	For all crops	3125	7	6	1	4007	2391
Methamidophos	angled gourd	150	7	6	0.3	58	
Methamidophos	Holy basil	150	14	3	1	96	
Methamidophos	Guava	150	10	5	1	135	
Methamidophos	Roseapple	150	10	5	1	135	
Methamidophos	Guava	150	7	6	1	192	
Methamidophos	Roseapple	150	7	6	1	192	
Methamidophos	Roseapple	150	7	6	1	192	
Methamidophos	Roseapple	150	7	6	1	192	70
Methomyl	Guava	87.5	10	5	1	79	
Methomyl	Guava	87.5	10	5	1	79	
Methomyl	Guava	87.5	10	5	1	79	
Methomyl	Roseapple	87.5	10	5	1	79	
Methomyl	Roseapple	87.5	7	6	1	112	
Methomyl	Roseapple	87.5	7	6	1	112	36
Mevinphos	Drumstick Moringa (Marum)	60	15	3	1	36	19
Profenofos	For all crops	93.75	7	6	0.3	36	
Profenofos	For all crops	93.75	7	6	0.3	36	
Profenofos	For all crops	93.75	7	6	1	120	
Profenofos	For all crops	93.75	5	9	1	169	
Profenofos	Chinese cabbage (Kwang tung)	93.75	3	15	1	281	
Profenofos	Chinese kale (Ka na)	93.75	3	15	1	281	
Profenofos	Lettuce (Pak kad horm)	93.75	3	15	1	281	27
Propineb	For all crops	262.5	7	6	1	53	
Propineb	Roseapple	262.5	10	5	1	53	
Propineb	Drumstick Moringa (Marum)	262.5	15	3	1	53	51
Prothiofos	Guava	93.75	10	5	1	84	18

mono-crop (recommended dosages)

Pesticide name	Crop name	Load g a.i./ha.	Application Interval days	Number of applications	Spray drift -	1st tier PEC µg/L	2nd tier PEC µg/L
Abamectin	Longan	2.25	15	2	1	1	
Abamectin	Tangerine	2.25	15	2	1	1	
Abamectin	Tangerine	2.25	10	3	1	1	
Abamectin	Tangerine	2.25	15	4	1	2	
Abamectin	Tangerine	2.25	7	9	1	4	
Abamectin	Tangerine	2.25	5	12	1	5	
Abamectin	Tangerine	2.25	7	26	1	12	1
Captan	Tangerine	156.25	15	4	1	125	
Captan	Tangerine	156.25	7	4	1	134	
Captan	Tangerine	156.25	7	26	1	804	31
Carbaryl	Tangerine	159.375	15	4	1	128	37
Carbendazim	Longan	187.5	15	2	1	75	
Carbendazim	Tangerine	187.5	30	2	1	75	
Carbendazim	Tangerine	187.5	15	2	1	75	
Carbendazim	Tangerine	187.5	10	3	1	113	
Carbendazim	Tangerine	187.5	10	3	1	113	
Carbendazim	Tangerine	187.5	15	4	1	150	
Carbendazim	Tangerine	187.5	7	4	1	161	
Carbendazim	Tangerine	187.5	5	6	1	225	
Carbendazim	Longbean	187.5	5	6	1	225	
Carbendazim	Tangerine	187.5	7	9	1	321	
Carbendazim	Tangerine	187.5	7	9	1	321	
Carbendazim	Longbean	187.5	7	9	1	321	
Carbendazim	Tangerine	187.5	7	9	1	321	
Carbendazim	Tangerine	187.5	7	9	1	321	189
Carbofuran	Coconut	2250	30	2	0.3	270	
Carbofuran	Coconut	2250	30	2	0.3	270	622
Cypermethrin	Tangerine	7.65625	15	4	1	6	
Cypermethrin	Tangerine	7.65625	15	4	1	6	
Cypermethrin	Tangerine	7.65625	7	4	1	7	
Cypermethrin	Tangerine	7.65625	7	4	1	7	2
Dicrotophos	Tangerine	61.875	30	2	1	25	
Dicrotophos	Tangerine	61.875	7	4	1	53	22
Dimethoate	Longan	50	15	2	1	20	
Dimethoate	Tangerine	50	30	2	1	20	
Dimethoate	Tangerine	50	15	2	1	20	
Dimethoate	Tangerine	50	15	2	1	20	
Dimethoate	Tangerine	50	30	2	1	20	
Dimethoate	Tangerine	50	15	2	1	20	
Dimethoate	Tangerine	50	10	3	1	30	
Dimethoate	Tangerine	50	30	3	1	30	
Dimethoate	Tangerine	50	10	3	1	30	
Dimethoate	Tangerine	50	10	3	1	30	
Dimethoate	Tangerine	50	20	3	1	30	
Dimethoate	Tangerine	50	20	3	1	30	
Dimethoate	Tangerine	50	10	3	1	30	
Dimethoate	Tangerine	50	10	3	1	30	

Pesticide name	Crop name	Load g a.i./ha.	Application Interval days	Number of applications	Spray drift -	1st tier PEC µg/L	2nd tier PEC µg/L
Dimethoate	Tangerine	50	10	3	1	30	
Dimethoate	Tangerine	50	10	3	1	30	
Dimethoate	Tangerine	50	15	4	1	40	
Dimethoate	Tangerine	50	15	4	1	40	
Dimethoate	Tangerine	50	15	4	1	40	
Dimethoate	Tangerine	50	15	4	1	40	
Dimethoate	Tangerine	50	15	4	1	40	
Dimethoate	Tangerine	50	15	4	1	40	
Dimethoate	Tangerine	50	15	4	1	40	
Dimethoate	Tangerine	50	15	4	1	40	
Dimethoate	Tangerine	50	7	4	1	43	
Dimethoate	Tangerine	50	7	4	1	43	
Dimethoate	Tangerine	50	7	4	1	43	
Dimethoate	Tangerine	50	7	4	1	43	
Dimethoate	Tangerine	50	7	4	1	43	
Dimethoate	Tangerine	50	7	4	1	43	
Dimethoate	Tangerine	50	7	4	1	43	
Dimethoate	Tangerine	50	7	4	1	43	
Dimethoate	Tangerine	50	7	4	1	43	
Dimethoate	Tangerine	50	7	4	1	43	
Dimethoate	Tangerine	50	7	4	1	43	
Dimethoate	Tangerine	50	7	4	1	43	
Dimethoate	Tangerine	50	7	4	1	43	
Dimethoate	Tangerine	50	10	6	1	60	
Dimethoate	Tangerine	50	10	6	1	60	
Dimethoate	Tangerine	50	5	6	1	60	
Dimethoate	Tangerine	50	5	6	1	60	
Dimethoate	Tangerine	50	15	6	1	60	
Dimethoate	Tangerine	50	5	6	1	60	
Dimethoate	Tangerine	50	7	9	1	86	
Dimethoate	Tangerine	50	7	9	1	86	
Dimethoate	Tangerine	50	7	9	1	86	
Dimethoate	Tangerine	50	7	9	1	86	
Dimethoate	Tangerine	50	7	9	1	86	
Dimethoate	Tangerine	50	7	9	1	86	
Dimethoate	Tangerine	50	7	9	1	86	
Dimethoate	Tangerine	50	10	9	1	90	
Dimethoate	Tangerine	50	15	12	1	120	
Dimethoate	Tangerine	50	15	12	1	120	
Dimethoate	Tangerine	50	10	12	1	120	
Dimethoate	Tangerine	50	7	13	1	129	
Dimethoate	Tangerine	50	7	13	1	129	
Dimethoate	Tangerine	50	10	18	1	180	
Dimethoate	Tangerine	50	7	26	1	257	
Dimethoate	Tangerine	50	7	26	1	257	13
EPN	Longbean	84.375	5	6	1	101	
EPN	Longbean	84.375	7	9	1	145	5
Mancozeb	Tangerine	250	15	2	1	100	
Mancozeb	Tangerine	250	15	2	1	100	

Pesticide name	Crop name	Load g a.i./ha.	Application Interval days	Number of applications	Spray drift -	1st tier PEC µg/L	2nd tier PEC µg/L
Mancozeb	Tangerine	250	20	3	1	150	
Mancozeb	Tangerine	250	7	4	1	214	
Mancozeb	Tangerine	250	7	4	1	214	
Mancozeb	Tangerine	250	10	6	1	300	
Mancozeb	Tangerine	250	10	6	1	300	
Mancozeb	Tangerine	250	7	9	1	429	49
Methamidophos	Tangerine	150	15	2	1	60	
Methamidophos	Tangerine	150	15	2	1	60	
Methamidophos	Tangerine	150	15	2	1	60	
Methamidophos	Tangerine	150	15	2	1	60	
Methamidophos	Guava	150	30	2	1	60	
Methamidophos	Guava	150	30	2	1	60	
Methamidophos	Tangerine	150	10	3	1	90	
Methamidophos	Tangerine	150	10	3	1	90	
Methamidophos	Tangerine	150	10	3	1	90	
Methamidophos	Tangerine	150	10	3	1	90	
Methamidophos	Tangerine	150	10	3	1	90	
Methamidophos	Tangerine	150	10	3	1	90	
Methamidophos	For all crop	150	15	4	1	120	
Methamidophos	Tangerine	150	15	4	1	120	
Methamidophos	Tangerine	150	7	4	1	129	
Methamidophos	For all crop	150	7	4	1	129	
Methamidophos	Tangerine	150	7	4	1	129	
Methamidophos	Tangerine	150	5	6	1	180	
Methamidophos	Tangerine	150	5	6	1	180	
Methamidophos	Tangerine	150	5	6	1	180	
Methamidophos	For all crop	150	5	6	1	180	
Methamidophos	Tangerine	150	5	6	1	180	
Methamidophos	Tangerine	150	7	9	1	257	
Methamidophos	Tangerine	150	7	13	1	386	
Methamidophos	Tangerine	150	7	13	1	386	
Methamidophos	Tangerine	150	7	17	1	514	87
Methomyl	Tangerine	87.5	7	4	1	75	
Methomyl	Tangerine	87.5	10	6	1	105	38
Profenofos	Tangerine	93.75	10	3	1	56	
Profenofos	Tangerine	93.75	10	3	1	56	
Profenofos	Tangerine	93.75	10	3	1	56	
Profenofos	Tangerine	93.75	10	3	1	56	
Profenofos	Tangerine	93.75	15	4	1	75	
Profenofos	Tangerine	93.75	15	4	1	75	
Profenofos	Tangerine	93.75	7	4	1	80	
Profenofos	Tangerine	93.75	7	4	1	80	
Profenofos	Tangerine	93.75	7	4	1	80	
Profenofos	Tangerine	93.75	7	4	1	80	
Profenofos	Tangerine	93.75	7	4	1	80	
Profenofos	Tangerine	93.75	15	6	1	113	
Profenofos	Tangerine	93.75	5	6	1	113	
Profenofos	Tangerine	93.75	5	6	1	113	
Profenofos	Tangerine	93.75	5	6	1	113	
Profenofos	Tangerine	93.75	7	9	1	161	

Pesticide name	Crop name	Load g a.i./ha.	Application Interval days	Number of applications	Spray drift -	1st tier PEC µg/L	2nd tier PEC µg/L
Profenofos	Tangerine	93.75	7	9	1	161	
Profenofos	Tangerine	93.75	7	9	1	161	
Profenofos	Tangerine	93.75	15	12	1	225	
Profenofos	Tangerine	93.75	7	13	1	241	15
Zineb	Tangerine	400	15	2	1	160	
Zineb	Tangerine	400	10	3	1	240	
Zineb	Tangerine	400	10	3	1	240	
Zineb	Tangerine	400	15	4	1	320	
Zineb	Tangerine	400	15	4	1	320	
Zineb	Tangerine	400	7	4	1	343	
Zineb	Tangerine	400	7	4	1	343	
Zineb	Tangerine	400	7	4	1	343	
Zineb	Tangerine	400	7	4	1	343	
Zineb	Tangerine	400	7	4	1	343	
Zineb	Tangerine	400	5	6	1	480	
Zineb	Tangerine	400	10	9	1	720	
Zineb	Tangerine	400	7	13	1	1029	
Zineb	Tangerine	400	10	18	1	1440	199
Tetradifon	Tangerine	18.8	7	4	1	16	
Tetradifon	Tangerine	18.8	7	4	1	16	
Tetradifon	Tangerine	18.8	10	6	1	23	
Tetradifon	Tangerine	18.8	10	12	1	45	11

Appendix 7: Examples of relative sensitivities of aquatic animals and some invertebrates to **carbendazim** (year 1995-2005).

Species (Appendix 7)	Effect	Duration (days)	Reported Effect Concentration (µg/L)	Reference
Cyprinus carpio	LC_{50}	2	0.008	Subramaniam *et al.* 1996
Asellus aquaticus (Aquatic sowbug)	LOEC	28	985	Cuppen *et al*., 2000
Asellus sp. (Aquatic sowbug)	LOEC	28	985	Cuppen *et al*., 2000
Proasellus coxalis (Isopod)	LOEC	28	985	Cuppen *et al*. ,2000
P. meridianus (Isopod)	LOEC	28	985	Cuppen *et al*. ,2000
A. aquaticus (Aquatic sowbug)	NOEC	28	319	Cuppen *et al*. ,2000
Asellus sp. (Aquatic sowbug)	NOEC	28	319	Cuppen *et al*. ,2000
P. coxalis (Isopod)	NOEC	28	319	Cuppen *et al*. ,2000
P. meridianus (Isopod)	NOEC	28	319	Cuppen *et al*. ,2000
Cyprinodon variegatus (Sheepshead minnow)	LC_{50}	4	1,160	Environmental Fate and Effects Division, U.S.EPA. 2000.
Lepomis macrochirus (0.6 g)	LC_{50}	4	1,850	Environmental Fate and Effects Division, U.S.EPA. 2000.
Oncorhynchus mykiss (1.0 g)	LC_{50}	4	650	Environmental Fate and Effects Division, U.S.EPA. 2000.
O. mykiss (2.7 g)	LC_{50}	4	1,320	Environmental Fate and Effects Division, U.S.EPA. 2000.
O. mykiss (0.8 g)	LC_{50}	4	230	Environmental Fate and Effects Division, U.S.EPA. 2000.
Crassostrea virginica (American or Virginia oyster) Spat	EC_{50} Immobility	4	1,160	Environmental Fate and Effects Division, U.S.EPA. 2000.
Bithynia leachi (Snail)	LOEC	28	319	Cuppen *et al*. ,2000
B. tentaculata (Snail)	LOEC	28	92.1	Cuppen *et al*. ,2000
Lymnaea sp.(Pond snail)	LOEC	28	30.5.	Cuppen *et al*. ,2000
L. stagnailis (Great pond snail)	LOEC	28	3.1	Cuppen *et al*. ,2000
Physa fontinalis (Bladder snail)	LOEC	28	30.5	Cuppen *et al*. ,2000
Physella acuta (European physa, bladder snail)	LOEC	28	30.5	Cuppen *et al*. ,2000
Segmentina nitida (Shining ram's-horn snail)	LOEC	28	3.1	Cuppen *et al*. ,2000
B. leachi (Snail)	NOEC	28	92.1	Cuppen *et al*. ,2000
Bithynia sp. (Snail)	NOEC	28	31.5	Cuppen *et al*. ,2000

Species (Appendix 7)	Effect	Duration (days)	Reported Effect Concentration (µg/L)	Reference
B. tentaculata (Snail)	NOEC	28	30.5	Cuppen *et al.* ,2000
Lymnaea sp. (Pond snail)	NOEC	28	3.1	Cuppen *et al.* ,2000
L. stagnalis (Great pond snail)	NOEC	28	3.1	Cuppen *et al.* ,2000
Physa fontinalis (Bladder snail)	NOEC	28	3.1	Cuppen *et al.* ,2000
Physella acuta (European physa, bladder snail)	NOEC	28	3.1	Cuppen *et al.* ,2000
Segmentina nitida (Shining ram's-horn snail)	NOEC	28	3.1	Cuppen *et al.* ,2000
Dugesia lugubris (Planarian, Vortex worm)	LOEC	28	30.5	Cuppen *et al.* ,2000
D. tigrina (Turbellarian, flatworm)	LOEC	28	30.5	Cuppen *et al.* ,2000
D. lugubris (Planarian, Vortex worm)	NOEC	28	3.1	Cuppen *et al.* ,2000
D. tigrina (Turbellarian, flatworm)	NOEC	28	3.1	Cuppen *et al.* ,2000
D. lugubris	EC_{50}	4	25	Van Wijngaarden *et al.*, 1998
D. lugubris	NOEC (reproduction)	21	11	Van Wijngaarden *et al.*, 1998
Stylaria lacustris (oligochaete)	LC_{50}	4	2035	Van Wijngaarden *et al.*, 1998
Dero digitata (oligochaete)	LC_{50}	4	980	Van Wijngaarden *et al.*, 1998
Daphnia magna	EC_{50}	2	110, 350	Environmental Fate and Effects Division, U.S.EPA. 2000.
D. magna	LC_{50}	2	320	Van Wijngaarden *et al.*, 1998
Americamysis bahia (Opossum shrimp)	LC_{50}	4	98	Environmental Fate and Effects Division, U.S.EPA. 2000.
Gammarus pulex	LOEC	1	985	Cuppen *et al.* ,2000
G. pulex	LOEC	2	319	Cuppen *et al.* ,2000
G. pulex	LOEC	3,6,13	92.1	Cuppen *et al.* ,2000
G. pulex	LOEC	20,27	3.1	Cuppen *et al.* ,2000
G. pulex	NOEC	1	319	Cuppen *et al.* ,2000
G. pulex	NOEC	2	92.1	Cuppen *et al.* ,2000
G. pulex	NOEC	3,6,13	31	Cuppen *et al.* ,2000
G. pulex	NOEC	20,27	3.1	Cuppen *et al.* ,2000
G. pulex (juvenile)	LC_{50}	2	77	Van Wijngaarden *et al.*, 1998
G. pulex (adult)	LC_{50}	2	1041	Van Wijngaarden *et al.*, 1998

Appendix 8 : Examples of relative sensitivities of aquatic animals and some invertebrates to **chlorpyrifos** (year 1995-2005).

Species (Appendix 8)	Effect	Duration (days)	Reported Effect Concentration (µg/L)	Reference
Oligochaeta (Annelid worm class)	NOEC	< 1 , 7, 14	44	Van den Brink, *et al.*, 1996.
Oligochaeta (Annelid worm class)	NOEC	28	0.9	Van den Brink, *et al.*, 1996.
Oligochaeta (Annelid worm class)	NOEC	56	6	Van den Brink, *et al.*, 1996.
Oligochaeta (Annelid worm class)	NOEC	84, 133, 168, 294, 329, 357 and 385	44	Van den Brink, *et al.*, 1996.
Stylaria lacustris (Oligochaete)	NOEC	< 1, 7, 14, 96, 133, 168, 294, 329, 357 and 385	44	Van den Brink, *et al.*, 1996.
S. lacustris (Oligochaete)	NOEC	28, 56	0.9	Van den Brink, *et al.*, 1996.
Paratya australiensis (Shrimp)	LOEC Enzyme activity	4	0.005, 0.01, 0.07, 0.09	Olima *et al.*, 1997.
Asellus aquaticus (Aquatic sowbug)	EC_{50}	2	3.4	Van Wijngaarden, *et al.*, 1996.
Oziotelphusa senex senex (Crab)Adult male, 10 g	LC_{50}	2	550	Radhakrishnaiah, *et al.*,1995.
P. australiensis (Shrimp)	LC_{50}	4	0.08, 0.10, 0.28, 0.15, 0.25	Olima, *et al.*,1997.
P. australiensis (Shrimp)	LC_{50}	3	0.1, 0.2	Olima, *et al.*,1997.
Penaeus vannamei (Whiteleg shrimp) Juvenile, 6-7 cm, 2.4-2.9 g	LC_{50}	2	4.8	Galindo, *et al.*,1996.
P. australiensis	LOEC	4	0.04, 0.09, 0.12, 0.15, 0.20, 0.25, 0.27	Olima, *et al.*, 1997.
Ostracoda Ostracod/seed shrimp subclass)	NOEC	< 1, 14, 28, 84, 105, 133	6	Van den Brink, *et al.*, 1996.
Ostracoda Ostracod/seed shrimp subclass)	NOEC	7 , 56,	0.9	Van den Brink, *et al.*, 1996.
Ostracoda Ostracod/seed shrimp subclass)	NOEC	168, 357, 385	44	Van den Brink, *et al.*, 1996.
Ostracoda Ostracod/seed shrimp subclass)	NOEC	329	0.1	Van den Brink, *et al.*, 1996.
Ostracoda Ostracod/seed shrimp subclass)	NOEC	7	0.9	Van Wijngaarden, *et al.*, 1996.
Gambusia affinis (Western mosquitofish) Adult	LC_{50}	1.5	215	Environmental Fate and Effects Division, U.S. EPA., 2000.

Species (Appendix 8)	Effect	Duration (days)	Reported Effect Concentration (µg/L)	Reference
Heteropneustes fossilis (Indian catfish) 42-53 g.	LC_{50}	4	2,200	Srivastav, *et al.*,1997
Ictalurus punctatus (Channel catfish) fingerling, 20-25 g	LC_{50}	4	2,077	Dalvi, *et al.*,1998.
Lepomis cyanellus (Green sunfish) Adult	LC_{50}	1.5	22.5	Environmental Fate and Effects Division, U.S. EPA., 2000.
L. macrochirus (Bluegill)	LC_{50}	4	1.30 (1.5 g fish) 5.80 (2.1 g fish)	Environmental Fate and Effects Division, U.S. EPA., 2000.
Oncorhynchus mykiss (rainbow trout) 83 Days, Fry, 0.671 g.	LC_{50}	4	45	Kikuchi, *et al.*, 1996.
O. mykiss (rainbow trout) 41-46 Days, Fry, 0.23 – 0.343 g.	LC_{50}	4	41	Kikuchi, *et al.*, 1996.
O. mykiss (rainbow trout)	LC_{50}	4	8 (1.0 g fish) 13.5 (1.5 g fish) 27 (0.7 g fish)	Environmental Fate and Effects Division, U.S. EPA., 2000.
Pimephales promelas (Fathead minnow) 0.1 G	LC_{50}	4	203	Environmental Fate and Effects Division, U.S. EPA., 2000.
Chironomus tentans (Midge) 4th instar larvae, 1.0 cm	EC_{50} Behavioral changes	4	0.51, 0.58, 0.75	Pape-Lindstrom and Lydy ,1997
C. tentans (Midge) 4th instar larvae, 0.63 -0.71 mm	EC_{50} swimming	4	150, 330, 580	Lydy, *et al.* ,1999
Chaoborus obscuripes (Midge)	EC_{50} Immobility	2	0.5	Van Wijngaarden, *et al.*,1996.
Cloeon dipterum (Mayfly)	EC_{50}	2	0.4	Van Wijngaarden, *et al.*,1996.
Aedes aegypti (Yellow fever mosquito) 3rd instar	LC_{50}	1	7.1	Environmental Fate and Effects Division, U.S. EPA., 2000.
A. albopictus (Mosquito) late 4th instar larva	LC_{50}	1	3.3	Ali, et a.,1995
C. tentans 10-13 days	LC_{50}	2	0.3	Moore, *et al.*,1998.
C. tentans 10-13 days	LOEC	2	0.38	Moore, *et al.*,1998.
Gammarus palustris	LC_{50}	2	5.21, 6.51	Leight and Van Dolar, 1999.
G. palustris	LC_{50}	3	0.6, 1.02	Leight and Van Dolar, 1999.
G. palustris	LC_{50}	4	0.19, 0.3	Leight and Van Dolar, 1999.
Crassostrea virginica (American or virginia oyster) Spat	EC_{50} Immobility	4	84	Environmental Fate and Effects Division, U.S. EPA., 2000.

Species (Appendix 8)	Effect	Duration (days)	Reported Effect Concentration (µg/L)	Reference
Mytilus galloprovincialis (Mediterranean mussel) 6.95 g soft tissue, 5.13 cm	LC_{50}	4	22,500	Serrano, *et al.*,1995
M. galloprovincialis (Mediterranean mussel) 6.95 g soft tissue, 5.13 cm	NOEC	4	4,900	Serrano, *et al.*, 1995
Palaemonetes pugio (Daggerblade grass shrimp) Embryos, 6-10 D	NOEC	4	0.25, 1	Wilson,1997.
P. pugio (Daggerblade grass shrimp) Embryos, 6-10 D	LOEC	4	0.5	Wilson,1997.
Brachionus plicatilis (Rotifer) Russian strain, <= 5 H, female	NOEC	1 hour	1,500	Juchelka and Snell, 1995
Ceriodaphnia dubia (<24 H, Female)	NOEC	1 hour	5	Juchelka and Snell, 1995
Paramecium Aurelia	NOEC	1 hour	3,000	Juchelka and Snell, 1995
Daphnia magna	EC_{50} Immobility	2	0.1 (flow through), 1.7 (Static)	Environmental Fate and Effects Division, U.S. EPA., 2000.
D. pulex	EC_{50}	2	0.42 (renewal)	Van der Hoeven and Gerritsen, 1997.
D. pulex	EC_{50}	3	0.09 (renewal)	Van der Hoeven and Gerritsen, 1997.
D. pulex	EC_{50}	6	0.17 (renewal)	Van der Hoeven and Gerritsen, 1997.
D. pulex	EC_{50}	8	0.19 (renewal)	Van der Hoeven and Gerritsen, 1997.
D. pulex	EC_{50}	10	0.17 (renewal)	Van der Hoeven and Gerritsen, 1997.
D. pulex	EC_{50}	2	0.25(Static)	Van der Hoeven and Gerritsen, 1997.
D. pulex	EC_{50}	1	0.42 (Static)	Van der Hoeven and Gerritsen, 1997.
Gammarus pulex	EC_{50}	2	0.3	Van Wijngaarden, *et al.*, 1996.
D. pulex	LOEC	2	0.2	Van der Hoeven and Gerritsen,1997
D. pulex	LOEC	17	0.1	Van der Hoeven and Gerritsen,1997
D. pulex	NOEC	2	0.2	Van der Hoeven and Gerritsen,1997
D. pulex	NOEC	17	0.05	Van der Hoeven and Gerritsen,1997
Artemia sp.	EC_{50}	1	1,900, 2,000	Guzzella, *et al.*, 1997.
Brachionus plicatilis	EC_{50}	1	1,400, 1,900, 1,700	Guzzella, *et al.*, 1997.
D. pulex Adult 7-8 days	EC_{50}	8	0.28	Van der Hoeven and Gerritsen,1997
Americamysis bahia (Opossum shrimp) Juvenile	LC_{50}	4	0.05	Environmental Fate and Effects Division, U.S. EPA., 2000.
Ceriodaphnia dubia Neonate, < 24 H	LC_{50}	4	0.06	Bailey, *et al.*,1996

Species (Appendix 8)	Effect	Duration (days)	Reported Effect Concentration (µg/L)	Reference
D. magna *< 24 h, 3rd to 5th brood neonate*	LC_{50}	2	344	Guihermino, *et al.*,2000
Hyalella azteca *Juvenile, 7-14 days*	LC_{50}	4	0.04	Ankley and Collyard, 1995.
H. azteca	LC_{50}	10	0.09	Phipps, *et al.*,1995
Palaemonetes pugio *Juvenile*	LC_{50}	4	83	Environmental Fate and Effects Division, U.S. EPA., 2000.
D. magna	LOEC	2	0.5	Moore, *et al.*, 1998
H. azteca	LOEC	2	0.1	Moore, *et al.*, 1998
Palaemonetes pugio *Embryos, 6-10 D*	LOEC	4	0.50	Wilson,1997
P. pugio *Embryos, 6-10 D*	NOEC	4	0.25	Wilson,1997

Appendix 9: Examples of relative sensitivities of aquatic animals and some invertebrates to **dimethoate**.

Species (Appendix 9)	Effect	Duration (days)	Reported Effect Concentration (µg/L)	Reference
Macrobrachium lamarrei(intermolt, 12-17 mm.)	LC_{50}	1	4,275	Mary *et al.*, 1986.
M. lamarrei(intermolt, 12-17 mm.)	LC_{50}	2	3,459	Mary *et al.*, 1986.
M. lamarrei (intermolt, 12-17 mm.)	LC_{50}	3	2,630	Mary *et al.*, 1986.
Metapenaeus monoceros (intermoult, 75 mm., 2.5 g)	LC_{50}	4	2,080, 2,470, 2,860	Reddy and Rao, 1992
Pandalus montagui (Aesop shrimp): Adult	LC_{50}	2	33	Portmann and Wilson, 1971.
Oncorhynchus mykiss (rainbow trout) 6 month	EC_{50} Behavioral changes	2	8,600	Canton *et al*., 1980
Poecilia reticulata (Guppy) 21-28 days	EC_{50} Behavioral changes	1	187,000	Canton *et al*., 1980
P. reticulata (Guppy) 21-28 days	EC_{50} Behavioral changes	2	135,000	Canton *et al*., 1980
P. reticulata (Guppy) 21-28 days	EC_{50}	3	135,000	Canton *et al*., 1980
P. reticulata (Guppy) 21-28 days	EC_{50}	4	120,000	Canton *et al*., 1980
Oryzias latipes (Medaka, high-eyes) ; 28-35 Days	EC_{50} Immobility	1	105,000	Canton *et al*., 1980
O. latipes (Medaka, high-eyes) ; 28-35 Days	EC_{50} Immobility	2	128,000	Canton *et al*., 1980
O. latipes (Medaka, high-eyes) ; 28-35 Days	EC_{50} Immobility	3	118,000	Canton *et al*., 1980
O. latipes (Medaka, high-eyes) ; 28-35 Days	EC_{50} Immobility	4	108,000	Canton *et al*., 1980
Auguilla japonica (Japanese eel)	LC_{50}	1	200,000	Yokoyama *et al.*, 1988.
Carassius auratus (Goldfish) 0.6 g	LC_{50}	2	180,000	Environmental Fate and Effects Division, U.S.EPA., 2000.
Cirrhinus mrigala (hawkfish) 30 mm. fingerling	LC_{50}	4	10,100	Kulshrestha *et al.*, 1986.
Clarias batrachus (walking catfish) 35-40 g, 20-22 cm.	LC_{50}	4	65	Begum and Vijayaraghavan,1995
C. batrachus (Walking catfish) 38 g, 20 cm.	LC_{50}	4	50,000	Begum *et al*.,1994
Cyprinodon variegatus (Sheepshead minnow) 0.52 g	LC_{50}	4	111,000	Environmental Fate and Effects Division, U.S.EPA., 2000.
Cyprinus carpio (Fingerling, 4.5-5.5 cm, 1.1-1.4 g)	LC_{50}	1	4,230 , 4,620	Dutt and Guha, 1988.
C. carpio (Fingerling, 4.5-5.5 cm, 1.1-1.4 g)	LC_{50}	2	4,000 , 4,550	Dutt and Guha, 1988.
C. carpio (Fingerling, 4.5-5.5 cm, 1.1-1.4 g)	LC_{50}	3	3,560 , 4,000	Dutt and Guha, 1988.
Danio rerio (Zebra danio)	LC_{50}	2	7,500 , 8,200	Beusen and Neven, 1989.

Species (Appendix 9)	Effect	Duration (days)	Reported Effect Concentration (µg/L)	Reference
D. rerio *(Zebra danio)*	LC_{50}	4	6,800 , 7,800	Beusen and Neven, 1989.
D. rerio *(Zebra danio)*	LC_{50}	7	6,200 , 7,000	Beusen and Neven, 1989.
Heteropneustes fossilis *(Indian catfish)* *20-40 g, 17-25 cm.*	LC_{50}	0.5	12,880	Chaturvedi and Agrawal, 1991.
H. fossilis *(Indian catfish)* *20-40 g, 17-25 cm.*	LC_{50}	1	12,130	Chaturvedi and Agrawal, 1991.
H. fossilis *(Indian catfish)* *20-40 g, 17-25 cm.*	LC_{50}	2	11,400	Chaturvedi and Agrawal, 1991.
H. fossilis *(Indian catfish)* *20-40 g, 17-25 cm.*	LC_{50}	4	10,550	Chaturvedi and Agrawal, 1991.
Labeo rohita *(30 mm. ,fingerling)*	LC_{50}	4	10,200	Kulshrestha *et al.*,1986
Lepomis macrochirus *(Bluegill)*	LC_{50}	4	6,000	Johnson and Finley, 1980.
L. macrochirus *(Bluegill) 0.3 g*	LC_{50}	1	28,000	Mayer and Ellersieck, 1986
L. macrochirus *(Bluegill) 0.3 g*	LC_{50}	4	6,000	Mayer and Ellersieck, 1986
Oncorhynchus mykiss *(rainbow trout)* *Juvenile*	LC_{50}	4	7,500	Environmental Fate and Effects Division, U.S.EPA., 2000.
Phoxinus phoxinus *(Minnow)*	LC_{50}	4	500	Grande *et al.*, 1994.
Poecilia reticulate *(Guppy)*	LC_{50}	2	10,400, 11,200, 13,000	Beusen and Neven, 1989.
P. reticulate *(Guppy)*	LC_{50}	4	10,400, 11,200, 13,000	Beusen and Neven, 1989.
Tilapia mossambica *(Mozambique tilapia) Fry, 1-1.5 cm.*	LC_{50}	2	31,260	Shafiei and Costa], 1990.
T. mossambica *(Mozambique tilapia) Fingerling, 2.5-4.5 cm.*	LC_{50}	2	28,510	Shafiei and Costa], 1990.
Aedes aegypti *(Yellow fever mosquito) 1st instar larvae, 24 h*	LC_{50}	2	5,040, 6,410	Song *et al.*,1997
A. taeniorhynchus *(Yellow fever mosquito) 1st instar larvae, 24 h*	LC_{50}	2	31	Song *et al.*,1997
Culex fatigans *(Mosquito) 4th instar larvae*	LC_{50}	1	460	Tasbassum *et al.*,1993
Biomphalaria alexandrina *(Snail) 11.6 mm.*	EC_{50} Immobility	4	3.10	Aboul-Ela and Khalil, 1987
Crassostrea virginica *(Virginia oyster)* *Spat*	EC_{50} Immobility	4	113,000	Environmental Fate and Effects Division, U.S.EPA., 2000.
Daphnia magna *(< 24 h)*	EC_{10}	16	210	Deneer *et al.*, 1988.
D. magna *(< 24 h)*	NOEC	16	29	Deneer *et al.*, 1988.

Species (Appendix 9)	Effect	Duration (days)	Reported Effect Concentration (µg/L)	Reference
D. magna *(neonate, < 24 h)*	EC_{50} Immobility	23	110, 150, 190	Beusen and Neven, 1989
D. magna *(neonate, < 24 h)*	EC_{50} Immobility	2	560, 740, 800, 880, 1,500, 1,800	Beusen and Neven, 1989
Artemia sp. Larvae	LC_{50}	1	297,000, 305,000, 308,000	Guzzella *et al.*,1997
Brachionus plicatilis (Rotifer) Neonate	LC_{50}	1	218,000, 251,000, 264,000	Guzzella *et al.*,1997
Americamysis bahia (Opossum shrimp)	LC_{50}	4	15,000	Environmental Fate and Effects Division, U.S.EPA., 2000.
D. magna *Neonate < 24 h*	LC_{50}	23	110 ,230	Beusen and Neven, 1989
D. magna *Neonate < 24 h*	LC_{50}	2	830, 1,260, 1,600, 1,700, 2,000,	Beusen and Neven, 1989
D. magna *< 1 D*	LC_{50}	2	6,400	Canton et.al., 1980
D. magna *< 1 D*	LC_{50}	20	310	Canton et.al., 1980
D. magna *(Newly hatched, < 24 h)*	LC_{50}	2	3,120	Song *et al.*, 1997
Gammarus italicus *(Adult male)*	LC_{50}	4	3,800	Pantani *et al.*,1997
G. lacustris *(adult)*	LC_{50}	4	200	Environmental Fate and Effects Division, U.S.EPA., 2000.

Appendix 10 : Examples of relative sensitivities of aquatic animals and some invertebrates to **profenofos.**

Species (Appendix 10)	Effect	Duration (days)	Reported Effect Concentration (µg/L)	Reference
Callinectes sapidus (Blue crab)	LC_{50}	4	33	Environmental Fate and Effects Division U.S.EPA.,2000.
Penaeus duorarum (Northern pink shrimp)	LC_{50}	4	4.6	Environmental Fate and Effects Division U.S.EPA.,2000.
Ameiurus melas (Black bullhead)	LC_{50}	4	20	Environmental Fate and Effects Division U.S.EPA.,2000.
Anguilla japonica (Japanese eel)	LC_{50}	2	3,000	Yokoyama *et al.*, 1988.
Carassius carassius (Crucian carp)	LC_{50}	4	90	Environmental Fate and Effects Division U.S.EPA.,2000.
Ictalurus punctatus (Juvenile)	LC_{50}	4	21	Environmental Fate and Effects Division U.S.EPA.,2000.
I. punctatus (0.6 g)	LC_{50}	2	20	Mayer and Ellersieck, 1986
I. punctatus (0.6 g)	LC_{50}	4	13.5	Mayer and Ellersieck, 1986
Lagodon rhomboids (Pinfish)	LC_{50}	4	7.7	Environmental Fate and Effects Division U.S.EPA.,2000.
Lepomis macrochirus (Bluegill) (0.35 g)	LC_{50}	4	19	Environmental Fate and Effects Division U.S.EPA.,2000.
L. macrochirus (Bluegill) (0.3 g)	LC_{50}	2	42	Mayer and Ellersieck, 1986
Oncorhynchus mykiss (rainbow trout) (1.0 g)	LC_{50}	4	21	Environmental Fate and Effects Division U.S.EPA.,2000.
O. mykiss (rainbow trout) (0.6 g)	LC_{50}	4	23.5	Mayer and Ellersieck, 1986
Pimephales promelas (Fathead minnow)	LC_{50}	3	30	Environmental Fate and Effects Division U.S.EPA.,2000.
Poecilia reticulate (Guppy)	LC_{50}	4	800	Environmental Fate and Effects Division U.S.EPA.,2000.
Chironomus plumosus (Midge) 3rd instar	EC_{50} Immobility	2	1.8	Mayer and Ellersieck, 1986
C. tentans (Midge)	LC_{50}	4	86	Environmental Fate and Effects Division U.S.EPA.,2000.
Crassostrea virginica (American oyster)	EC_{50} Immobility	4	263	Environmental Fate and Effects Division U.S.EPA.,2000.

Species (Appendix 10)	Effect	Duration (days)	Reported Effect Concentration (µg/L)	Reference
Physa pumilia (Pouch snail)	LC_{50}	4	1.15	Environmental Fate and Effects Division U.S.EPA.,2000.
Daphnia magna *(< 24 h)*	EC_{50} Immobility	2	0.93, 1.06, 2.8	Environmental Fate and Effects Division U.S.EPA.,2000.
D. magna *(1st instar)*	EC_{50} Immobility	2	0.5, 1.4	Mayer and Ellersieck, 1986
Gammarus pseudolimnaeus (Scud) (Immature)	LC_{50}	4	0.8, 1.8	Mayer and Ellersieck, 1986

Appendix 11: Water quality and pesticide concentration on deployment day in carbendazim microcosm and results of the bioassays.

Treatment	Total Ammonia (mg/L)	Unionized ammonia (mg/L)	Nitrite (mg/L)	Hardness (mg/L $CaCO_3$)	Chlorophyll a (mg/L)
Control 1	0.561	0.395	0.029	156.76	7.20
Control 2	0.240	0.135	0.097	129.73	2.88
Control 3	0.388	0.223	0.068	154.95	2.88
Control 4	0.000	0.000	0.007	122.52	30.22
M-3.3 rep 1	0.265	0.172	0.131	126.58	4.32
M-3.3 rep 2	0.400	0.182	0.114	123.42	2.88
M-33 rep 1	0.178	0.090	0.075	163.51	1.44
M-33 rep 2	0.611	0.249	0.210	135.14	2.88
M-100 rep 1	0.821	0.338	0.082	148.65	10.07
M-100 rep 2	0.002	0.002	0.064	131.53	5.76
M-1000 rep 1	0.017	0.008	0.119	166.67	5.76
M-1000 rep 2	0.000	0.000	0.014	126.13	28.78

Appendix 11: (continued) Water quality and pesticide concentration in carbendazim microcosm and results of the bioassays.

Treatment	TSS (mg/L)	TVS (mg/L)	Water temperature	Dissolved Oxygen (mg/L)	% oxygen saturation
Control 1	25.5	14.0	31.0	11.4	156
Control 2	30.0	25.5	31.7	9.1	126
Control 3	13.0	11.5	31.5	10.2	140
Control 4	39.5	8.5	31.3	12.5	172
M-3.3 rep 1	14.0	8.0	31.4	10.0	137
M-3.3 rep 2	24.5	32.5	31.5	8.9	122
M-33 rep 1	10.0	3.0	31.1	9.4	129
M-33 rep 2	15.0	1.0	31.4	9.1	125
M-100 rep 1	38.0	11.0	31.0	8.9	121
M-100 rep 2	21.0	13.5	31.5	11.9	164
M-1000 rep 1	12.0	5.0	31.2	8.4	115
M-1000 rep 2	59.0	23.0	31.4	11.6	159

Appendix 11: (continued) Water quality and pesticide concentration in carbendazim microcosm and results of the bioassays.

Treatment	Conductivity (µS)	pH	Mortality after exposure	Post-exposure feeding rate	Carbendazim Conc. (µg/L)
Control 1	750	9.4	35	14.93	0
Control 2	772	9.2	20	14.93	0
Control 3	770	9.2	30	14.93	0
Control 4	786	10.2	90	14.93	0
M-3.3 rep 1	775	9.3	40	12.8	2.57
M-3.3 rep 2	752	9.0	5	12.8	2.57
M-33 rep 1	816	9.1	25	8.44	26.13
M-33 rep 2	783	8.9	20	8.44	26.13
M-100 rep 1	737	8.9	35	7.28	85.6
M-100 rep 2	785	9.8	70	7.28	85.6
M-1000 rep 1	773	9.0	55	8.1	777.36
M-1000 rep 2	742	9.9	35	8.1	777.36

Appendix 12: Mortality (%) and post-exposure feeding rate (number of *Artemia*/prawn/hr) of prawn after 24 hours exposure in the field.

Season	Date	Farm name	Mortality (%)	Feeding rate	SE
Wet	9 June 04 (bioassay 1)	Chuchart (veg1)	36.0	7.82	1.02
		Chuchart (supply canal as ref.)	100.0	c.d.	c.d.
		Jars (soft ASTM) Chuchart	6.7	14.75	0.66
		Surat (veg 2)	58.0	6.85	1.69
		Surat (supply canal as ref.)	100.0	c.d.	c.d.
		Jars (soft ASTM) Surat	6.7	6.0	2.29
		Chanchai (veg 3)	67.0	11.45	2.90
		Chanchai (supply canal as ref.)	80.0	18.0	1.81
		Jars (soft ASTM) Chanchai	13.3	16.67	4.08
Wet	22 June 04 (bioassay 2)	Organic farm (Ref)	55.0	15.82	1.65
		Jars (soft ASTM) organic farm	8.9	13.32	1.54
Wet	26 Aug 04 (bioassay 3)	Mali (Tan 1)	37.0	15.75	1.61
		Mali (supply canal as ref.)	95.0	16.25	1.25
		Jars (soft ASTM) Mali	10.0	> 25	n.a.
		Tew (Tan 2)	75.0	12.45	1.35
		Tew (supply canal as ref.)	90.0	14.06	4.28
		Jars (soft ASTM) Tew	0.0	> 25	n.a.
Dry	25 Nov 04 (bioassay 4)	Chumpon (veg 4)	30.0	14.30	2.24
		Chumpon (supply canal as ref.)	10.0	14.03	1.28
		Jars (soft ASTM) Chumpon	10.0	4.5	2.38
		Somjit (veg 5)	42.5	12.92	2.08
		Somjit (supply canal as ref.)	67.5	14.30	1.48
		Jars (soft ASTM) Somjit	6.7	18.67	4.87
Dry	2 Dec 04 (bioassay 5)	Add (Tan 3)	25.0	12.80	1.97
		Add (supply canal as ref.)	30.0	14.58	0.83
		Jars (soft ASTM) Add	16.7	10.67	1.40
		Organic farm (Org 2)	73.8	8.62	0.80
		Jars (soft ASTM) organic farm	10.0	11.58	2.89
Dry	9 Feb 05 (bioassay 6)	Chuchart (veg 6)	62.5	7.38	1.18
		Jars (soft ASTM) Chuchart	26.7	13.7	1.66
		Reference AIT	80.0	9.32	1.58
		Jars (soft ASTM) reference AIT	30.0	12.44	1.03
Dry	14 Feb 05 (bioassay 7)	Samnieng (Fr 1)	100.0	c.d.	c.d.
		Jars (soft ASTM) Samnieng	20.0	17.75	1.00
		Reference AIT (Ref 1)	70.0	17.75	1.72
		Jars (soft ASTM) Lab	13.3	20.36	0.79
Dry	17 Feb 05 (bioassay 8)	Samnieng (Fr 1)	100.0	c.d.	c.d.
		Jars (soft ASTM) Samnieng	3.3	> 25	n.a.
		Reference AIT (H) (Ref 2)	65.0	17.25	0.63
		Jars (soft ASTM) Lab	20.0	20.75	2.0
Dry	21 Feb 05 (bioassay 9)	Samnieng (Fr 1)	30.0	7.91	2.79
		Jars (soft ASTM) Samnieng	20.0	> 25	n.a.
		Reference AIT (H)	68.3	9.0	1.60
		Jars (soft ASTM) Lab	16.7	8.12	1.54

Note : c.d. – could not detect
n.a. - no *Artemia* left after 24 hours

Appendix 13: Water quality in the field study and reference sites

Season	Date	Farm name	Water temp. (˚C)	pH	Conductivity (µS)
Wet	9 June 04	Chuchart (veg1)	32.7	9.0	112
		Chuchart (supply canal as ref.)	32.5	7.1	105
		Surat (veg 2)	36.9	8.2	401
		Surat (supply canal as ref.)	33.9	6.8	146
		Chanchai (veg 3)	37.6	8.9	146
		Chanchai (supply canal as ref.)	32.5	7.2	715
Wet	22 June 04	Organic farm (Ref)	30.1	6.9	650
Wet	26 Aug 04	Mali (Tan 1)	32.5	6.5	354
		Mali (supply canal as ref.)	30.0	6.0	334
		Tew (Tan 2)	34.5	6.7	356
		Tew (supply canal as ref.)	33.0	6.9	367
Dry	25 Nov 04	Chumpon (veg 4)	30.0	8.0	1080
		Chumpon (supply canal as ref.)	28.3	7.4	804
		Somjit (veg 5)	28.8	8.7	2500
		Somjit (supply canal as ref.)	31.1	7.4	839
Dry	2 Dec 04	Add (Tan 3)	29.1	6.9	374
		Add (supply canal as ref.)	30.4	7.0	383
		Organic farm (Org 2)	27.7	7.1	490
Dry	9 Feb 05	Chuchart (veg 6)	32.7	9.0	832
		Reference AIT	32.0	7.0	480
Dry	14 Feb 05	Samnieng (Fr 1)	32.8	6.9	853
		Reference AIT (Ref 1)	29.0	7.3	507
Dry	17 Feb 05	Samnieng (Fr 1)	33.8	8.2	1380
		Reference AIT (H) (Ref 2)	30.0	6.9	502
Dry	21 Feb 05	Samnieng (Fr 1)	33.1	8.1	1210
		Reference AIT (H)	31.0	7.1	510

Appendix 13: (continued) Water quality in the field study and reference sites.

Season	Date	Farm name	D.O. (mg/L)	% Saturation	Hardness (mg $CaCO_3$)	Nitrite (mg/L)
Wet	9 June 04	Chuchart (veg1)	2.0	28	432.9	0.07
		Chuchart (supply canal as ref.)	0.1	1	225.2	0.03
		Surat (veg 2)	0.2	3	467.9	0.03
		Surat (supply canal as ref.)	1.7	25	167.7	2.21
		Chanchai (veg 3)	18.6	291	307.8	0.00
		Chanchai (supply canal as ref.)	4.8	68	275.3	0.58
Wet	22 June 04	Organic farm (Ref)	4.5	61	211.0	0.00
Wet	26 Aug 04	Mali (Tan 1)	3.4	48	114.1	0.03
		Mali (supply canal as ref.)	1.8	24	164.2	0.02
		Tew (Tan 2)	4.5	66	174.2	0.02
		Tew (supply canal as ref.)	0.5	7	164.2	0.03
Dry	25 Nov 04	Chumpon (veg 4)	5.8	78	326.2	0.21
		Chumpon (supply canal as ref.)	4.8	63	243.8	0.06
		Somjit (veg 5)	7.9	104	549.8	0.43
		Somjit (supply canal as ref.)	2.0	27	274.2	0.65
Dry	2 Dec 04	Add (Tan 3)	2.4	32	101.5	0.05
		Add (supply canal as ref.)	3.4	46	101.8	0.06
		Organic farm (Org 2)	0.7	9	96.1	0.13
Dry	9 Feb 05	Chuchart (veg 6)	9.9	140	192.8	0.00
		Reference AIT	0.7	10	171.2	0.03
Dry	14 Feb 05	Samnieng (Fr 1)	1.2	17	370.3	0.02
		Reference AIT (Ref 1)	3.2	42	178.3	0.06
Dry	17 Feb 05	Samnieng (Fr 1)	7.0	101	416.2	0.02
		Reference AIT (H) (Ref 2)	3.8	51	184.5	0.06
Dry	21 Feb 05	Samnieng (Fr 1)	8.8	126	387.0	0.001
		Reference AIT (H)	1.8	25	173.9	0.04

Appendix 13: (continued) Water Quality in the field study and reference sites.

Season	Date	Farm name	Un-ionized Amonia (mg/L)	Chlorophyll a (mg/L)	TSS (mg/L)	TVS (mg/L)
Wet	9 June 04	Chuchart (veg1)	0.1918	54.7	122.5	26.1
		Chuchart (supply canal as ref.)	0.0081	30.2	24.9	7.3
		Surat (veg 2)	0.2526	24.5	95.1	23.7
		Surat (supply canal as ref.)	0.0080	5.0	23.7	4.3
		Chanchai (veg 3)	0.1854	14.4	316.7	60.8
		Chanchai (supply canal as ref.)	0.0122	24.5	21.8	5.5
Wet	22 June 04	Organic farm (Ref)	0.0004	9.90	-	-
Wet	26 Aug 04	Mali (Tan 1)	0.0002	0.003	13.6	3.2
		Mali (supply canal as ref.)	0.0001	0.004	5.7	1.3
		Tew (Tan 2)	0.0004	0.003	18.6	11.3
		Tew (supply canal as ref.)	0.0005	0.003	7.7	3.4
Dry	25 Nov 04	Chumpon (veg 4)	0.0037	1.8	101.9	1.94
		Chumpon (supply canal as ref.)	0.0007	19.1	30.0	1.25
		Somjit (veg 5)	0.0108	27.1	18.2	1.82
		Somjit (supply canal as ref.)	0.0021	11.0	10.8	0.40
Dry	2 Dec 04	Add (Tan 3)	0.0004	3.26	12.2	2.00
		Add (supply canal as ref.)	0.0005	10.6	30.2	12.7
		Organic farm (Org 2)	0.0005	4.8	16.4	2.44
Dry	9 Feb 05	Chuchart (veg 6)	0.0000	0.6	168	62
		Reference AIT	0.0030	0.04	30.0	15.2
Dry	14 Feb 05	Samnieng (Fr 1)	0.0019	0.06	176	42
		Reference AIT (Ref 1)	0.0064	0.05	32.0	18.7
Dry	17 Feb 05	Samnieng (Fr 1)	0.0042	0.07	104	38
		Reference AIT (H) (Ref 2)	0.0013	0.03	35.0	14.8
Dry	21 Feb 05	Samnieng (Fr 1)	0.0311	0.05	64	34
		Reference AIT (H)	0.0020	0.05	38.0	15.8

Appendix 14: Pesticide concentration during field deployment

Season	Date	Farm name	Profenofos (µg./L)	Dimethoate (µg /L)	Chlorpyrifos (µg /L)	Carbendazim (µg /L)
Wet	9 June 04	Chuchart (veg1)	0	0	0	0
		Chuchart (supply canal as ref.)	0	0	0	0
		Surat (veg 2)	0	0	0	0
		Surat (supply canal as ref.)	0	0	0	0
		Chanchai (veg 3)	0	0	0	0
		Chanchai (supply canal as ref.)	0	0	0	0
Wet	22 June 04	Organic farm (Ref)	0	0.15	0	0
Wet	26 Aug 04	Mali (Tan 1)	0	0.16	0	0
		Mali (supply canal as ref.)	0	0.07	0	0
		Tew (Tan 2)	0	0	0	0
		Tew (supply canal as ref.)	0	0.15	0	0
Dry	25 Nov 04	Chumpon (veg 4)	11.9	0	0	0
		Chumpon (supply canal as ref.)	11.5	0	0	0
		Somjit (veg 5)	19.8	0	0	0
		Somjit (supply canal as ref.)	18.8	0	0	0
Dry	2 Dec 04	Add (Tan 3)	15.9	0	0	0
		Add (supply canal as ref.)	16.7	0	0	0
		Organic farm (Org 2)	15.5	0	0	0
Dry	9 Feb 05	Chuchart (veg 6)	29.0	0	0	0
		Reference AIT	0	0	0	0
Dry	14 Feb 05	Samnieng (Fr 1)	0	0	0	8.9
		Reference AIT (Ref 1)	0	0	0	0
Dry	17 Feb 05	Samnieng (Fr 1)	0	0	0	0
		Reference AIT (H) (Ref 2)	0	0	0	0
Dry	21 Feb 05	Samnieng (Fr 1)	0	0	0	0
		Reference AIT (H)	0	0	0	0

Appendix 15: Field water quality, pesticide concentration and results of the bioassays. Only sites with dissolved oxygen saturation higher than 60 % are included.

Farm name	Survival rate (%)	Feeding rate	Water temp.	pH
Chanchai (veg 3)	33.0	11.45	37.6	8.9
Chanchai (supply canal as ref.)	20.0	18.00	32.5	7.2
Organic farm (Ref)	45.0	15.82	30.1	6.9
Tew (Tan 2)	35.0	12.45	34.5	6.7
Chumpon (veg 4)	70.0	14.30	30.0	8.0
Chumpon (supply canal as ref.)	90.0	14.03	28.3	7.4
Somjit (veg 5)	57.5	12.92	28.8	8.7
Chuchart (veg 6)	37.5	7.38	32.7	9.0
Samnieng (Fr 1) 17 Feb	0	0	33.8	8.2
Samnieng (Fr 1) 21 Feb	70.0	7.91	33.1	8.1

Appendix 15: (continued) Field water quality and pesticide concentration and results of the bioassays. Only sites with dissolved oxygen saturation higher than 60 % are included.

Farm name	Conductitvity(µS)	DO(mg/L)	%Saturation	Hardness ($mgCaCO_3$)
Chanchai (veg 3)	146	18.6	291	307.8
Chanchai (supply canal as ref.)	715	4.8	68	275.3
Organic farm (Ref)	650	4.5	61	211.0
Tew (Tan 2)	356	4.5	66	174.2
Chumpon (veg 4)	1080	5.8	78	326.2
Chumpon (supply canal as ref.)	804	4.8	63	243.8
Somjit (veg 5)	2500	7.9	104	549.8
Chuchart (veg 6)	832	9.9	140	192.8
Samnieng (Fr 1) 17 Feb	1380	7.0	101	416.2
Samnieng (Fr 1) 21 Feb	1210	8.8	126	387.0

Appendix 15: (continued) Field water quality and pesticide concentration and results of the bioassays. Only sites with dissolved oxygen saturation higher than 60 % are included.

Farm name	Nitrite (mg/L)	Unionized Ammonia (mg/L)	Chlorophyll a (mg/L)	TSS (mg/L)
Chanchai (veg 3)	0.00	0.18545	14.4	316.7
Chanchai (supply canal as ref.)	0.58	0.01222	24.5	21.8
Organic farm (Ref)	0.00	0.00045	9.9	-
Tew (Tan 2)	0.02	0.00038	0.003	18.6
Chumpon (veg 4)	0.21	0.00373	1.8	101.9
Chumpon (supply canal as ref.)	0.06	0.00071	19.1	30.0
Somjit (veg 5)	0.43	0.01084	27.1	18.2
Chuchart (veg 6)	0.00	0.00000	0.6	168.0
Samnieng (Fr 1) 17 Feb	0.02	0.00425	0.07	104.0
Samnieng (Fr 1) 21 Feb	0.001	0.03113	0.05	64.0

Appendix 15: (continued) Field water quality and pesticide concentration and results of the bioassays. Only sites with dissolved oxygen saturation higher than 60 % are included.

Farm name	TVS (mg/L)	Profenofos (µg/L)	Dimethoate (µg/L)
Chanchai (veg 3)	60.8	0	0
Chanchai (supply canal as ref.)	5.5	0	0
Organic farm (Ref)	-	0	0.15
Tew (Tan 2)	11.3	0	0
Chumpon (veg 4)	1.94	11.9	0
Chumpon (supply canal as ref.)	1.25	11.5	0
Somjit (veg 5)	1.82	19.8	0
Chuchart (veg 6)	62.0	29.0	0
Samnieng (Fr 1) 17 Feb	38.0	0	0
Samnieng (Fr 1) 21 Feb	34.0	0	0