Interactive toxicity of a triazole-derivative fungicide and an organophosphate pesticide in the marine shrimp *Litopenaeus vannamei* (Boone, 1931)

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.

Candidate:

ABSTRACT

Varied agricultural activities integrate with the shrimp farms in the state of Sinaloa on the North-Western Pacific coast of Mexico. Out of many wild species found in this area, the marine shrimp *Litopenaeus vannamei* was selected for this study as it is highly sensitive to a wide range of pollutants and because it is the principal marine shrimp for aquaculture in this region. *L. vannamei* plays an important ecological role in estuarine environments along this coast where it supports one of the most important fishery resources in Mexico.

In this study, a tropical marine bioassay was developed to assess the toxicity of pesticides in the shrimp *L. vannamei*. A flow-through system (FTS) was designed to provide the optimal conditions for shrimp juveniles, which were individually placed in cages to evaluate the moulting rate and behaviour during the experiments, as well as mortality. The FTS was a simple apparatus, consisting of a 16-channel peristaltic pump which delivered the test solution from reservoirs into exposure chambers under controlled conditions (flow rate, temperature and photoperiod). Time-independent exposures were carried out in flow-through conditions for the organophosphate pesticide methyl-parathion, and a triazole-derivative fungicide in two preparations: Tilt (commercial formulation, 25% propiconazole) and technical grade propiconazole. The threshold median lethal concentrations (threshold LC50) were determined for methyl-parathion (4.4 $\mu g.l^{-1}$), Tilt (1137 $\mu g.l^{-1}$), and propiconazole (1716 $\mu g.l^{-1}$). During these tests, the comparative toxicity of the pesticides was examined,

and in particular, the toxic contribution of propiconazole in the commercial formulation, Tilt, was established.

The toxicity data of each one of the toxicants was also used to assess the interactive toxicity for each form of the fungicide (technical grade and formulation) with methyl-parathion. When methyl-parathion was combined with Tilt, a less than additive effect was found. On the other hand, when methyl-parathion and technical grade propiconazole were combined, results ranged from a more than additive to a simple additive effect.

On the whole, exposure to methyl-parathion produced erratic movements and hypersensitivity, and no indication of moult retardation was observed. On the other hand, exposure to fungicides caused lethargy and slight moult retardation was observed. Feeding inhibition was frequently observed in all tests.

When Tilt was used in a subchronic test, several morphological abnormalities occurred in the test organisms in relation to exposure concentration. Other effects observed during this exposure were changes in feeding and motor behaviour, and alterations in moulting rate.

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CHAPTER 1.

GENERAL INTRODUCTION

Some decades ago, pesticides were considered as a final solution to the ever-increasing demand for agricultural products and to control insect vectors of human diseases. However, as the use of pesticides intensified, negative impacts were revealed (Carson, 1962). It was then evident that pesticides not only control and eliminate pests, but can also have effects upon a wide variety of non-target species, including humans. Moreover, it is now understood that the indiscriminate use of pesticides led to the development of pesticide resistance, which in turn resulted in the need to develop more toxic compounds and formulations and, to a steady increase in the volume of application (Crow, 1957; Pimentel *et al.*, 1992).

Although there have been advances in non-chemical alternatives to pesticides such as biological control or utilisation of pest-resistant genetic varieties of plants, the production of high yields of high-quality crops cannot be achieved without the aid of pesticides (Eidt *et al.,* 1989), and pests still represent a major cause of crop losses, both in the field and in storage (Nimmo, 1985; Pimentel *et al.*, 1992). For instance, the total crop losses from insect damage in the United States have nearly doubled (7% to 13%) from 1945 to 1989 despite a tenfold increase in insecticide use (Pimentel *et al.*, 1991). The economic and environmental costs associated with pest resistance to pesticides are significantly greater in tropical developing countries because pesticides are used there not only to control agricultural pests but also for the control of

disease vectors (Pimentel *et al.*, 1992). This is particularly true in Latin America, which has become one of the fastest growing regions for agrochemical sales (PANUPS, 1996). The consequences of pesticide use in agricultural-based export economies are clearly evident, as pesticide poisoning has been recognised as an important public health problem in developing countries (Wesseling *et al.*, 1993; Tinoco-Ojanguren and Halperin, 1998; Van der Hoek *et al.*, 1998; Wesseling *et al.*, 1997). The World Health Organization estimates that, annually, the majority of the 3 million cases of severe poisoning and almost the 220 000 fatalities worldwide occur in developing countries (WHO/UNEP, 1990). The population growth rates and economic constraints of many developing countries are reflected in the steady expansion of the proportion of land in agriculture production in the tropics, contrasting sharply with the constant or declining proportion in developed countries (World Resources Institute, 1994). Although Mexico does not depend heavily on exporting raw agricultural products (Lacher and Goldstein, 1997), its market for pesticides has grown from $199 million U.S. dollars in 1980 to $351 million in 1985 and had reached $565 million by 1990 (Ortega *et al.*, 1994).

Nevertheless, the environmental consequences of agricultural expansion and other human activities in the tropics are difficult to predict from our current state of knowledge. In fact, Bourdeau *et al.* (1989) consider that the understanding of the fate and transport of environmental contaminants in northern latitudes has little applicability in the tropics. For instance, the combinations of temperature and precipitation in temperate regions are substantially different than those encountered in the tropics, which also sustain a considerably higher biological

diversity that is translated in more possible interactions among species. Therefore, the possible ramifications of the indirect effects of contaminants are likely to be more complex (Lacher and Goldstein, 1997). Indeed, the physico-chemical characteristics and environmental fluctuations occurring in the tropics can influence the interaction between biological responses and pollutants differently than in temperate regions (see Johannes and Betzer, 1975; Bourdeau *et al.*, 1989). For instance, higher temperatures can favour greater solubility of pollutants. This, in combination with the generally higher metabolic processes of tropical organisms, can result in higher uptake rates of contaminants, although it normally corresponds to higher release rates as well. The biological and physico-chemical degradation of pollutants can be considered higher in the tropics as well. Although tropical ecosystems represent around 75% of global diversity, ecotoxicological studies have been performed mainly in countries and ecosystems in temperate zones (Lacher and Goldstein, 1997). As a direct consequence, most toxicity test and bioassays have been developed for organisms native to Europe and North America (Traunspurger and Drews, 1996).

1.1. Pesticides in the aquatic environment

The chemical nature of pesticides ranges from simple inorganic compounds to extremely complex organic molecules (Nimmo, 1985) with remarkably diverse modes of action and metabolism (Hassall, 1990). Pesticides may enter the aquatic environment in several ways; as runoff from agricultural fields, through internal soil drainage, aerial overspray, washing from the atmosphere by precipitation (Edwards, 1977; Eidt *et al.*, 1989) and through direct use for pest

control in aquaculture (Baird, 1994). Drift of pesticides outside the spraying area occurs with almost all methods of application, including both ground and aerial equipment, sometimes affecting non-target organisms located several miles downwind (Barnes *et al.*, 1987). The high levels of production of bulk organic pesticides also increases the risk of accidental spills during their transportation and production (Badawy *et al.*, 1984).

The physical and chemical characteristics of pesticides are significant in determining their activity and effects in the aquatic environment (Nimmo, 1985). Some pesticides remain in the water column while others may bind to suspended particles and bottom sediments (Edwards, 1977; Nimmo, 1985). Stability and persistence are governed by the chemical structures of the compounds, which range from very stable ones that may persist as residues for several years, to unstable compounds that can break down in few hours (Edwards, 1977; Nimmo, 1985). Even with the use of apparently biodegradable, contemporary pesticides, there is still a potential hazard posed for those organisms in proximity to the site of application, and also through the cumulative and indirect effects arising from repeated exposures to chemicals at sublethal concentrations (Clark *et al.*, 1993). The result of such exposures may be to predispose organisms to a variety of potentially deleterious biological effects (Malins and Ostrander, 1991).

Pollution became a matter of public and scientific concern in fresh water bodies several decades before marine pollution was apparent. As a consequence, studies of pesticide ecology have a much longer history in freshwater than in

the marine environment (Abel, 1991a). However, studies of the transport and fate of pollutants have shown that habitats such as coastal wetlands, estuaries (Hart and Fuller, 1979; Clark *et al.,* 1993) and the marine environment in general (Abel and Axiak, 1991) are threatened in similar ways. In fact, atmospheric processes like evaporation and condensation explain the considerable levels of pesticides, polychlorinated biphenyls (PCB's) and other pollutants registered in fish, mammals, birds and other marine life in the Arctic region (Bidleman *et al.*, 1993; Pearce, 1997; Muir *et al.*, 1999). In the sea, the behaviour and characteristics of the pollutant are primarily influenced by the sea water matrix and the concentrations of toxicants are usually present at low levels, with the exception of accidental (or intentional) spills, heavily polluted harbours or coastal areas with industrial waste discharges (Abel, 1991a).

1.2. Mixtures of pollutants in the environment

Historically, the continuous and sometimes erratic development of the industrialised world has surpassed the ability of researchers to study and predict the effects of pollution. After World War II, the rapid economic and population growth, combined with a minimal environmental awareness, produced an enrichment of complex mixtures of pollutants (Luoma, 1996). Nowadays, some of these chemicals, particularly the so called 'persistent pollutants", are widely distributed due to their long-half lives in the environment and their ability to be transported by air, water and through food chains (Walker and Livingstone, 1992). Although important efforts have been focused in the regulation of hazardous chemicals and waste treatment (mainly in developed

countries), significant numbers of new compounds are produced every year and eventually released into the environment.

In the field, pollutants almost always occur as mixtures, rather than as single compounds alone (Axiak and Abel, 1991; Kraak *et al.*, 1994), and the mechanisms of interactive toxicity of these mixtures are generally poorly understood (Marking, 1985). Mixtures of pesticides may occur when they are used to enhance the effects of single compounds for use in agriculture and pest control (Marking, 1985) or because of a random *in situ* combination. The effects of pesticide mixtures in aquatic systems depend not only on the characteristics of the pesticides and their concentrations, but also on the nature and biology of the aquatic system (Edwards, 1977).

The joint action of chemicals was first characterised by Plackett and Hewlett (1952) with respect to quantal responses as similar or dissimilar depending on whether the sites of primary action of two chemicals are the same or different, and as interactive or non-interactive depending on whether one chemical does or does not influence the biological action of the other. For instance, the effects of mixtures can be predicted in LC50 experiments by using compounds with simple similar action, normally producing concentration-addition effects (van Leeuwen, 1995). However, the difficulty in predicting the joint action of two or more chemicals with different modes of action is a consequence of the possible interaction of the chemicals themselves and also of interactions between the effects on the organisms (Hermens *et al.*, 1984a,b; van Leeuwen, 1995).

Although there are several procedures for assessing the toxicity of mixtures, only a few are truly quantitative and no single approach can accommodate all types of multiple exposures (Marking, 1985). Marking and Dawson (1975) developed a quantitative 'additive index' adapting existing methods based in the isobole theory to assess the toxicity of mixtures of chemicals in water. This method considers that the toxic strength of a mixture of compounds may be determined by summing the strengths of individual compounds, or toxic units. Assuming that a combination of equitoxic chemicals with similar modes of action produces a simple additive toxicity, the additive index can then be used to measure the deviations of this pattern to indicate less than additive (antagonisms) or greater than additive (synergism) toxicity. Calamari and Marchetti (1973) applied a similar approach that considers the toxic units as fractions of the lethal threshold concentration and used them to construct and compare toxicity curves of the toxicants individually and in mixture. Both methods allow the assessment of more than two chemicals in the combination. On the other hand, quantitative structure-activity relationships (QSARs) can be useful in the classification of many pollutants into a small number of groups of similar acting compounds, but have limitations when applied to mixtures of more than two chemicals due to the fact that the different combinations can fall into multiple classes of joint action (van Leeuwen, 1995).

Most of the studies with mixtures of pollutants have been focused on the combination of two chemicals, most commonly with freshwater organisms. Based predominantly in laboratory studies with fish, a review of the available data on mixture toxicity with freshwater organisms was published by the

European Inland Fisheries Advisory Commission (EIFAC, 1987). Based mostly in laboratory studies, the report indicates that the joint action of toxicant mixtures may be influenced by factors such as the type of toxicant and its proportional contribution to the mixture, water quality characteristics and its effects on toxicant speciation, test species (life stages, acclimation and experimental conditions), the measured response (e.g. lethal or non-lethal) and the magnitude of the response. A general consensus among researchers is that for most toxicants their joint toxic action is additive when two or three substances are tested in equitoxic concentrations (e.g. 0.5 of the respective lethal median concentrations in a mixture of two compounds), which together constitute a common threshold lethal level. However, as their concentrations are reduced towards the level of no observable effect (NOEC), their potential for addition is also generally reduced (EIFAC, 1987; Lloyd, 1991). This is an important issue in contemporary ecotoxicology as the information on the mechanisms of joint sublethal action of mixtures of chemicals with different types of toxic action is still scarce and fragmentary (Lloyd, 1991). The most important conclusions from experimental studies is that mixture toxicity occurs, and that certain chemicals can exert their detrimental effects at very low concentrations in complex equitoxic mixtures (EIFAC, 1987; van Leeuwen, 1995). The latter can be specially applied to some organic pollutants with a common and well defined Quantitative Structure-Activity Relationship (QSAR or relationship between biological activity and chemical properties) for which there is apparently no lower concentration at which individual toxicants do not contribute their full toxic potential in a mixture (EIFAC, 1987).

There has been relatively little research on the effects of combinations of pollutants in marine organisms, and direct measures of adverse biological effects are needed to assess the significance of chemical contamination in coastal zones (Axiak and Abel, 1991, Long, 1996). Most of these studies have been performed in point sources like effluents and industrial discharges (Lloyd, 1991). Synergism, or greater than additive effect, has been rarely reported in marine organisms, mostly related with shorter survival times recorded for organisms exposed to lethal concentrations (Lloyd, 1991). However, strong synergism was demonstrated in mixtures of organophosphate and carbamate pesticides on several marine species using acetylcholinesterase activity as endpoint (Bocquené *et al.*, 1995). No references were found on the interactive effects of triazole fungicides and organophosphate pesticides in marine organisms.

The influence of pollutant mixtures in both freshwater and marine environments has become a matter of concern in pollution regulation. This is led by a common consensus on the possible inefficacy of conventional water quality criteria based on studies for specific pollutants. Indeed, it has been recognised that the presence of other pollutants may modify the corresponding quality standards set for the protection of fisheries (EIFAC, 1987). In fact, some water quality criteria have failed to predict adverse effects of mixtures of heavy metals on crustaceans and fish (Spehar and Fiandt, 1986; Enserink *et al.*, 1991). The inclusion of ecotoxicological data involving the recognition of joint toxic action of pollutants has already been used in the establishment of water quality criteria

(Enserink *et al.*, 1991) or used to demonstrate the limitations of risk management programmes (van Leeuwen, 1995).

The development and application of pesticide mixtures in agriculture and pest control has been encouraged as a way to reduce the pesticide inputs to save costs, reduce environmental loads and lower the selection pressures resulting in rapid development of resistance (Gressel, 1993). In general, the selection of mixtures is based on considerations of the mode of action of the chemicals involved. One approach to selectively decrease the phytotoxicity of herbicides against crops is through the use of some compounds, known as 'safeners', that presumably can accelerate the herbicide detoxification (Scalla, 1991). On the other hand, some combinations are designed to act synergistically by enhancing the pesticide penetration, and block pesticide degradation and detoxification (Gressel, 1993). This has been proved valid for some herbicides (Scalla, 1991; Gressel, 1993) and with mixtures of fungicides, sometimes even under field conditions (Zeun and Buchenauer, 1991). However, due to registration requirements, far more is known about pesticide metabolism in crops than in target species (Gressel, 1993). Actually, it has been suggested that the use of some synergistic mixtures of fungicides may favour pest resistance as they select for dual resistance approximately twice as fast as the sequential use of the components in the mixture (Shaw, 1993). The effects of mixtures on non-target species have been almost completely neglected in pest control practices. Synergistic combinations, though they can help to reduce the amount of pesticides applied, are also more toxic to pests and are likely to have similar or even greater effects on non-target species. Moreover, particular

combinations of chemicals may show multiple types of toxic interactions depending on the species and the environmental conditions, while every released chemical contributes to the toxic burden of the environment (Marking, 1985) and therefore can engage in random *in situ* combinations with unpredictable consequences. Considering that the distribution of pesticides in the aquatic environment is not homogeneous, and that any new added compound could increase the toxic burden of the water, it is necessary to evaluate areas of critical accumulation (Duke, 1977; Marking, 1985), with special emphasis on the most sensitive species (Cairns, 1989).

1.3. Effects of pesticides in marine and estuarine ecosystems

Environmental effects of pesticides have been reported upon estuarine ecosystems. Studies carried out by the Gulf Breeze EPA laboratory determined that the runoff of pesticides used on vegetables crops adjacent to estuarine marshes was related to mortalities of fish and crustacean residents of tidal creeks, as well as test animals exposed *in situ* (Clark *et al.*, 1993). In these studies, the runoff of pesticides into the tidal creeks following rain was reportedly able to generate pulse exposures which exceeded acute LC50 for several local species, while field assessment showed that altered bioenergetic metabolism of some organisms could be related to sublethal concentrations. In recent years, several events involving massive mortalities of marine organisms have been reported in coastal waters in the Northwest of Mexico (La Jornada, 1997; El Universal, 1997, 1999), which have been linked to environmental pollution generated by agricultural practices (including the disposal of pesticide containers in rivers), mining, industry or poorly-managed sewage treatment

plants. Although this has raised the public awareness about environmental issues, the problem illustrate the need for a better understanding of when contamination translates into effects. So far no etiologies have been proved for the mortalities, but some research efforts have been focused in the levels, dynamics and fate of most used pesticides like organochlorines, organophosphates, carbamates and pyrethroids (Rosales and Escalona, 1983; Rosales *et al.*, 1985; Michel and Gutiérrez-Galindo, 1989; Mee *et al.*, 1992; Readman *et al.*, 1992; Carvalho *et al.*, 1996; González-Farias *et al.*, 1997) or heavy metals (Páez-Osuna and Ruiz-Fernandez, 1995; Páez-Osuna and Tron-Mayen, 1996). This information is however rather fragmentary and dispersed in time, highlighting the importance of assessing the potential effects of pollutants in locals species and the need for a concerted approach to tackle the problem. In particular, there is growing concern about the potential effects of contaminants on commercially important species. For instance, the white shrimp *Litopenaeus vannamei* (the species used in this study) plays a very important ecological role in estuarine environments along the Pacific coast of Mexico, Central, and South America (Wyban and Sweeney, 1991), while supporting one of the most important fisheries in Mexico, and it is the most popular shrimp for culture in North, Central, and South America (Escobar, 1985; Rosenberry, 1992). The production of this crustacean in the Northwest of Mexico increased from 200 tons in 1985 to 10,000 tons in 1993 (SEPESCA, 1994). As consequence, in the last two decades, the economic value of most penaeid species has encouraged important research efforts on their ecology, biology and physiology.

The existence of considerable infrastructure regarding shrimp culture, and the current state of knowledge about its biology makes this group of animals ideal for laboratory and field ecotoxicological studies. Nowadays, it is possible to optimally rear and reproduce shrimps in laboratory conditions. With adequate conditions provided, these organisms can tolerate a great deal of handling and have demonstrated high sensitivity to pollutants. In fact, Couch (1978) reports that, after several years of testing, the U. S. Environmental Protection Agency Laboratory, Gulf Breeze, Florida, has found that penaeid shrimps are generally far more sensitive to the toxic and ecological effects of most pesticides than fishes and molluscs (Couch, 1978), and have also been found to accumulate certain pesticide compounds, both in the laboratory and in the field (Couch, 1979). The same author proposed the use of penaeid shrimps as indicators of estuarine health, due to their world wide distribution throughout temperate, subtropical and tropical regions. Penaeid shrimps have relatively complex life cycles (Fig. 1.1) that bring metamorphic stages into several environments, ranging from open ocean waters to tidal estuaries. In a typical penaeid life cycle, gravid females spawn offshore, and early metamorphic stages (nauplii, protozoea, and mysis) occur in high salinity coastal or offshore waters. Postlarvae migrate to estuaries and become juveniles, then young adults return offshore upon reaching sexual maturity (Edwards, 1977; Couch, 1979). This sort of information is ecotoxicologically relevant since shrimps could have different sensitivity to toxicants depending on their life stages (Kobayashi *et al.*, 1991).

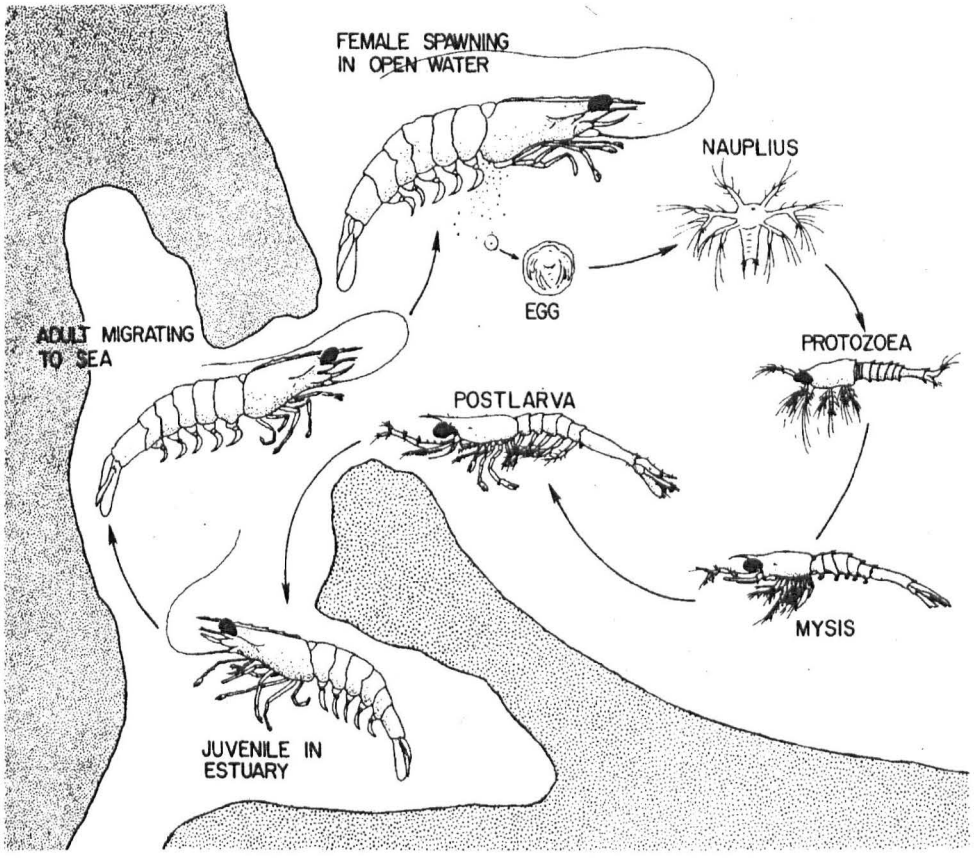


Fig. 1.1. Life cycle of the penaeid shrimp. From Couch (1979).

1.4. Aquaculture and the environment

In aquaculture, a polluted environment is undesirable since this generally leads to a potential decrease in production, with an increased possibility of bioaccumulation of pollutants and increased risk of disease outbreaks within stock (Couch, 1974; Couch and Courtney, 1977; Roque *et al.*, unpublished). Aquaculture however could itself pose an ecological hazard when it involves the use of natural and synthetic agents for pest control (Baird, 1994), displacement of key habitats (as mangroves and marshes), infiltration of foreign diseases to wild organisms, or acceleration of the natural process of eutrophication through the use of fertilisers and increased deposition of wastes (Primavera, 1997; Boyd and Clay, 1998; Guo *et al.*, 1999), and the use of chemicals in disease control (see below) .

In addition to the accidental introduction of pesticides and other pollutants through air, water or food, many chemicals are employed in the management of aquaculture systems depending on the species and the characteristics of the culture. For example, oxidants, mainly inorganic salts, peroxides and chlorine, are used to kill bacteria and to oxidise organic compounds; aluminium sulphate for removing colloids from water and reducing the pH; calcium hydroxide for removing carbon dioxide; sodium or calcium chloride for counteracting nitrite toxicity; ethylenediaminetetraacetic acid disodium salt (Na EDTA) often is applied to hatchery water to chelate heavy metals; copper sulphate, simazine, and solricin 135, among others, are used as herbicides and algicides; application of detergents and oils is a common method of aquatic insect control; formalin, copper sulphate, potassium permanganate and some dyes are applied

for the control of parasites and fungi (Boyd, 1990; Chien, 1992; Baird, 1994) while a wide range of antibiotics are used to control or prevent bacterial diseases (Brown, 1989). To eliminate undesirable fishes from the ponds, compounds like rotenone, saponins, croton oil and some other plant-derived toxins are frequently used (Boyd, 1990; Baird, 1994). Organic pesticides, as organophosphates and organochlorines, have been also used in aquaculture as pest control agents despite the problems of hazard to the user, unpredictable biodegradability, persistence, development of resistance, and lack of specificity (Baird, 1994). A comprehensive review of drugs, vaccines and pesticides used in aquaculture was published by the Texas Agricultural Extension Service (1994).

Information regarding the accumulation and effects of chemicals in shrimp aquaculture practices is rather scarce. The performance of shrimp farming practices are strongly influenced by natural environmental conditions, and since it is restricted to coastal waters, it can also be subjected to the effects of urban, industrial and agricultural pollution (Hirono, 1992; Weidner and Rosenberry, 1992). This makes complicated to establish definitive cause-and-effect linkages. However, in countries like China and Thailand, the decrease in aquaculture production has been attributed to generalised environmental pollution (Weidner and Rosenberry, 1992). Recently, in Ecuador, the identification and characterisation of mortality-causing agents in shrimp ponds provoked great controversy. While in 1992 the overall production of farmed shrimp reached record levels, during the same year the pond survival dropped dramatically in the region which depended on water from the Taura river and nearby estuaries

(Stern, 1995). The phenomenon received the name of Taura Syndrome, reaching catastrophic dimensions in 1993 and 1994 as the mortality events included other regions of Ecuador with losses estimated from US$45 million (Comite Tecnico Camara Nacional de Acuacultura, 1994, in Lacher and Goldstein, 1997) to more than US$100 million (Rosenberry, 1993, 1994a, 1994b). The common consensus among several researchers in 1994 was that no infectious etiology was involved in the Taura syndrome, suggesting that a probable toxic agent was causing the mortalities (Acuacultura del Ecuador, 1994). This question later became hotly contested. A review of the pesticide market revealed major increases in agrochemical sales of the fungicides Tilt (active ingredient: propiconazole) and Calixin (active ingredient: tridemorph), which were then used to combat a fungal disease outbreak known as *sigatoka negra* in banana plantations upstream in the Taura river (Stern, 1995). Presence of trace amounts of propiconazole was also reported in sediments, water and hepatopancreas tissue from ponds previously affected by Taura syndrome (Brock *et al.*, 1995). Based in this information, the Ecuadorian government took steps to control the use of the involved fungicides and offered economic incentives to banana farmers willing to abandon their farming activities (Wigglesworth, 1994). The toxic etiology was later supported by local producers and scientists which reportedly were able to induce the Taura syndrome in healthy shrimps with combined challenges of the fungicides (Stern, 1995). However, further experimentation failed to link such fungicides with the symptoms and histological lesions initially described (Enriquez *et al.*, 1996). The toxic hypothesis became difficult to sustain as the syndrome became widespread in shrimp farms of South, Central and North America (Brock, 1997),

and was eventually proven experimentally to be caused by a virus (Hasson *et al.*, 1995; Lightner *et al.*, 1995). As a consequence of this issue, results of studies testing chemicals on wildlife on Ecuadorian banana plantations may be sceptically examined by industry in the future (Lacher and Goldstein, 1997).

1.5. Crustaceans as test organisms in toxicity testing with pesticides

Studies regarding pesticide effects in marine crustaceans are relatively abundant. This is due to the fact that arthropods and fishes have been traditionally the most used animals in toxicity testing (Hansen, 1984). Still, our knowledge of the effects on realistic exposure scenarios is rather limited as most toxicity studies have been carried out in controlled laboratory conditions. Nevertheless, the data obtained in toxicity tests could represent a starting point in the hazard evaluation process. Considering the complexity of biological systems, the bioassays to assess the toxicity of pollutants vary in complexity, from short-term tests with single species to multi-species test in field conditions, depending on the research purposes (OECD, 1993). These tests, in particular acute toxicity tests, can be used to measure the toxicity of substances or sensitivity of species to substances, but normally fail to predict environmental hazard directly. However, the data obtained in the tests can be further used to select exposure concentrations for chronic toxicity, bioconcentration and other tests used in the evaluation process (Hansen, 1984). For laboratory tests, the exposure conditions are characterised by time and concentration of the toxicant. Ideally, the test organisms should be maintained in optimal and constant environmental conditions and later be exposed to a range of toxicant concentrations. To determine whether an adverse effect occurs or not, the

responses from the exposed organisms are compared with control (or unexposed) organisms. From the toxicity testing procedures available, three general types of toxicant delivery systems can be identified (Abel, 1991b; OECD, 1993; Van Leeuwen, 1995): static, renewal and flow-through. In a static test, the toxicant is added once into the system and the test medium is not changed during the entire experiment, while in a renewal (or semi-static) test, the test medium and test compound are periodically replaced. In the flow-through systems (also called continuous-flow systems), the test solutions are replaced at constant rate and concentration in the exposure chambers. In general, flow-through systems are aimed to maintain constant exposure levels and are considered useful for the assessment of poorly soluble, volatile or unstable substances (Diamantino *et al.*, 1997). Examples of flow-through systems reported in the literature have been developed for single or multi-species tests (i.e. seagrass, macroinvertebrates, fish, etc.) and were used to perform either acute or chronic exposures (Walsh *et al.*, 1982; Benoit *et al.*, 1982; Green and Williams, 1983; Diamond *et al.*, 1995; Diamantino *et al.*, 1997; Sanni *et al.*, 1998).

Based in the data available, the potential effects of pesticides in the biological processes of crustaceans depend on their mode of action, the stage of the organisms and the environmental conditions. Organochlorines are persistent pesticides generally insoluble in water and much more soluble in lipids (Williams and Duke, 1979). Due to their persistent nature, these pesticides tend to accumulate and remain in the tissues of organisms. Schimmel *et al.* (1979) found that the Kepone assimilated by the blue crab *Callinectes sapidus* in

exposure experiments remained in the tissues after the animals were fed 28 days with Kepone-free oysters, and occurred in detectable concentrations after 90 days of the experiment. The uptake route can be an important factor influencing the accumulation of organochlorines. Epifanio (1973) reported that larvae of the crab *Cataleptodius floridanus* was able to accumuate dieldrin 19.1 times as fast from 0.5 ppb in seawater than from 213 ppb in food. Dieldrin is commonly found in estuaries and has been reported as extremely toxic to crustaceans. Concentrations as low as 10 ppb of dieldrin in water prevented the normal development of the estuarine crabs *C. floridanus* and *Panopeus herbsii* (Epifanio, 1971), and was lethal to the pink shrimp, *Penaeus duorarum*, at 0.9 ppb (96-hour LC50) (Parrish *et al.*, 1973). Reported chronic effects of the pesticides DDT, hexachlorobenzene and Mirex on estuarine shrimps include paralysis and lethargy that can be associated with anorexia and inability to escape from predators (Lowe *et al.*, 1971; Parrish *et al.*, 1974; Couch, 1979). It has been reported that DDT can cause accelerated limb regeneration, shortening in moulting time and a possible excitation of the central nervous system in fiddler crabs (Weis and Mantel, 1976), while toxaphene can be more toxic to the pink shrimp at the early metamorphic stages (Courtenay and Roberts, 1973). Organophosphates and carbamates are considered the "second generation" of pesticides, designed to be more specific to target organisms than organochlorines (Williams and Duke, 1979; Hassall, 1990). Although organophosphates and carbamates have relatively short half lives in the environment, eventual pulse exposures can be extremely detrimental to non-target organisms such crustaceans (Couch, 1979; Williams and Duke, 1979; Clark *et al.*, 1993). Several studies have investigated the toxic effects of

organophosphates and carbamates in crustaceans. Both types of pesticides are reported to be potent acetylcholinesterase (AChE) inhibitors (Couch, 1979; Hassall, 1990). However, realistic exposure conditions are difficult to simulate in the laboratory as concentrations and bioavailability of these pesticides can change considerably over time in the environment (Hansen, 1984). Organophosphates can show differential toxicity in the different developmental stages of shrimp (Kobayashi *et al.*, 1991) and produce an impairment of its cell-mediated immunological responses (Bodhipaksha and Week-Perkins, 1994). The observed effects could have important ecological implications. For example, Farr (1978) found a relative increase in the consumption of the grass shrimp *Palaemonetes pugio* by the fish *Fundulus grandis*, when the shrimp was exposed to sublethal concentrations of methyl-parathion, suggesting that the pesticide could decrease the ability of the shrimp to avoid predation. Dithiocarbamates have been found to affect limb regeneration in shrimp, though they did not alter the duration of the time to moulting (Conklin and Rao, 1982). Vogt (1987) found heavy histological damage in the midgut gland of *Penaeus monodon* when exposed to sublethal concentrations of the insecticide dimethoate. The organophosphate insecticide for mosquito control, fenthion, has been proved to affect the metabolism, growth and/or reproduction of mysid shrimp at non-lethal concentrations (see Clark *et al.*, 1987). Some organic pesticides that are routinely used in aquaculture can produce non-desirable effects in shrimp. *P. monodon* was found to be adversely affected in terms of shell quality (softening) in exposure experiments with Gusathion A, rotenone and saponin, all of them used as piscicides in pond cultures, suggesting a possible deleterious effect on chitin synthesis (Baticados and Tendencia, 1991;

Cruz-Lacierda, 1993). Other types of pesticides, like pyrethoids and fungicides, can reach the estuarine and marine environment after applications to agricultural crops. In particular, pyrethroids could be extremely toxic to crustaceans in laboratory tests, while fish are less sensitive and oysters relatively insensitive (Clark *et al.*, 1989). Like DDT, pyrethroids appear to cause hyperexcitability within the nervous system by holding the neuronal sodium channels open (Chambers and Carr, 1995). Although pyrethroids are environmentally labile (Chambers and Carr, 1995), they have a significant potential for bioaccumulation (Hill, 1985), but also readily bind to organic and inorganic particulate matter, making difficult to predict their effects in the aquatic biota (Sharom and Solomon, 1981; Muir *et al.*, 1985; Clark *et al.*, 1989). In turn, detectable levels of fungicides have been reported after rain in water samples near banana plantations where they are frequently applied (Mortensen *et al.*, 1994; Henriques *et al.*, 1997). However, studies of the potential adverse effects of these pollutants on crustaceans and other aquatic life are needed to assess the significance chemical contamination.

1.6. Crustacean moulting as response to pollutants

In ecotoxicology, the hazard assessment of pollutants sometimes involves the study of measurable responses of an organism in order to obtain sensitive indication that a toxicant has actually entered the organism, been distributed throughout the tissue or body fluids, and is causing a toxicological effect (McCarthy and Shugart, 1990; Depledge, 1993). The challenge for researchers is to discover which responses, called 'biomarkers', indicate if the population of organisms is suffering increased stress in environments contaminated with

pollutants (reviewed by Callaghan and Holloway, 1999). In crustaceans, moulting is probably one of the most relevant responses to assess the effects of toxicants. Moulting is actually the only way that crustaceans have to increase in size, it is intimately related in defining the periods in which reproduction occurs, and influence crustacean morphology, physiology and behaviour (Skinner, 1985; Dall *et al.,* 1990). It has been reported that environmental factors such as predation, cannibalism, temperature, salinity, light, and pollutants, alter the moulting process and limb regeneration (Conklin and Rao, 1982; Mothershead and Hale, 1992; Weis *et al.,* 1992; Engel and Brouwer, 1993). Toxicity test data have shown that crustaceans have different responses depending on the moult stage, and this is likely to be due to physiological changes which occur during moulting (Robertson *et al.,* 1987). An understanding of the physiological, biochemical and behavioural mechanisms involved in moulting can help to understand how a given pollutant affects the moulting metabolism, and also provide evidence to link the exposure levels with the effects in the organism's performance, to ultimately predict and assess the potential effects in the environment.

1.7. Aims

In this study, a tropical marine bioassay was developed to assess the toxicity of pesticides in the shrimp *Litopenaeus vannamei.* Time-independent exposures were performed in a flow-through system for the organophosphate insecticide methyl-parathion, and a triazole-derivative fungicide in two presentations: Tilt (commercial formulation, 25% propiconazole) and technical grade propiconazole. Mortality data was used to estimate the toxicity parameter LC50

(median lethal concentration) at different exposure times in order to establish the threshold LC50. As a result from these tests, the comparative toxicity of the pesticides was examined, and in particular, the toxic contribution of propiconazole in the commercial formulation, Tilt, was established. The toxic interaction between the pesticides was also investigated. As an organophosphate insecticide, the toxicity of methyl-parathion is based in the oxidative enzymatic attack of the parental molecule when it enters the organism, which derives a more potent acetylcholinesterase inhibitor called methyl-paraoxon (Hassal, 1990). On the other hand, the mode of action of the fungicide propiconazole is based on interference with sterol synthesis, and belongs to the group generically called ergosterol biosynthesis inhibitors (EBIs) (Hartley and Kidd, 1987; Hassal, 1990; Kwok and Loeffler, 1993). Propiconazole is also reportedly able to induce the oxidative cytochrome P-450 metabolism (Hassall, 1990). If both pesticides are placed simultaneously, the oxidative induction by propiconazole could, in theory, enhance the oxidative-dependent activity of methyl-parathion. To investigate this, the toxicity data of the single toxicants was used to assess the interactive toxicity of each form of the fungicide (technical grade and formulation) with methyl-parathion. Apart of mortality, the toxicity was also evaluated using feeding and locomotory behaviour, and moulting rate of the shrimp with the intention of identifying specific responses depending on the type and mode of action of the pesticides. In a long-term subchronic exposure to Tilt, the effects were also assessed using alterations of morphological characteristics as endpoints. All together, this study was aimed to establish a tropical marine bioassay using the marine shrimp *Litopenaeus vannamei*, and to study the potential toxic interactions

between pesticides using several endpoints simultaneously. This study can be considered a preliminary attempt to tackle a very important issue in tropical coastal areas with intense agricultural activity.

CHAPTER 2.

GENERAL MATERIAL AND METHODS

2.1. Introduction

Penaeid shrimps play a significant role in the middle of the trophic web in the estuaries and inner oceanic littoral zones of temperate, subtropical and tropical regions of the world (Couch, 1979). Because penaeid shrimps spend a considerable part of their life cycles in coastal waters, they are exposed to all types of contaminants that are released in these areas. Unlike many other tropical species, the ecology and biology of shrimps have been studied extensively as consequence of penaeids' economic value due to fisheries and aquaculture. This places penaeids as promising candidates to be used as test species to study the effects of pollutants in tropical marine and estuarine environments.

The information presented in this chapter describes the design and implementation of a tropical marine bioassay to assess toxicity of pesticides on the marine shrimp *Litopenaeus vannamei*. In order to provide the appropriate experimental conditions for the test organisms, a flow-through system was designed. Additionally, the flow-through system was aimed to perform reliable and constant exposures in a time-independent fashion.

Common procedures like procurement of test organisms, and maintenance and operation of the flow-through system are described in this chapter. Material and

methods specific to individual experiments are described in the relevant chapters.

2.2. Procurement and maintenance of test organisms

All test organisms used in this study were *Litopenaeus vannamei* acquired in postlarvae stage (PL 10 to 15), which is characterised by the development of swimming setae, forward swimming and benthic lifestyle (Wyban and Sweeney, 1991). The organisms were donated from two local shrimp hatcheries: Maricultura del Pacifico (Teacapan, Sinaloa) and Cultivos Morales (San Blas, Nayarit). Both hatcheries were selected for their reputed adequate laboratory practices and good larvae quality. At regular intervals, the larvae were transported to the Centro de Investigación en Alimentación y Desarrollo (CIAD) in Mazatlan, Sinaloa, Mexico. The animals were placed in plastic bags with seawater and carried in an insulated box. If necessary, ice was used to maintain the temperature around 20°C. As normal procedure for the shipment, the water was bubbled with oxygen and some *Artemia* nauplii (rations decided by personnel of the hatcheries) introduced to prevent oxygen deprivation and starvation in the postlarvae respectively.

Once at CIAD, the postlarvae were gradually acclimatised to room temperature (28 ± 1°C). The procedure was to introduce the plastic bags containing the postlarvae in tanks with water at the room temperature until the temperature was the same outside and inside the bag. After that, not more than 5000 postlarvae were placed per 40-liter aquarium. The animals were fed *ad libitum* with *Artemia* nauplii and pellets of commercial formulation (Rangen, 30 %

protein) with daily water renewals, until they reached approximately 0.1 g. At that point, animals were transferred to a recirculatory system (Fig. 2.1). This system was designed to allow separate maintenance and growth of batches of shrimps that were arriving periodically at the lab in order to ensure a constant supply of animals for the bioassays. The shrimp were fed daily with Rangen pellets (30 % protein). A further set of 600-litre tanks were used only on occasions when the recirculatory system was totally occupied, and animals were held under the same feeding conditions with daily water renewals.

2.3. Toxicants

All toxicant stock solutions were prepared prior to the start of each experiment. The solutions were always kept at 4° C in foil-wrapped flasks and shaken prior to the preparation of test solutions. Stock solution and exposure solution nominal concentrations are given in the relevant chapters.

Technical grade methyl-parathion (80.3 % purity, Fig. 2.2) was donated by TEKCHEM, S. A. de C.V. Stock solutions were prepared in deionised water to the desired concentration and shaken in an orbital mixer (Mistral Multi-mixer, model 4600) for 1 hour at room temperature.

Tilt 250E (commercial grade, 25 % propiconazole as active compound) was provided by CIBA-Geigy Mexicana (now Novartis Mexico). Stock solutions of the required concentration were made up in deionised water and shaken for 1

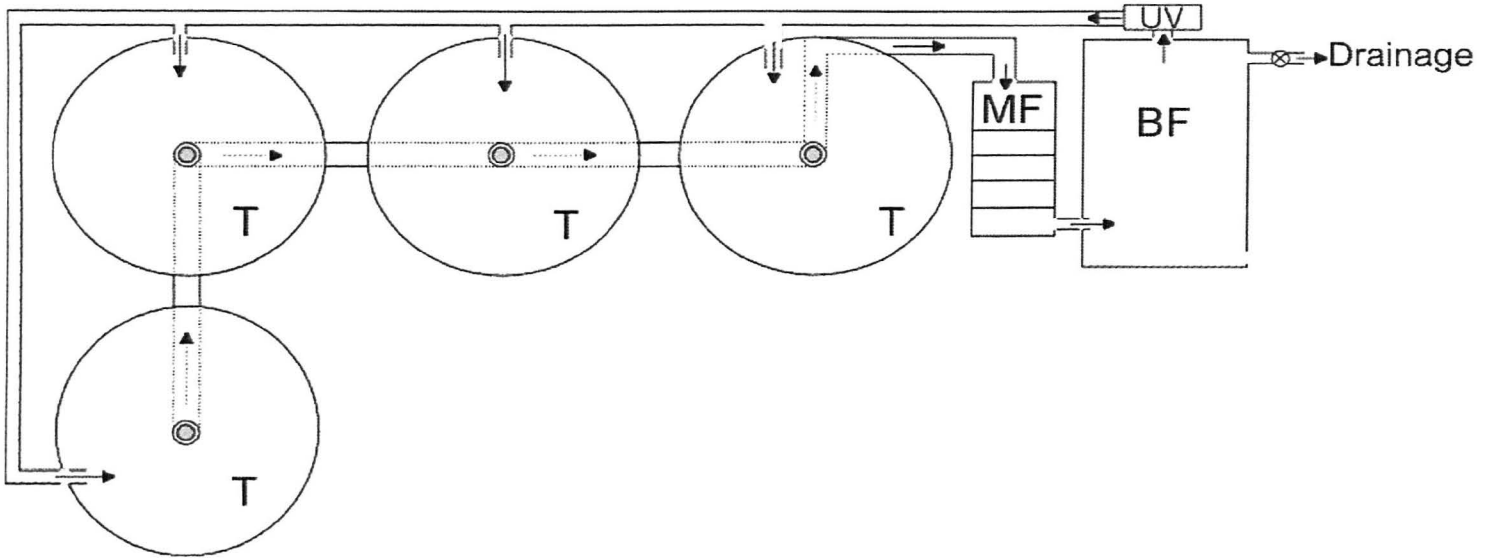
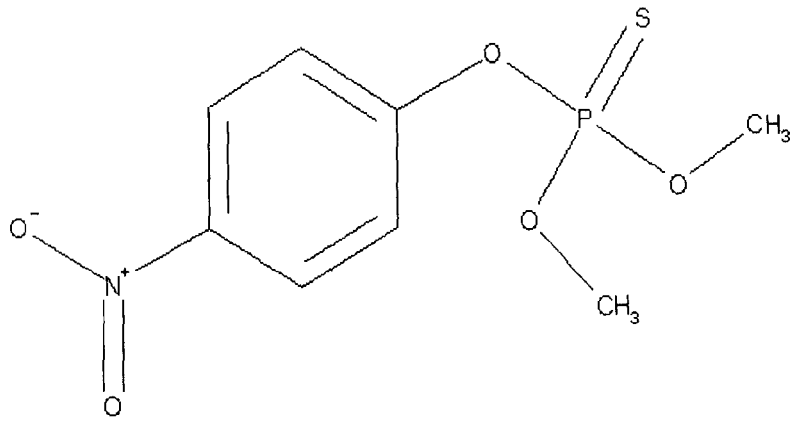


Fig. 2.1. Schematic representation of the recirculatory system for the maintenance of the test organisms. T) 600 litres tanks; MF) mechanical filter; BF) biological filter; UV) ultra-violet filter. Arrows indicate the flow in the system.



•Name: Methyl-parathion
•Chemical class: Organophosphate pesticide
•Water solubility: 55 - 60 mg/l at 20^0 C
•Decomposition temperature: 100^o C
•Breakdown in seawater: 100% degradation within 2 weeks to 1 month or more

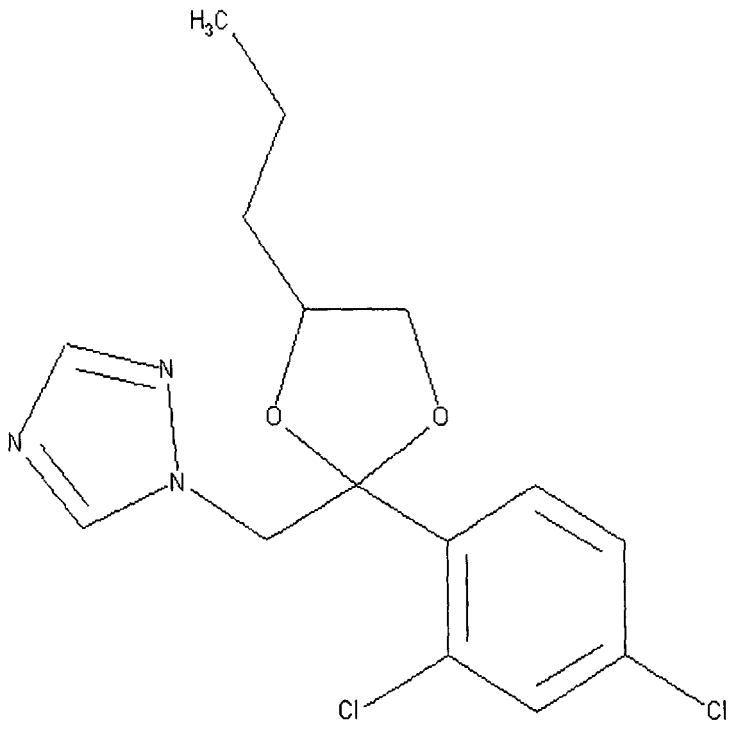
Fig. 2.2. Chemical structure and properties of methyl-parathion. From EXTOXNET (1998a)

hour at room temperature. Other ingredients present in Tilt 250E formulation are emulsifiers (3.5% - calcium dodecylphenylsulfonate, 4.5% - castor-oil polyglycolether, 2% - oleyl polyethoxyethanol), 4% of deionised water and 60.2% of solvents (a mixture of unspecified aromatic hydrocarbons).

Technical grade propiconazole (92.4 % purity, Fig. 2.3) was supplied by Novartis Crop Protection, Basel, Switzerland, through a confidential research agreement. Stock solutions were made up in HPLC grade acetonitrile (Sigma Chemicals Ltd.).

2.4. Design and operation of a flow-through toxicity system

A flow-through system was designed for toxicity tests on juvenile shrimps. A basic representation and dimensions of the flow-through system are shown in Fig. 2.4, which consisted of the following elements: Sixteen 18-litres glass bottles were used as reservoirs of the test solution with or without toxicants. Plastic bags were used to wrap the reservoirs to prevent photodegradation of the toxicants, in such a way that they could be pulled down to see the water level during the refilling. The exposure chambers (Fig. 2.5) consisted of sixteen oval-shaped baking trays (Pyrex, 4.5-litres capacity). Each exposure chamber had ten cylindrical perforated cages (PVC, 10x10 cm), to allow test organisms to be held individually. Transparent plastic covers were placed in the top of exposure chambers to prevent organisms escaping. A 16-channel variable-speed peristaltic pump (Masterflex, model 7553-60) employing 8 roller pump head and using silicone-peroxide cured tubing of 2.79-mm internal diameter was used to deliver the test solution from the reservoirs into the independent



•Name: Propiconazole
•Chemical name: 1-[[2(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]1-H-1,2,4-triazole
•Chemical class: Triazole fungicide
•Water solubility: 100 mg/l at 20^0 C
•Breakdown in water: 20% photolysis after 12 days to natural light. Rapid photodegradationin presence of photosynthetisers (<1 day)

Fig. 2.3. Chemical structure and properties of propiconazole. From EXTOXNET (1998b)

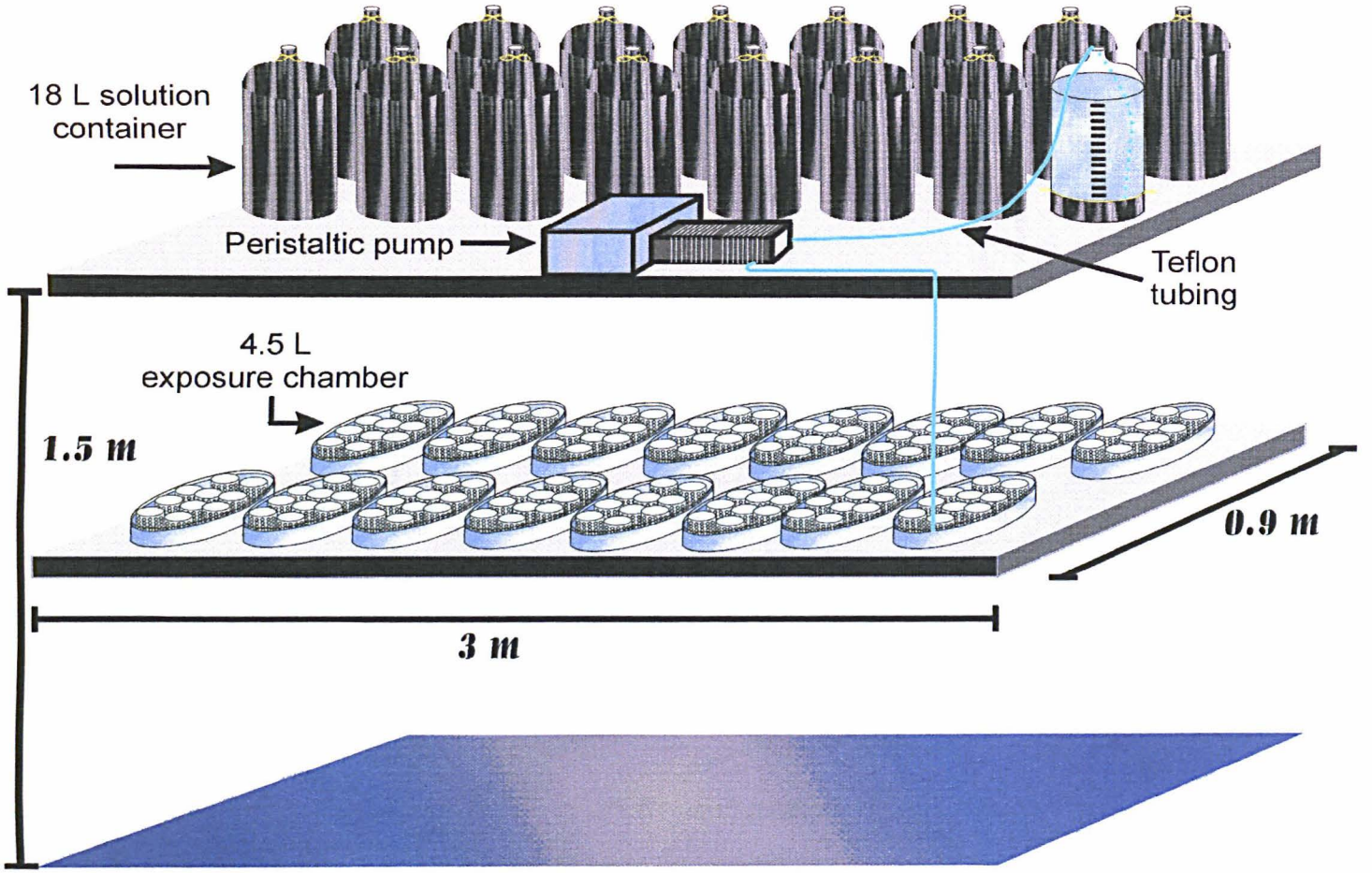


Fig. 2.4. Schematic representation and dimensions of the Flow-through system.

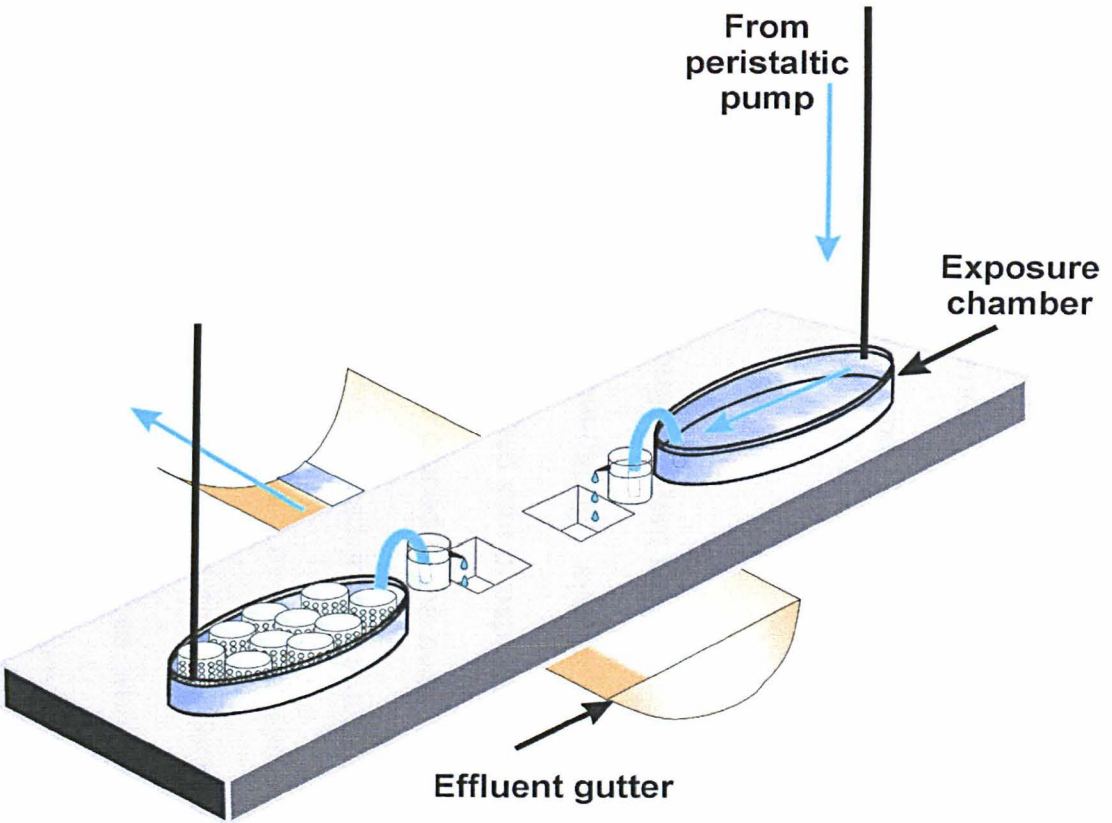


Fig. 2.5. Diagram showing the water flow in the exposure chambers (indicated by blue arrows).

exposure chambers. Teflon FEP tubing (3.2-mm internal diameter) was used to connect the peristaltic pump with the reservoirs and the exposure chambers (Teflon tubing was selected to minimise pesticide loss and allowed its reutilization in consecutive experiments). A U-shaped glass tube (2-cm internal diameter) was used to connect each exposure chamber to a 250-ml plastic flask, which later delivered the test solution into a PVC effluent gutter. Pads of fibrous material were placed along the gutter for toxicant recovery and disposal. The flow-through system was operated in a constant temperature room at 28 ± 1°C, with a 12/12 light/dark photoperiod. Fig. 2.6 (a to d) shows the set-up and operation of the flow-through system.

2.5. Preparation of the flow-through system for experiments

Prior to each experiment, the flow-through system was disassembled for cleaning. All glass material was washed with phosphate-free detergent, rinsed with 95% ethanol and still water, and placed in the oven at 250 ° C overnight. Plastic covers and PVC cages were placed in a 200-litre outdoor tank in a detergent solution for several days, followed by several cycles of rinsing with tap water, to be finally washed with phosphate-free detergent, and then rinsed with 95% ethanol and distilled water. Teflon tubes were cleaned by slowly passing though a sulphuric acid and potassium dichromate (3:1) mixture for six hours, followed by 95 % ethanol for 1 hour and finally distilled water overnight. The silicone tubing connecting the Teflon tubing was always replaced with new tubes prior the start of each experiment.

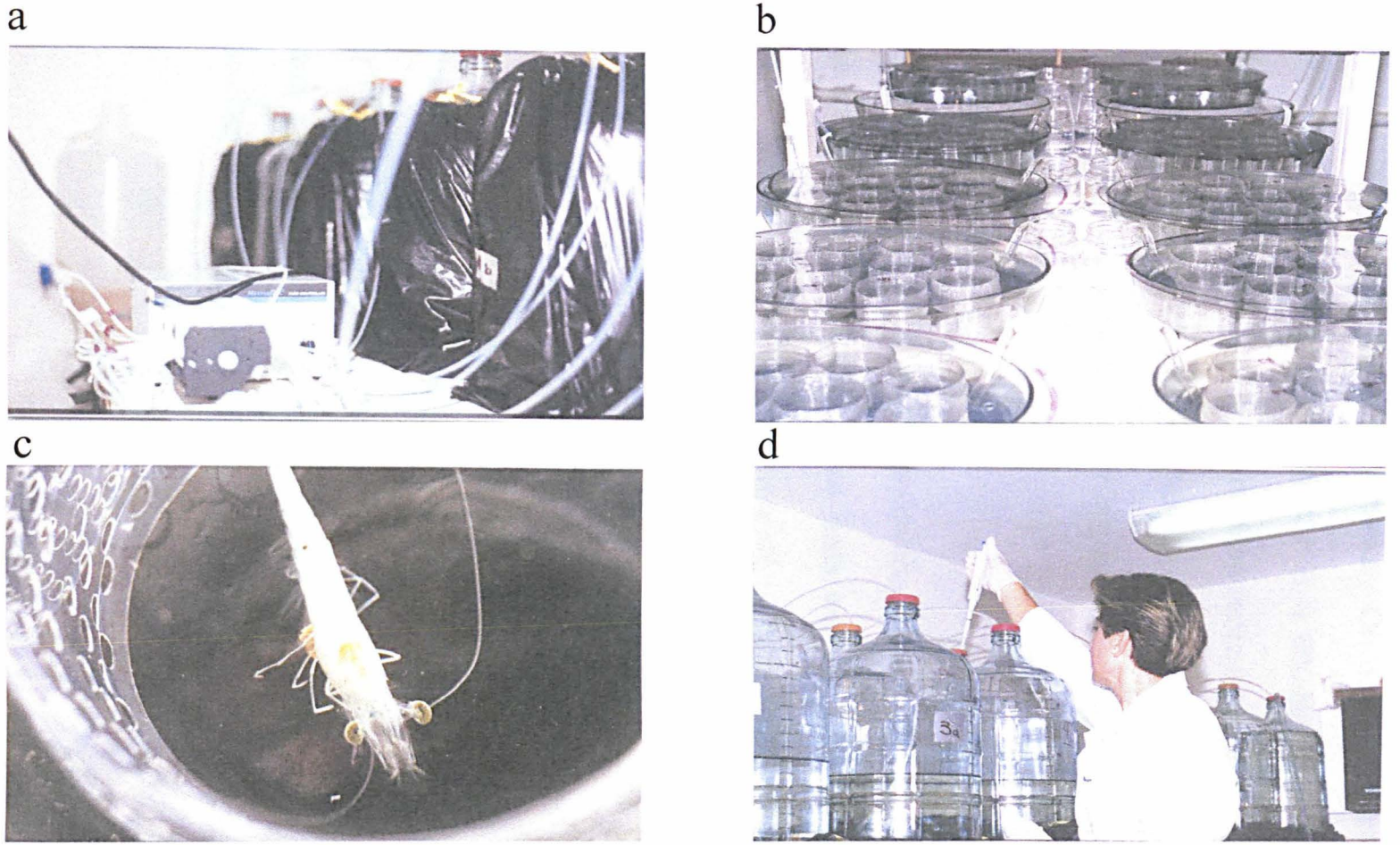


Fig. 2.6. Set-up and operation of the FTS. a) Test solution reservoirs and peristaltic pump; b) Exposure chambers; c) Shrimp in cage; d) Application of toxicants.

The flow-through system was assembled and run for 24 hours with seawater before introducing the shrimps into the exposure chambers. All the seawater used in the experiments was pumped from a nearby beach, sand filtered and stored in 10 thousand-litre dark outdoor tanks. On demand, the water was filtered through a 5μm cartridge and placed in a 600-litre dark tank in the constant temperature room. The water was then recirculated for two hours with a submersible pump through an UV filter in order to reduce the microbial content and kept with artificial aeration for 24 hours prior to utilisation.

2.6. Range finding tests

Prior to definitive experimentation with any compound, a series of range finding tests was carried out. The aim of these tests was to establish the lowest toxicant concentration which could effectively kill 100 % of the test organisms in the first 24 hours, as well as the highest concentration that resulted in no mortality during the expected time of exposure. These two concentrations would set the boundaries for the definitive experiments. A first approach was to place four or five organisms in 3-litres simple glass containers in order to test a broad range of concentrations. This allowed an approximate estimation of the toxicity. Final confirmation and tuning of concentrations were carried out in the flow-through system with four or five animals and without replicates. Mortality records from all range finding tests are presented in Appendix A (Table A.1 to A.5).

2.7. Acclimation

Before introducing test substances in any experiment, animals were allowed to acclimate to the flow-through system. To do this, approximately 180 *L. vannamei* juveniles were transferred from the maintenance system (see section 2.2.1) to the room where the flow-through system was installed and placed in 300-litres tanks with aeration. When the flow-through system was fully assembled, the animals were randomly placed in the exposure chambers and observed, to determine whether they were able to survive under the conditions. During this period, organisms that were injured, moribund, behaving abnormally or not feeding normally were rejected and replaced with organisms from the same batch. A maximum of 20% mortality during the acclimation period was the limit to consider a batch appropriate for experimentation (Abel, 1991b). Moulted carapaces were recorded daily. Feed pellets were individually offered to organisms for about 1 hour every day. After this, faeces and food remaining were siphoned out of the exposure chambers. In all the experiments, the flow was set at 18 litres per day (12.5 $ml.min^{-1}$), which resulted in a total daily consumption of 288 litres of seawater for the sixteen channels. This flow was selected in order to comply with recommended values of water replacement in the exposure chambers every 6 to 8 hours (Abel, 1991b), or a 90 % of replacement every 8-12 hours calculated by a nomograph (Sprague, 1969). During the first days of acclimation, observation of the water levels in the flow-through system reservoirs was carried out to make the necessary adjustments in the peristaltic pump cassettes in order to assure homogeneous flow in all channels. Acclimation times and flow rate in relation to loading of organisms are mentioned in the relevant chapters.

2.8. Exposure in the flow-through system

After acclimation, a concentrated stock solution of the toxicant was prepared. Test concentrations were obtained by pipetting the stock solution into the reservoirs that were then refilled up to 18 litres with seawater. No analytical determinations of toxicants were done in the definitive experiments. The water was transferred from the 600-litres storage tank to the reservoirs with the help of a submersible pump. Generally, a single-compound acute toxicity test was run with seven nominal test concentrations spaced logarithmically with a control (variations from this approach in other experiments are explained in the relevant chapters). The sample size was ten animals per concentration with two replicates. As in acclimation, moulting was recorded daily.

During the exposure period, pellets were offered daily, and excess food and faecal matter siphoned after one hour. To avoid cross-contamination among treatments, each exposure chamber was individually siphoned out using assigned 1x15-cm glass tubes that could be attached to a common siphoning hose.

For the first day of exposure, the procedure was set as follows: After the photoperiod light was on, the first step was recording and removal of moults, followed by feeding of animals. The peristaltic pump was stopped and the plastic bags that cover the reservoirs were pulled down. Test solutions were made up by pippeting stock solution into each reservoir during the refilling up to 18 litres. This proved to be a good method to thoroughly mix the toxicants with

the seawater. Plastic bags were then pulled up. Before starting the pump again, the water of each exposure chamber was lowered to its minimum level and the toxicant solution was allowed to flow from the reservoirs by opening the channels in the peristaltic pump. After the exposure chambers reached their maximum level, the channels were closed and the peristaltic pump switched on at 12.5 $ml.min^{-1}$. This indicated the initial time of exposure. After one hour of feeding, the remaining pellets and faecal matter were siphoned out.

For the following days of exposure, moults were recorded and removed, and food pellets offered for one hour followed by siphoning. The peristaltic pump was stopped and the bags were pulled down to see if there was any test solution in the reservoirs. All remaining test solution was drained out of the system, so fresh solution could be prepared. New test solutions were prepared as described previously. Plastic bags were then pulled up and the peristaltic pump was switched on.

For single-compound and mixture toxicity tests, mortalities were recorded at 0.25, 0.5, 0.75, 1, 2, 4, 8, 14, 24, 33 and 48 hours, and thereafter at daily intervals until the end of the experiment (exposure times are mentioned in the relevant chapters). Partially immobilised organisms which were able to move their limbs were not considered as 'responding'. Dead organisms were removed (as soon as they were detected), weighed and registered in relation to the time of death. At the end of the experiments, all the remaining animals were weighed and preserved in Davidson's AFA fixative (Bell and Lightner, 1988).

Mortality results from all definitive experiments are presented in Appendix B (Table B.1 to B.5). The analysis of the data and statistical methods will be mentioned in the relevant chapters.

2.9. Results and discussion

The information presented and discussed in this section is focused in the overall results on the flow-through system utilisation for the toxicity tests.

The simple and modular characteristics of the flow-through system allowed an easy operation and uncomplicated daily maintenance, and its construction was relatively inexpensive. The utilisation of a continuous-duty peristaltic pump was an essential feature to perform reliable time-independent exposures and long-term experiments. Including acclimation, the longer experimental period was of 42 days for the Tilt subchronic test (see Chapter 4). With some modifications, the system could be readily adapted for lower or higher organisms loading due to the variable-speed capability of the peristaltic pump. For all experiments, the flow-through system had an overall daily consumption of 288-litres equally distributed among sixteen flow channels. According with the nomograph provided by Sprague (1969), this represents a 90% of replacement of the test solution in each exposure chamber (4.5 litres) every 15 hours. The rate of flow of test solution per gram of shrimp in the experiments (see Chapters 3, 4 and 5) ranged from 1.32 to 2.22 $l.g^{-1}.day^{-1}$ (Table 2.1), close to the values recommended by Sprague (1969) of 2 to 3 $l.g^{-1}.day^{-1}$ for fish in continuous-flow tests. This flow rate proved to be adequate to optimally maintain 10 shrimp

Table 2.1. Daily flow rates of test solution per gram of shrimp in the FTS. Standard deviation indicated in parenthesis.

Toxicant(s)	Section	Average weight (g)	Flow rate per gram ($l.g^{-1}.day^{-1}$)
Methyl-parathion	3.3.1	Not determined	Not determined
Tilt	3.3.2	0.97 (0.29)	1.85
Propiconazole	3.3.3	1.37 (0.71)	1.32
Tilt	4.3	1.14 (0.42)	1.58
Methyl-parathion – Tilt	5.3.1	0.81 (0.26)	2.22
Methyl-parathion – Propiconazole	5.3.2	0.81 (0.26)	2.22

juveniles per exposure chamber as 100% survival was achieved in the control channels in all experiments.

The design of the flow-through system has some basic advantages in comparison with other systems. The multi-channel peristaltic pump permits adjustment of the flow rate in relation to the loading of organisms and volume of exposure chamber. The toxicants were individually placed in reservoirs, which later delivered the test solution into the exposure chambers. This feature offers benefits over more sophisticated systems such as proportional diluters or split channel designs, which lack truly independent channels required for inferential statistical analysis (Hurlbert, 1984). On the other hand, more simple designs such as static or water-renewal systems are ineffective in maintaining a constant exposure level (Diamond *et al.*, 1995; Van Leeuwen, 1995). In addition, static systems could present problems when dealing with oil-formulated chemicals or hydrocarbons, which tend to form a film in the water surface preventing the oxygen exchange. A solution could be to place artificial aeration, but this could facilitate in turn the volatilisation of certain compounds. The organisms in a flow-through systems are less likely to suffer from oxygen deprivation since there is a continuous and automatic replacement of test solution, which also prevents the build up of waste metabolic products. This allows the feeding of test organisms during the experiment, therefore minimising the starvation factor in the toxic response.

For the experiments performed in this study, each exposure chamber had 10 cylindrical cages, which were aimed to perform individual observations on the

test organisms. The cages, however, presented a potential binding surface for pesticides. However, this would be compensated with the constant replacement of the test solution through the exposure chambers. Further analytical corroboration of toxicant levels in the flow-through system is necessary. On the other hand, the use of cages allowed the assessment of individual endpoints. For instance, moult frequency can be particularly difficult to study in shrimps since tags are lost after ecdysis and cast moults are rapidly eaten when the animals are placed in groups. If shrimps are confined to individual cages, most moult casts can be easily collected and recorded during the first hours of the photoperiod light (moulting occurs mostly during the night and shrimps normally do not eat immediately after ecdysis). This also applies to the study of other endpoints that need periodic measurements from a given organism (i.e. growth, development, reproductive parameters, etc.), or non-destructive sampling procedures (i.e. of haemolymph).

CHAPTER 3.

ACUTE TOXICITY TESTS: SINGLE-COMPOUNDS

3.1. INTRODUCTION

The prediction of ecological effects of pollutants and other stressors constitutes the scientific basis for the use of bioassays and toxicity tests in ecotoxicology (Cairns and Pratt, 1989). Ideally, this goal can be achieved through a comprehensive understanding of the whole ecosystem, but in practice this is not often achievable. Thus ecotoxicological research has focused on the effects at different levels of biological organisation, with attempts being made to link effects at lower levels to consequences at higher levels. Frequently, the aim is to assess the degree of hazard of chemicals to man and the environment by establishing dose-response relationships (WHO, 1978; OECD, 1993). Such approach, although it helps to simplify the problem, does not consider ecosystem dynamics and thus may fail to predict the effects of stressors on the "ecosystem health" (Cairns and Pratt, 1989), although attempts have been made to extrapolate laboratory aquatic toxicity data to the real environment (OECD, 1992). As an alternative, Hebel *et al.* (1997) have proposed a holistic approach to study simultaneously a repertoire of responses at several levels of organisation of the organisms in order to perform an integrative assessment of the biological responses to chemicals. These concepts are likely to continue evolving in the years to come as risk assessment of new and existing pollutants requires a combination of better understanding of the ecological processes with the continuous improvement of the toxicity tests.

Observing the above arguments, the information provided by conventional dose-response bioassays (such as the acute toxicity tests performed in this study), should be considered as a basic outline for ecotoxicological research (Abel, 1991a; Van Leeuwen, 1995). Alternatively, toxicity tests could be useful as screening tools to characterise the toxicity of chemicals (i.e. comparative toxicity, threshold levels, specific effects), to establish quantitative structure-activity relationships (QSAR) and to understand the mode of action of pollutants.

Acute toxicity can be defined as the severe effects suffered by organisms from relatively short exposures to toxic chemicals (Van Leeuwen, 1995). It assumes that an individual organism's fitness is diminished in the presence of sufficiently high concentration of a toxicant (Newman and Dixon, 1996). Thus, the objective of a toxicity test is to determine the concentration of a particular chemical that will elicit a specific response or measurable endpoint from a test species in a given time (Van Leeuwen, 1995).

Mortality is the most commonly used endpoint for aquatic organisms, and is usually expressed in terms of the median lethal concentration (LC50, defined as the concentration of the toxicant in water that kills 50% of the population observed). Analogously, an EC50 (or median effect concentration) can be determined for effects other than lethality. If calculated at different exposure times, LC50 values can be used to construct a toxicity curve, which describes the relationship between the poison concentration and survival time of the test

organisms exposed to the toxicant (Abel, 1991b). Eventually, when the exposure time is prolonged, the toxicity curve becomes asymptotic to the time axis, which means that further increases in exposure period cause no change in mortality (Abel, 1991b; Walker *et al.*, 1996). This steady level is called the lethal threshold concentration (or incipient lethal level) (Abel, 1991b; Van Leeuwen, 1995). It is reasonable to assume that when the threshold LC50 is reached the tissue concentration in the organisms is also no longer increasing with time (Walker *et al.*, 1996). In marine animals, this uptake (of organic pollutants) is a simple non-mediated transport through the integument, respiratory surfaces (gills) and gut (Lee, 1984). In some cases, the highest concentration of a chemical that does not have any significant adverse effect on the organisms can be estimated. Such a concentration is generically designated as the no observed effect concentration or NOEC (OECD, 1993; Van Leeuwen, 1995).

Studies with methyl-parathion on aquatic animals have shown that its toxicity varies considerably between species. In 1993, the WHO published the Environmental Health Criteria on methyl-parathion. The report indicates that methyl-parathion is highly toxic for aquatic invertebrates, with LC50's ranging from <1 µg to about 40 $\mu g.l^{-1}$. In the same review, the LC50's of fresh and seawater fish are reported between 6 and 25 $mg.l^{-1}$. For marine and estuarine species, the acute effects of methyl-parathion are summarised in Table 3.1. In general, crustaceans were found to be far more sensitive than annelids, molluscs and fishes. The LC50 values for penaeid shrimps varied between 0.5

Table 3.1. Acute effects of methyl-parathion on marine and estuarine animals in laboratory studies. Modified from WHO (1993).

Species	Life stage	Test period (h)	Experimental conditions	Criterion effect measured[a]	Concentration ($\mu g.l^{-1}$)	Remarks[b]	References
ANNELIDA							
Branchiura sowerbyi	n.s.	72	st.; 4.4°C	m, 100%	4 000	P: techn. gr. s: acetone	Naqvi (1973)
	n.s.	72	st.; 21°C	m, 0%	4 000	P: techn. gr. s: acetone	Naqvi (1973)
	n.s.	72	st.; 32°C	m, 100%	4 000	P: techn. gr. s: acetone	Naqvi (1973)
MOLLUSCA **Oyster**							
Crassostrea virginica	larvae	48	st.; seawater, 25°C	d, EC50	12 000	P: 99% s: TEG	Mayer (1987)
Clam							
Mercenaria mercenaria	adult	96	st.; wellwater (24°/oo), pH 8	no effect	25 000	s: acetone	Mayer (1987)
Nassa dosoleta	adult	96	st.; wellwater (24°/oo), pH 8	no effect	25 000	s: acetone	Mayer (1987)
CRUSTACEA **Copepod**							
Acartia tonsa	n.s.	96	st.; seawater (22°/oo); 22°C; pH 8.1-8.2; DO 7-7.6	m, LC50	28	P: 99% s: TEG	Mayer (1987)
	adult	96	st.; synthetic seawater (22°/oo); 17°C	m, LC50	890	P: 80%	Khattat and Farley (1976)
Sand shrimp							
Crangon setemspinosa	2.6 cm 0.25 g	24	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	11	s: acetone	Eisler (1969)
	2.6 cm 0.25 g	48	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	3	s: acetone	Eisler (1969)
	2.6 cm 0.25 g	96	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	2	s: acetone	Eisler (1969)

Table 3.1. (continued)

Species	Life stage	Test period (h)	Experimental conditions	Criterion effect measured[a]	Concentration ($\mu g.l^{-1}$)	Remarks[b]	References
Mysid shrimp							
Mysidopsis bahia	24 h old	96	st.; seawater (20°/oo); 25°C; DO 4.3-5.5	m, LC50	0.98	P: 99% s: TEG	Mayer (1987)
	24 h old	96	st.; seawater (20°/oo); 25°C; DO 4.3-5.5	no effect	0.32	P: 99% s: TEG	Mayer (1987)
	24 h old	96	flow-through; seawater (20°/oo); 25°C; DO 4.3-5.5	m, LC50	0.77	P: 99% s: TEG	Mayer (1987)
	<24 h old	96	flow-through; 14°/oo; 19.5°C	m, LC50	0.78	P: 99% s: TEG	Mayer (1987)
	juvenile	96	flow-through; 22-28°C	m, LC50	0.77	s: TEG	Nimmo *et al.* (1981)
	juvenile	96	flow-through; 22-28°C	MATC	0.11-0.16	s: TEG	Nimmo *et al.* (1981)
Hermit crab							
Pangurus longicarpus	3.5 mm 0.28 g	24	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	23	s: acetone	Eisler (1969)
	3.5 mm 0.28 g	48	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	7	s: acetone	Eisler (1969)
	3.5 mm 0.28 g	96	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	7	s: acetone	Eisler (1969)
Crab							
Portunus trituberculatus	Zoea IV stage	24	25°C	m, LC50	0.17-0.5	-	Hirayama and Tamaoi (1980)
Grass shrimp							
Palaemonetes vulgaris	31 mm 0.47 g	24	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	15	s: acetone	Eisler (1969)
	31 mm 0.47 g	48	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	10	s: acetone	Eisler (1969)
	31 mm 0.47 g	96	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	3	s: acetone	Eisler (1969)

Table 3.1. (continued)

Species	Life stage	Test period (h)	Experimental conditions	Criterion effect measured[a]	Concentration ($\mu g.l^{-1}$)	Remarks[b]	References
Penaeid shrimp							
Penaeus aztecus	adult	24	flow-through; 29°/oo; 25°C	m, LC50	5.5	s: acetone	Butler (1964)
	adult	48	flow-through; 29°/oo; 25°C	m, LC50	5.5	s: acetone	Butler (1964)
Penaeus duorarum	n.s.	n.s.	flow-through; 17-31°/oo; 7.6-28.8°C	m, LC50	1.9	s: acetone	Schoor and Braush (1980)
	post-larvae	96	flow-through; seawater; 20°/oo; 25°C	m, LC50	1.2	P: 99% s: TEG	Mayer (1987)
Penaeus japonicus	post-larvae	24	25°C	m, LC50	0.5-0.9	-	Hirayama and Tamaoi (1980)
Penaeus stylirostris	post-larvae	96	st.; seawater (20°/oo); 25°C; DO 5.6-6.3	m, LC50	1.4	P: 99% s: TEG	Mayer (1987)
Penaeus monodon	adult	96	st.; 15°/oo; 23°C; pH 7.3	m, LC50	148	-	Reddy and Rao (1986)
Penaeus indicus	adult	96	st.; 15°/oo; 23°C; pH 7.3	m, LC50	98	-	Reddy and Rao (1986)
	inter-moult, 2.5 g	48	st.; 15°/oo; 23°C; pH 7.1	m, LC50	95	-	Reddy and Rao (1986)
Metapenaeus monoceros	inter-moult, 2.5 g	48	st.; 15°/oo; 23°C; pH 7.1	m, LC50	120	-	Reddy and Rao (1988)
	adult	96	st.; 15°/oo; 23°C; pH 7.3	m, LC50	102	-	Reddy and Rao (1986)
Metapenaeus dopsoni	adult	96	st.; 15°/oo; 23°C; pH 7.3	m, LC50	115	-	Reddy and Rao (1986)
FISH **American eel**							
Anguilla rostrata	59 mm 0.14 g	24	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	27 600	P: act. ingred. s: acetone	Eisler (1970a)
	59 mm 0.14 g	48	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	22 400	P: act. ingred. s: acetone	Eisler (1970a)
	59 mm 0.14 g	96	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	16 900	P: act. ingred. s: acetone	Eisler (1970a)

Table 3.1. (continued)

Species	Life stage	Test period (h)	Experimental conditions	Criterion effect measured[a]	Concentration ($\mu g.l^{-1}$)	Remarks[b]	References
Anguilla rostrata	< 1 year	24	st.; 20°C; pH 7.2; DO 6; H 50	m, LC50	42 600	P: act. ingred. s: acetone	Rehwoldt *et al.* (1977)
	< 1 year	48	st.; 20°C; pH 7.2; DO 6; H 50	m, LC50	37 200	P: act. ingred. s: acetone	Rehwoldt *et al.* (1977)
	< 1 year	96	st.; 20°C; pH 7.2; DO 6; H 50	m, LC50	6 300	P: act. ingred. s: acetone	Rehwoldt *et al.* (1977)
Sheepshead minnow							
Cyprinodon variegatus	28 days old	96	st.; seawater (20°/oo); 25°C; DO 4.6-5.7	m, LC50	12 000	P: 99% s: TEG	Mayer (1987)
	28 days old	96	st.; seawater (20°/oo); 25°C; DO 4.6-5.7	no effect	10 000	P: 99% s: TEG	Mayer (1987)
Mummichog							
Fundulus heteroclitus	55 mm 1.7 g	24	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	> 85 100	P: act. ingred. s: acetone	Eisler (1970a)
	55 mm 1.7 g	48	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	85 200	P: act. ingred. s: acetone	Eisler (1970a)
	55 mm 1.7 g	96	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	58 000	P: act. ingred. s: acetone	Eisler (1970a)
	42 mm	96	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	8 000	P: act. ingred. s: acetone	Eisler (1970b)
	42 mm	96 (+ 240 obser-vation)	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	4 000	P: act. ingred. s: acetone	Eisler (1970b)
	42 mm	96	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	1 210	Solution aged for 96 h	Eisler (1970b)

Table 3.1. (continued)

Species	Life stage	Test period (h)	Experimental conditions	Criterion effect measured[a]	Concentration ($\mu g.l^{-1}$)	Remarks[b]	References
Striped killifish							
Fundulus majalis	84 mm 6.5 g	24	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	29 000	P: act. ingred. s: acetone	Eisler (1970a)
	84 mm 6.5 g	48	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	19 400	P: act. ingred. s: acetone	Eisler (1970a)
	84 mm	24	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	13 800	P: act. ingred. s: acetone	Eisler (1970a)
Spot							
Leiostomus xanthurus	84 mm 6.5 g	96	st.; seawater (20°/oo); 25°C; DO 3.2-4.5	m, LC50	93	P: 99% s: TEG	Mayer (1987)
	84 mm 6.5 g	96	st.; seawater (20°/oo); 25°C; DO 3.2-4.5	no effect	56	P: 99% s: TEG	Mayer (1987)
	84 mm 6.5 g	96	flow-through; 20°/oo; 25°C	m, LC50	59	P: 99% s: TEG	Mayer (1987)
Atlantic silverside							
Menidia menidia	50 mm 0.8 g	24	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	24 800	P: act. ingred. s: acetone	Eisler (1970a)
	50 mm 0.8 g	48	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	21 900	P: act. ingred. s: acetone	Eisler (1970a)
	50 mm 0.8 g	96	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	5 700	P: act. ingred. s: acetone	Eisler (1970a)
Striped bass							
Morone saxatilis	1 year	24	st.; 20°C; pH 7.2; DO 6; H 50	m, LC50	16 800	P: act. ingred. s: acetone	Rehwoldt *et al.* (1977)
	1 year	48	st.; 20°C; pH 7.2; DO 6; H 50	m, LC50	14 200	P: act. ingred. s: acetone	Rehwoldt *et al.* (1977)
	1 year	96	st.; 20°C; pH 7.2; DO 6; H 50	m, LC50	14 000	P: act. ingred. s: acetone	Rehwoldt *et al.* (1977)
	adult	96	interm. flow; 12.8°C	m, LC50	790	P: 99%	Earnest (1970)
	juvenile	96	flow-through; 30°/oo; 13°C	m, LC50	790	P: 86% s: ethanol	Korn and Earnest (1974)

Table 3.1. (continued)

Species	Life stage	Test period (h)	Experimental conditions	Criterion effect measured[a]	Concentration ($\mu g.l^{-1}$)	Remarks[b]	References
Black mullet							
Mugil cephalus	48 mm 0.78 g	24	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	39 000	P: act. ingred. s: acetone	Eisler (1970a)
	48 mm 0.78 g	48	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	26 300	P: act. ingred. s: acetone	Eisler (1970a)
	48 mm 0.78 g	96	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	5 200	P: act. ingred. s: acetone	Eisler (1970a)
Northern puffer							
Sphaeroides maculatus	196 mm 153 g	24	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	100 000	P: act. ingred. s: acetone	Eisler (1970a)
	196 mm 153 g	48	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	91 000	P: act. ingred. s: acetone	Eisler (1970a)
	196 mm 153 g	96	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	75 800	P: act. ingred. s: acetone	Eisler (1970a)
Bluehead							
Thalassoma bifasciatum	90 mm 7 g	24	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	98 000	P: act. ingred. s: acetone	Eisler (1970a)
	90 mm 7 g	48	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	88 000	P: act. ingred. s: acetone	Eisler (1970a)
	90 mm 7 g	96	st.; wellwater (24°/oo); 20°C; pH 8; DO 7.1-7.7	m, LC50	12 300	P: act. ingred. s: acetone	Eisler (1970a)

[a] Criterium: m = mortality; d = development; LC50 = median lethal concentration; EC50 = median effect concentration MATC = maximum acceptable toxic concentration

[b] P = purity; s = solvent; TEG = triethylene glycol; techn. gr. = technical grade

st = static system; °/oo = parts per thousand (salinity); H = hardeness ($mg.l^{-1}$ $CaCO_3$); DO = dissolved oxygen (mg $O_2.l^{-1}$); n.s. = data not specified.

$\mu g.l^{-1}$ for post-larvae of *Penaeus japonicus* (24 h) to 148 $\mu g.l^{-1}$ for adults of *Penaeus monodon* (96 h).

The toxicological information of propiconazole on aquatic organisms is presented in Table 3.2. Dr. C. Neumann from Novartis Crop Protection, Basel, Switzerland, complemented some of the toxicological information presented. Although the information is scarce, it can be appreciated that both freshwater and marine invertebrates are in general more sensitive to propiconazole than fishes. However, the toxicity varies considerably between species, with LC50 or EC50 generally ranging between 1 to 10 $mg.l^{-1}$. Marine crustaceans, *Mysidopsis bahia* and *L. vannamei*, are among the most sensitive organisms to propiconazole.

In this chapter, the acute toxicity of methyl-parathion, propiconazole and Tilt was determined with juveniles of *Litopenaeus vannamei* in flow-through conditions. Using mortality as endpoint, the experiments were performed in a time-independent fashion in order to establish the threshold LC50 for each toxicant. Moulting and behaviour were evaluated during the experiments.

3.2. MATERIAL AND METHODS

3.2.1. Range finding tests with methyl-parathion, Tilt and propiconazole.

Range finding tests were conducted for methyl-parathion, Tilt and propiconazole as described in the section 2.6. In all cases, test organisms were randomly allocated in the exposure system and kept in acclimation for 2

Table 3.2. Toxicological information of propiconazole on aquatic organisms in laboratory studies.

Species	Life stage	Test period (h)	Experimental conditions	Criterion effect measured	Concentration ($\mu g.l^{-1}$)	References
FRESHWATER						
ALGAE						
Scenedesmus subspicatus	n.s.	72	st.	EC50	4 800	Novartis (1997)
	5 days preculture	120	st.	EC50	800	Novartis (1999b)
Navicula seminumum	11 days old	264	st.	EC50	900	Novartis (1999b)
Anabaena sp	8 days old	264	st.	EC50	13 600	Novartis (1999b)
Lemma gibba	7 days old	336	st.	EC50	900	Novartis (1999b)
CRUSTACEA						
Daphnia magna	<24-h.	48	st.	EC50	4 800	Novartis (1997)
	<24-h.	48	st.	EC50	11 500	Neumann (com. pers.)
	n.s.	48	n.s.	m, LC50	3 200	Novartis (1999a)
	<24-h.	504	Flow-though; life cycle	MATC	> 310 and < 690	Novartis (1999a)
	n.s.	n.s.	n.s.	EC50 – LC50	3 200 – 11 500	Novartis (1999b)
Procamarus sp	n.s.	96	Flow-through, 9 weeks acclimation	LC50	42 000	Novartis (1999b)
Gammarus kischineffensis	adults	96	Daily renewal	LC50	1 800	Meliyan (1991)
FISH						
Trout	n.s.	96	st.; 12-d acclimation	LC50	5 300	Novartis (1997)
Rainbow trout	n.s.	96	st.	LC50	5 200	Novartis (1999a)
Salmo trutta fario	n.s.	n.s.	n.s.	EC50 – LC50	900 – 13 200	Novartis (1999b)
Salmo gairdneri	n.s.	n.s.	n.s.	LC50	3 300	Novartis (1999b)
Carp	n.s.	96	st.; 16-d acclimation	LC50	6 800	Novartis (1997)
Cyprinus carpio	n.s.	n.s.	n.s.	EC50 – LC50	6 800 – 21 000	Novartis (1999b)
Bluegill	n.s.	96	st; 20-d acclimation	LC50	6 400	Novartis (1997)
	n.s.	96	n.s.	LC50	> 100 000	Novartis (1999a)
Lepomis macrochirus	n.s.	n.s.	n.s.	EC50 – LC50	1 300 – 10 200	Novartis (1999b)

Table 3.2. (continued).

Species	Life stage	Test period (h)	Experimental conditions	Criterion effect measured[a]	Concentration ($\mu g.l^{-1}$)	References
Catfish	n.s.	96	st.; 18-d acclimation	LC50	5 100	Novartis (1997)
Ictaluris melas	n.s.	n.s.	n.s.	EC50 – LC50	2 000 – 5 100	Novartis (1999b)
Fathead minnow	<24-h	31 days	Flow-through	MATC	> 430 and < 970	Novartis (1999a)
Pimephales promelas	n.s.	n.s.	n.s.	LC50	7 600	Novartis (1999b)
MARINE						
ALGAE						
Skeletonema costatum	8 days	264	st.	EC50	2 200	Novartis (1999b)
MOLLUSCA						
Crassostrea virginica	n.s.	96	Flow-through, 3 weeks acclimation	EC50	300	Novartis (1999b)
CRUSTACEA						
Mysidopsis bahia	6-8 days	96	Flow-through	EC50 – LC50	500 – 1 400	Novartis (1999b)
Litopenaeus vannamei	Post-larvae	48	st; 35°/oo; 28°C, Tilt	LC50	500	Enriquez *et al.* (1996)
	Post-larvae	48	st; 2°/oo; 24°C , Tilt	LC50	1 300	Enriquez *et al.* (1996)
FISH						
Leiostomus xanthurus	n.s.	n.s.	n.s.	LC50	2 200	Novartis (1999b)

m = mortality; LC50 = median lethal concentration; EC50 = median effect concentration; MATC = maximum acceptable toxic concentration;

n.s. = data not specified; st = static system; °/oo = parts per thousand (salinity);

The information from Novartis (1999a, 1999b) was complemented by Dr. C. Neumann (Novartis Crop Protection, Basle, Switzerland).

or 3 days. Test solutions were prepared (as described in section 2.3) by mixing the appropriate amounts of stock solution of methyl-parathion, Tilt, and propiconazole with sea water into the exposure system. For methyl-parathion and Tilt, the concentration of stock solution, tested concentrations and exposure system are shown in Table 3.3.

Preliminary experiments were run with only with methanol and acetonitrile to evaluate the "safe levels" of carrier to be used in the propiconazole experiments. The conditions for solvent and propiconazole range finding tests are shown in Table 3.4.

The test solutions were renewed daily in both the static and flow-through system. In the static system, food was offered before the water renewal and moderate aeration was placed in each chamber. In the flow-through system the organisms were fed as described in 2.2.7. All tests were carried in a constant temperature room at 28 ± 1° C, with a 12/12 light/dark photoperiod as described in 2.2.3. Mortality was recorded daily and death animals were removed as soon as they were detected.

3.2.2. Acute toxicity tests with methyl-parathion, Tilt and propiconazole.

Based in the information from the range finding test, definitive acute toxicity tests were carried out. Shrimps were transferred to the flow-through system for acclimation as described in 2.2.6, followed by exposure as described in 2.2.7. For each acute test, the time of acclimation and exposure, concentration of stock solution and tested concentrations are shown in Table 3.5. For

Table 3.3. Range finding test conditions for methyl-parathion and Tilt. Concentrations based in the active compound content.

Toxicant	Stock solution ($mg\ l^{-1}$)	Tested concentrations ($\mu g\ l^{-1}$)	Exposure system
Methyl-parathion	80.3	0.1, 1, 5, 30, 50	Static
Tilt	25 000	100, 150, 200, 300, 500, 700, 900	Static
	7 000	900, 1 200, 1 700, 2 200, 2 700	Flow-through

Table 3.4. Range finding test conditions for carriers and propiconazole. Propiconazole concentrations based in the active compound content.

Solvent	**Tested concentrations ($\mu l.l^{-1}$)**	**Exposure system**
Ethanol	277, 554, 833	Flow-through
Acetonitrile	139, 277	Flow-through

Toxicant	**Stock solution ($mg\ l^{-1}$)**	**Tested concentrations ($\mu g.l^{-1}$)**	**Exposure system**
Propiconazole	78 540	900, 2 000, 6 000, 8 000, 10 000, 13 000, Solvent control	Flow-through
	74 500	800, 1 200, 4 300, 10 000, Solvent control	Flow-through

Table 3.5. Experimental conditions for definitive acute toxicity tests with methyl-parathion, Tilt and propiconazole in the flow-through system. Concentrations based in the active compound content. Concentrations placed in duplicates with 10 organisms per channel.

Toxicant	Acclimation / Exposure (hours)	Stock solution (mg l^{-1})	Tested concentrations (µg l^{-1})
Methyl-parathion	552 / 240	80.3	0.1, 0.5, 2.0, 10, 20, 35
Tilt	288 / 240 240 / 768 312 / 240	2 483 2 500 8 000	100, 150, 220, 320, 420, 500, 600 400, 500, 600, 690, 770, 850, 900 950, 1 000, 1 055, 1 110, 1 170, 1 235, 1 300
Propiconazole	240 / 336	78 000	800, 1 250, 2 000, 3 100, 4 900, 7 600, 12 000, Solvent control (acetonitrile: 155.5 µl.l^{-1})

propiconazole, a solvent control was included with the maximum amount of carrier solvent used in the treatments.

Dead organisms were removed and weighed as soon as they were detected. For Tilt and propiconazole tests, the time of death was recorded for each organism. The remaining organisms were also weighed at the end of the experiment prior to being preserved in Davidson's AFA fixative (Bell and Lightner, 1988). Moult occurrences were recorded during the acclimation and exposure periods.

3.2.3. Statistical analysis

The time–independent exposures performed in the flow-through system were aimed at establishing the lethal threshold concentration (threshold LC50) by calculating the median lethal concentration (LC50) at different times of exposure using Probit analysis with a maximum likelihood regression algorithm (Toxicologist software, version 1.0, 1990). Thresholds LC50's were established as the lowest LC50 determined for each exposure. Additional analyses were performed using the statistical software SigmaStat for Windows (version 1.01) and Statistica for Windows (release 4.2).

The average wet weight (± standard deviation) of the organisms was used to calculate the flow rate in relation to loading of organisms in the exposure chamber. In order to evaluate if the lethal responses occurred in a size-dependent pattern in the flow-through system, the time of death was analysed for correspondence with the wet weight by correlation analysis (Zar, 1996).

For each channel, moulting was evaluated by registering the number of moult casts produced by the organisms (moult frequency) and by calculating the time between consecutive moults (intermoult duration). For acclimation, moult frequency was calculated per channel and subsequently used to estimate the overall moulting frequency. In a similar way, intermoult duration was calculated per organism and per channel, and then used to estimate the overall intermoult duration. The intermoult data was ordered in class intervals to graphically assess its distribution of frequencies and normality by a Kolmogorov-Smirnov test. For exposure, the intermoult data was also ordered in class intervals, and when the data was appropriated, the cumulative frequencies were compared graphically.

3.3. RESULTS

The median lethal concentrations for methyl-parathion, Tilt and propiconazole on juveniles of *Litopenaeus vannamei* are shown in Table 3.6.

3.3.1. Methyl-parathion experiments

In the range finding test with methyl-parathion, the 50 % of responses occurred between 1 and 30 $\mu g.l^{-1}$ at 192 hours. In the definitive test (range from 0.1 to 35 $\mu g.l^{-1}$), the 50 % of mortality was obtained between 15 to 25 $\mu g.l^{-1}$ at the first 24 hours and between 0.1 to 10 $\mu g.l^{-1}$ at the 240 hours (Fig. 3.1). The toxicity curve (Fig. 3.2) shows that at 8 hours the LC50 occurs at 29 $\mu g.l^{-1}$, later to be situated around 20 $\mu g.l^{-1}$ from 14 to 72 hours. As the exposure continues, the

Table 3.6. Acute toxicity of methyl-parathion, Tilt and propiconazole on *Litopenaeus vannamei* in the flow-through system. Concentrations based in the active compound content. 95% confidence intervals are shown in parenthesis.

Time (Hours)	LC50 ($\mu g.l^{-1}$)		
	Methyl-parathion	**Tilt**	**Propiconazole**
8	29.16 *		
14	21.45 (17.14 – 25.63)	1598 *	
24	20.76 (16.42 – 24.94)	1272 (1201 – 1511)	9212 (6921 – 14595)
33	19.90 (16.42 – 24.4)		6991 (5306 – 8813)
48	19.02 *	1145 (1115 – 1175)	6204 (4551 – 7622)
58	17.87 *		
72	17.87 *	1137 (1110 – 1164)	5449 (4201 – 6666)
96	13.73 *		2539 (2063 – 3047)
120	10.45 (5.34 – 16.73)		1867 (1343 – 2339)
144	7.72 (3.77 – 12.15)		1780 (1231 – 2261)
168	6.33 (3.07 – 10.1)		1716 (1158 – 2196)
192	5.89 (2.85 – 9.46)		1716 (1158 – 2196)
216	5.09 (2.47 – 8.27)		1716 (1158 – 2196)
240	4.38 (2.13 – 7.20)		

* unreliable confidence limits.

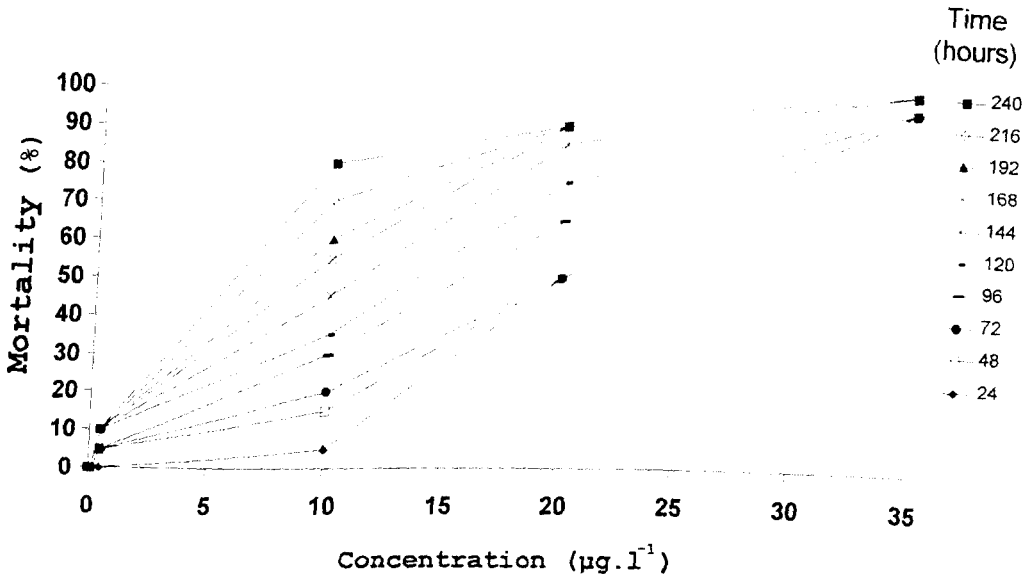


Fig. 3.1 Mortality pattern of *Litopenaeus vannamei* exposed to methyl-parathion. Group size = 20.

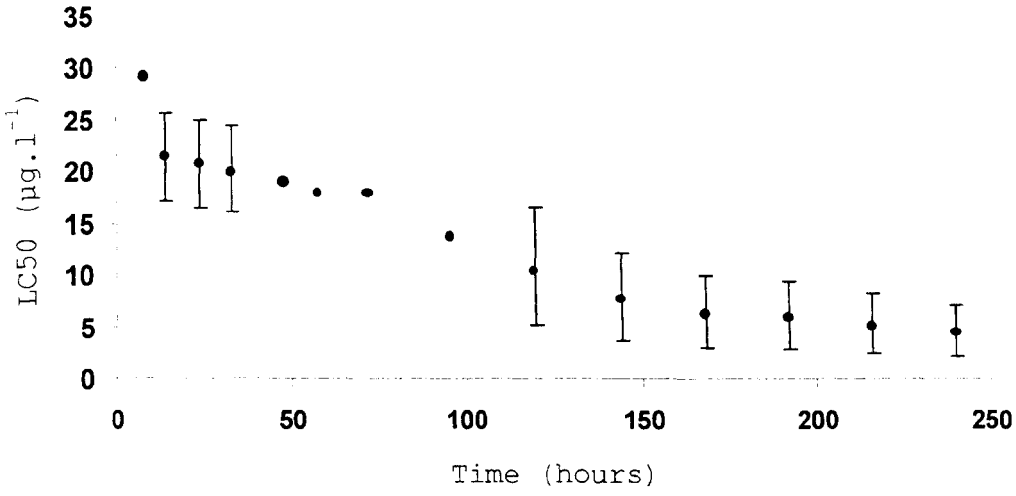


Fig. 3.2 LC50 values and 95% confidence limits of methyl-parathion for *Litopenaeus vannamei*. Threshold LC50 of 4.4 $\mu g.l^{-1}$.

LC50 gradually decreases to 10.5 $\mu g.l^{-1}$ at 120 hours and becomes asymptotic to the concentration axis in a threshold LC50 of 4.4 $\mu g.l^{-1}$ at 240 hours. The wet weight of test organisms was not recorded for the definitive test with methyl-parathion.

During acclimation, the average rate of moulting per organism occurred in average 0.98 times per ten days of acclimation. Table 3.7 shows the average of moult frequency per channel. For the same period, the organisms showed an average intermoult duration of 186 hours (≈8 days). The histogram in Fig. 3.3 shows the frequency of occurrence of intermoult data from all organisms, which failed the normality test ($P < 0.0001$).

The average rate of moulting per organism during the exposure is shown in Table 3.8, occurring on average 0.4 times in ten days for the control channels. The intermoult duration data are presented in Fig. 3.4. These data were not appropriate for statistical analysis since few organisms registered more than two moults during the exposure.

Some differences in behaviour were observed in the exposed animals in comparison with the controls. At the beginning of the exposure, most animals in all methyl-parathion treatments showed unusual irritability to light (some mortality counts were performed during the night) and handling (cleaning of chambers) in relation to control organisms. Symptoms of disorientation and spasms were observed at the highest concentrations, while in general, the exposed animals showed reduced food consumption, although no

Table 3.7. Moulting frequency per channel during ten days of acclimation for methyl-parathion definitive experiment. 10 shrimps in all channels. Conf. int. = 95% confidence interval for the population median.

Channel	1	2	3	4	5	6	7
Moults in 10 days	1.22	1.04	1.09	0.78	0.74	1.04	1.04
Median	3	2	2.5	2	2	2.5	2
Conf. int.	2-4	2-3	1-4	1-3	0-3	2-3	1-4
Channel	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Moults in 10 days	0.83	1	0.87	0.61	1.48	1.13	0.87
Median	2	3	2	1	3	2	2
Conf. int.	1-3	0-3	1-3	0-3	3-4	2-4	1-3

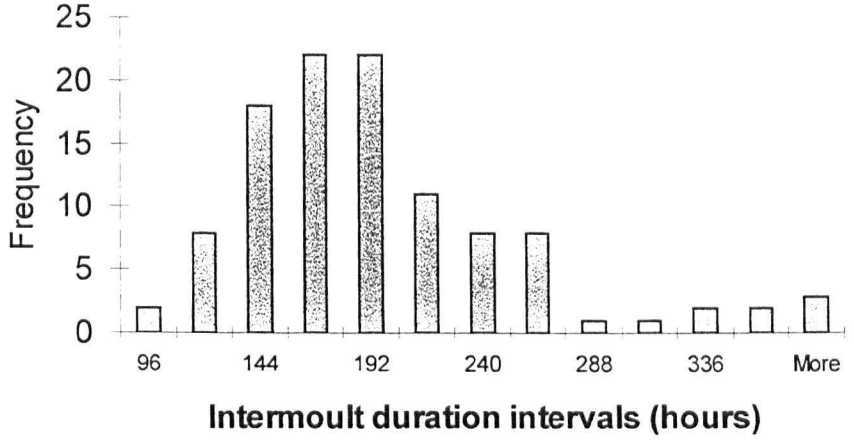


Fig. 3.3 Frequency distribution of intermoult duration during acclimation for the methyl-parathion definitive experiment.

Table 3.8. Moulting frequency per channel during ten days of exposure to methyl-parathion in the FTS. Concentrations based in the active compound content. Conf. int. = 95% confidence interval for the population median.

Channel	1	2	3	4	5	6	13	14
Conc. ($\mu g.l^{-1}$)	0.1	0.1	0.5	0.5	2	2	0	0
n	10	10	9	9	10	10	10	10
Moults in 10 days	0	1.3	0.4	0.3	0.7	0.4	0.4	0.4
Median	0	1	0	0	0.5	0	0	0
Conf. int.	0-0	1-2	0-1	0-1	0-1	0-1	0-1	0-1

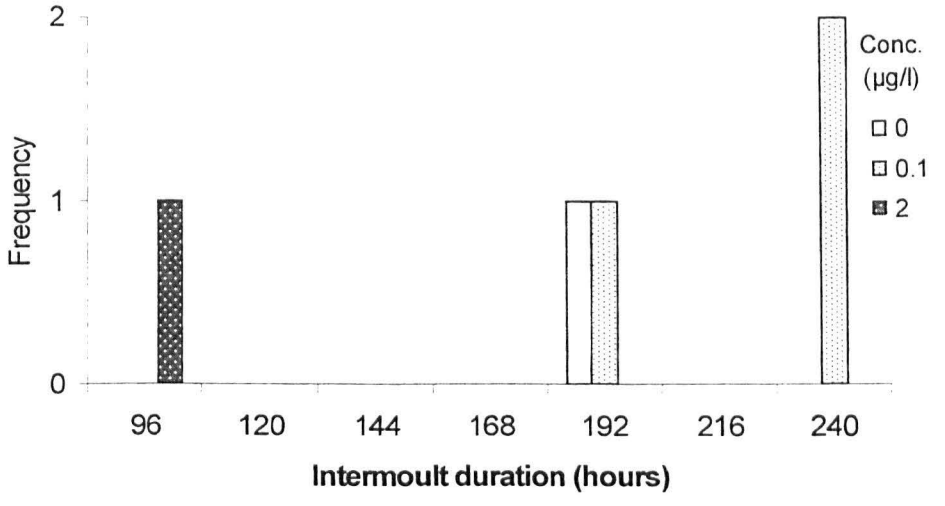


Fig. 3.4. Frequency distribution of intermoult duration data during exposure to methyl-parathion.

quantification was performed. In some cases, the organisms were able to detect the food when offered, but were unable to collect the pellets with their pereopods, nor to retain them once collected.

3.3.2. Fungicide experiments

3.3.2.1. Tilt

The mortalities recorded in the range finding tests for Tilt in the static system during 96 hours indicated that the lethal concentration lay between 150 and 500 $\mu g.l^{-1}$. A definitive test was set in the flow-through system with concentrations ranging from 100 to 600 $\mu g.l^{-1}$. Very few mortalities were recorded in this experiment over the first 240 hours. Further range finding tests performed in the static system confirmed the occurrence of the 96 hours LC50 between 150 and 500 $\mu g.l^{-1}$, so a second definitive acute test was carried from 400 to 900 $\mu g.l^{-1}$. This time the exposure was prolonged until 758 hours, but again few mortalities were recorded. This test, however, produced notable alterations in the exposed shrimps, which are described in Chapter 4.

After this, the range finding test was carried out in the flow-through system with concentrations ranging between 900 to 2700 $\mu g.l^{-1}$. The results of this test indicated that the 72-h LC50 lied between 900 and 12 000 $\mu g.l^{-1}$.

The definitive acute toxicity test for Tilt was set up with seven concentrations ranging from 950 till 1300 $\mu g.l^{-1}$, and showed 50 % of mortality between 1 200 and 1 300 $\mu g.l^{-1}$ at 24 hours, and between 1 000 and 1 200 $\mu g.l^{-1}$ at 72 hours

(Fig. 3.5). The toxicity curve is presented in Fig. 3.6, with a 14-h LC50 of 1 598 $\mu g.l^{-1}$ followed by values around 1 200 $\mu g.l^{-1}$ between 24 and 48 hours, to finally reach an asymptotic line at 72-h with a LC50 of 1 137 $\mu g.l^{-1}$. This value can be considered the threshold since no further mortalities were recorded during 240 hours of exposure.

The wet weight from the shrimps used in the toxicity test was on average 0.97 ± 0.29 g. Thus, the flow rate in the flow-through system was of 1.85 $l.g^{-1}$ per day, considering ten animals per exposure chamber and a daily consumption of 18 litres per channels (see section 2.9). Mortalities did not occur in a size-dependent pattern in the system. Fig. 3.7 shows the average wet weight of the organisms per Tilt concentration in relation with the time of death. No correlation was found between wet weight and time of death for the Tilt treatments (r = correlation coefficient; $P<0.05$): 1 300 $\mu g.l^{-1}$ ($r = 0.081$, $n = 20$); 1 235 $\mu g.l^{-1}$ ($r = 0.39$, $n = 20$); 1 170 $\mu g.l^{-1}$ ($r = -0.757$, $n = 5$); and 1 110 $\mu g.l^{-1}$ ($r = 0.274$, $n = 9$, P).

During acclimation, the average moulting rate was 1.19 times per 10 days. The moulting frequency per flow-through system channel is shown in Table 3.9. For the same period, the average intermoult duration from the shrimps was of 160 hours (≈ 7 days). The frequency distribution of the intermoult data from all organisms (Fig. 3.8) failed the normality test ($P < 0.0001$).

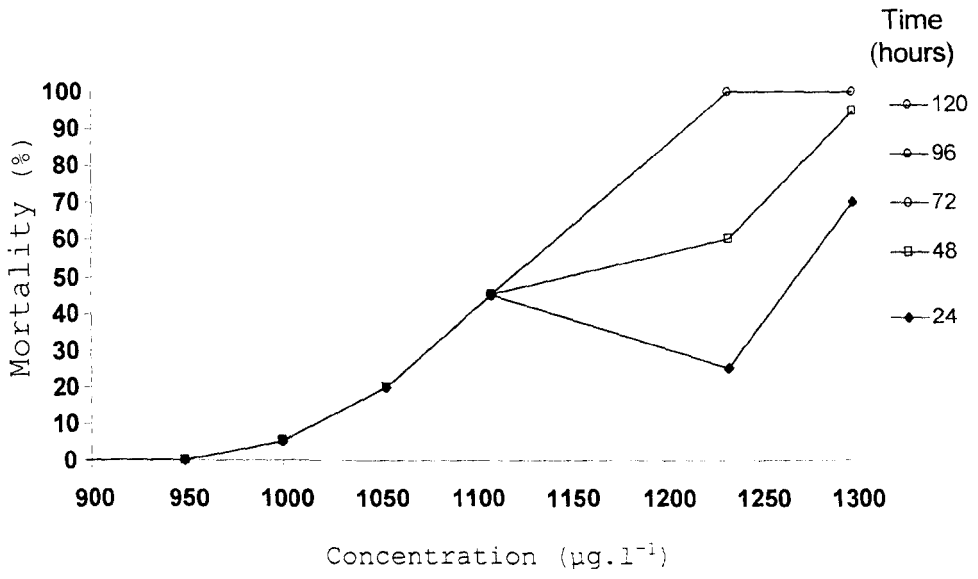


Fig. 3.5. Mortality pattern of *Litopenaeus vannamei* exposed to Tilt. Group size = 20.

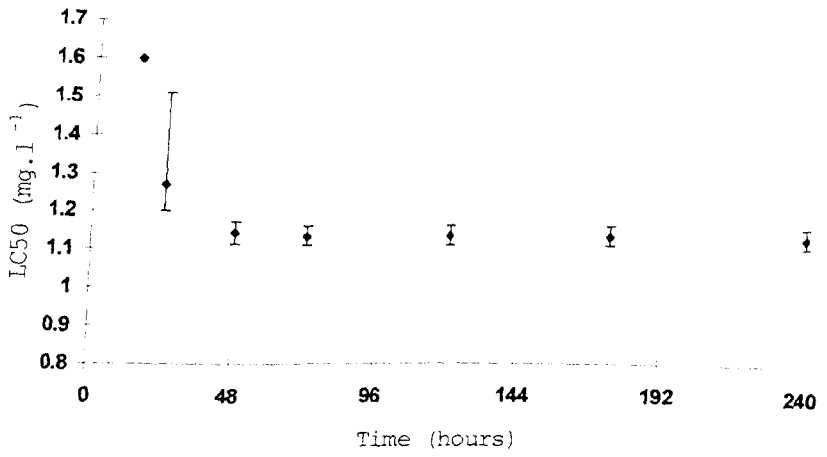


Fig. 3.6. LC50 values and 95% confidence limits of Tilt for *Litopenaeus vannamei*. Threshold LC50 of 1137 µg.l^{-1}.

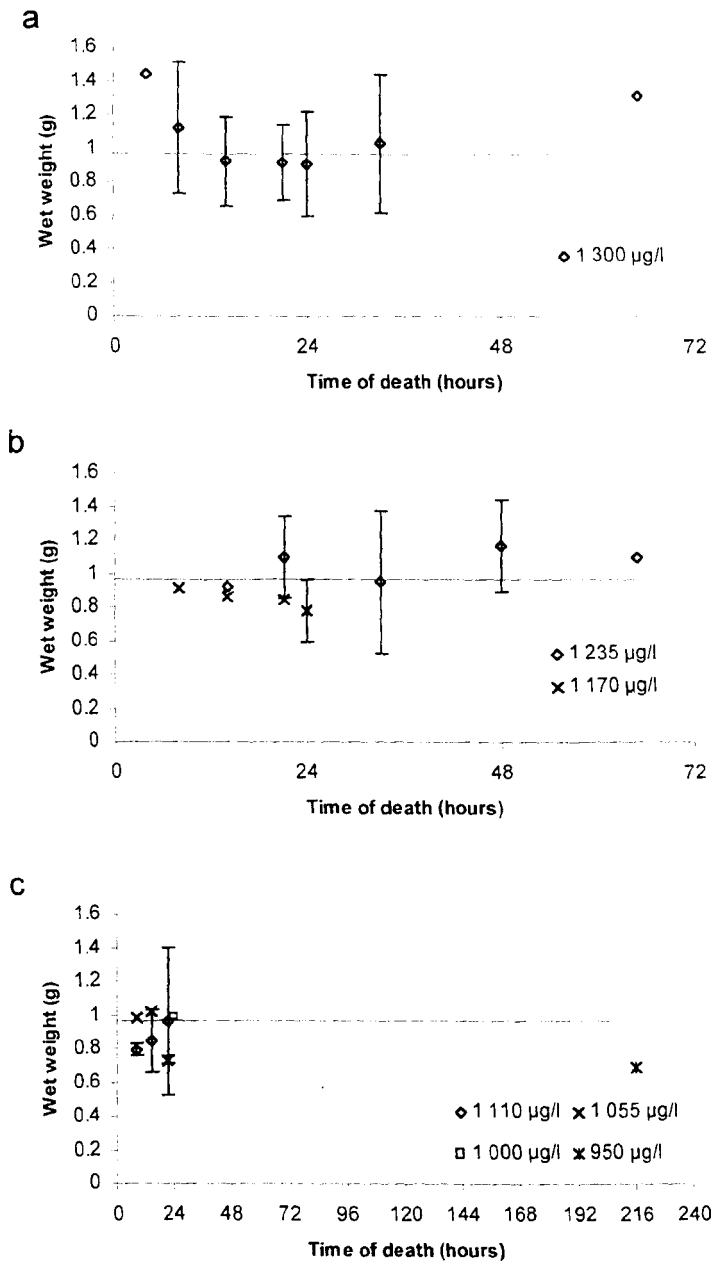


Fig. 3.7 (a, b and c). Average wet weight of organisms in relation to time of death for the Tilt acute experiment. Bars represent standard deviation and dashed line the average of all organisms used in the experiment.

Table 3.9. Moulting frequency per channel during ten days of acclimation for Tilt definitive experiment. Conf. int. = 95% confidence interval for the population median.

Channel	1	2	3	4	5	6	7	8
n	10	10	9	10	9	10	9	10
Moults in 10 days	1.38	1.15	1.20	1.23	1.28	1.08	1.11	0.85
Median	2	2	2	2	2	1.5	1	1
Conf. int.	1-2	1-2	1-2	1-2	1-2	1-2	1-2	0-2
Channel	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
n	10	10	10	10	10	10	10	9
Moults in 10 days	1.15	1.08	1.15	1.23	1.31	1.23	1.31	1.28
Median	1.5	1.5	2	2	2	2	2	2
Conf. int.	1-2	1-2	0-2	1-2	1-2	1-2	1-2	1-2

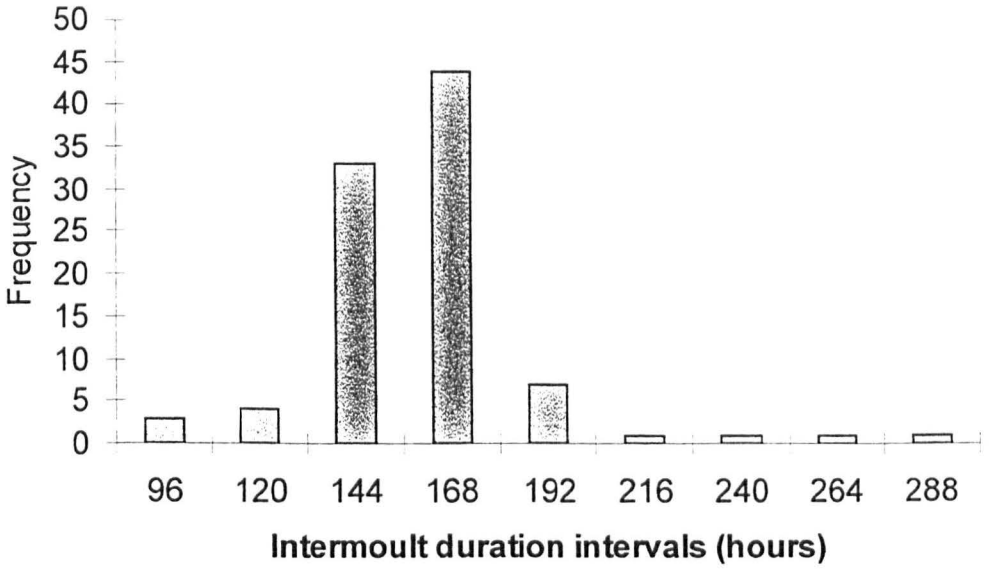


Fig. 3.8. Frequency distribution of intermoult duration during acclimation for the Tilt definitive experiment.

During exposure to Tilt, the organisms in control channels moulted 0.9 times in ten days. The moulting frequency per flow-through system channel is shown in Table 3.10. The intermoult duration during exposure was calculated only for five organisms (Fig. 3.9) and no statistical analysis was performed.

Some differences of behaviour were observed between organisms of exposed and control treatments. In general, the exposed animals showed less activity in non-perturbed conditions (observations not involving handling or light), but showed more irritability to stimuli such as light (night observations) and handling (cleaning of chambers). After the initial 3 days of exposure, the animals at the lowest concentrations consumed less food consumption for some days, ignoring (or not detecting) the pellet when offered, but regained normal appetite levels at the end of the experiment.

3.3.2.2. Technical grade propiconazole

The results of preliminary experiments with solvents showed that methanol concentrations of 277, 554 and 833 $\mu l.l^{-1}$ in seawater produced 100% mortalities at 48 hours. In turn, acetonitrile was less toxic to the test organisms. A concentration of 277 $\mu l.l^{-1}$ produced 100 % mortality at 144 hours, while 139 $\mu l.l^{-1}$ did not produce any mortalities during until the end of the exposure.

The range finding tests with propiconazole in the flow-through system indicated that 50 % of the responses at 48 hours occurred between 8 000 and 10 000 $\mu g.l^{-1}$, and at 144 hours between 1 900 and 4 300 $\mu g.l^{-1}$. The maximum amount

Table 3.10. Moulting frequency per channel during ten days of exposure to Tilt. Concentrations based in the active compound content. Conf. int. = 95% confidence interval for the population median.

Channel	**1**	**2**	**3**	**4**	**5**	**6**
n	10	9	10	9	7	9
Conc. (**µg.l^{-1}**)	950	950	1000	1000	1055	1055
Moults in 10 days	0.5	1.11	0.9	0.89	1.14	0.56
Median	0.5	1	1	1	1	1
Conf. int.	0-1	1-1	1-1	0-1	1-2	0-1
Channel	**7**	**8**	**9**	**10**	**15**	**16**
n	5	6	10	5	10	10
Conc. (**µg.l^{-1}**)	1110	1110	1170	1170	0	0
Moults in 10 days	0.8	0.83	0.9	1	0.9	0.9
Median	1	1	1	1	1	1
Conf. int.	0-1	0-1	1-1	1-1	0-1	1-1

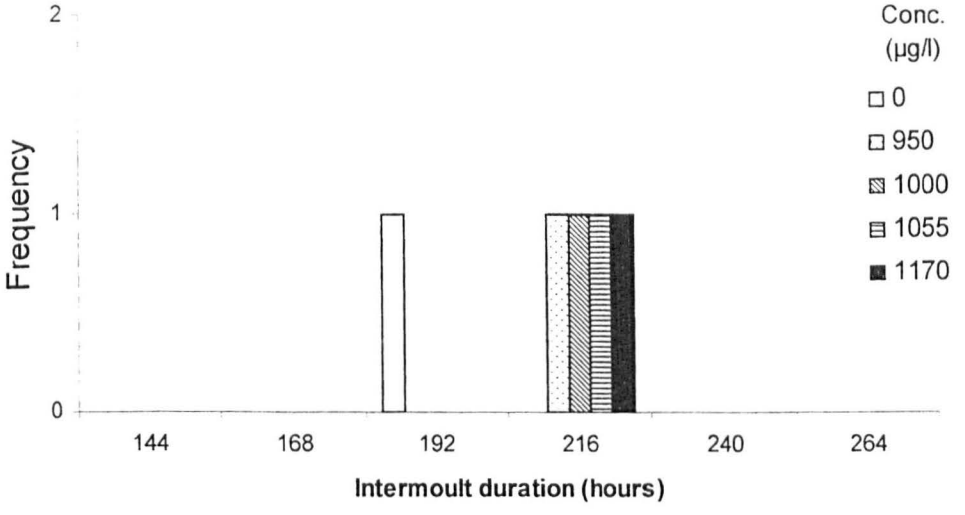


Fig. 3.9. Frequency distribution of intermoult duration data during exposure to Tilt.

of carrier used for the propiconazole tests was always keep near the 140 $\mu l.l^{-1}$ (approximately 0.01%) to minimise the toxic contribution of the solvent.

The definitive acute toxicity test for propiconazole was performed in the flow-through system with concentrations ranging from 800 to 12 000 $\mu g.l^{-1}$. 50% of the responses occurred in the initial 24 hours between 7 000 and 10 000 $\mu g.l^{-1}$, and subsequently between 800 and 3 000 $\mu g.l^{-1}$ at 216 hours (Fig. 3.10). The toxicity curve (Fig. 3.11) showed an initial 24-h LC50 of 9212 $\mu g.l^{-1}$, which decreases to levels between 7 000 to 5 000 $\mu g.l^{-1}$ from 33 to 72 hours. The curve then shows a second decrement to a concentration of 1716 $\mu g.l^{-1}$ from 96 hours onwards, which can be considered the threshold since it becomes asymptotic until the end of the experiment (336 hours).

The average wet weight from all the organisms in this test was 1.37 $\pm$ 0.71 g, which correspond to a flow rate of 1.32 $l.g^{-1}$ per day, considering ten animals per exposure chamber and a consumption of 18 litres per flow-through system channel per day (see section 2.9). No correlation was found between wet weight and time of death, indicating that mortalities did not occur in a size-dependent pattern in the exposure to propiconazole (Fig. 3.12; r = correlation coefficient; $P<0.05$): 12 000 $\mu g.l^{-1}$ ($r = 0.218$, $n = 20$); 7 600 $\mu g.l^{-1}$ ($r = 0.039$, $n = 20$); 4900 $\mu g.l^{-1}$ ($r = 0.212$, $n = 20$); 3 100 $\mu g.l^{-1}$ ($r = 0.418$, $n = 19$); 2 000 $\mu g.l^{-1}$ ($r = -0.024$, $n = 11$); and 1 250 $\mu g.l^{-1}$ ($r = 0.758$, $n = 8$).

In this experiment, the overall moulting rate was 1.24 times during the ten days of acclimation. The moulting frequency per flow-through system channel is

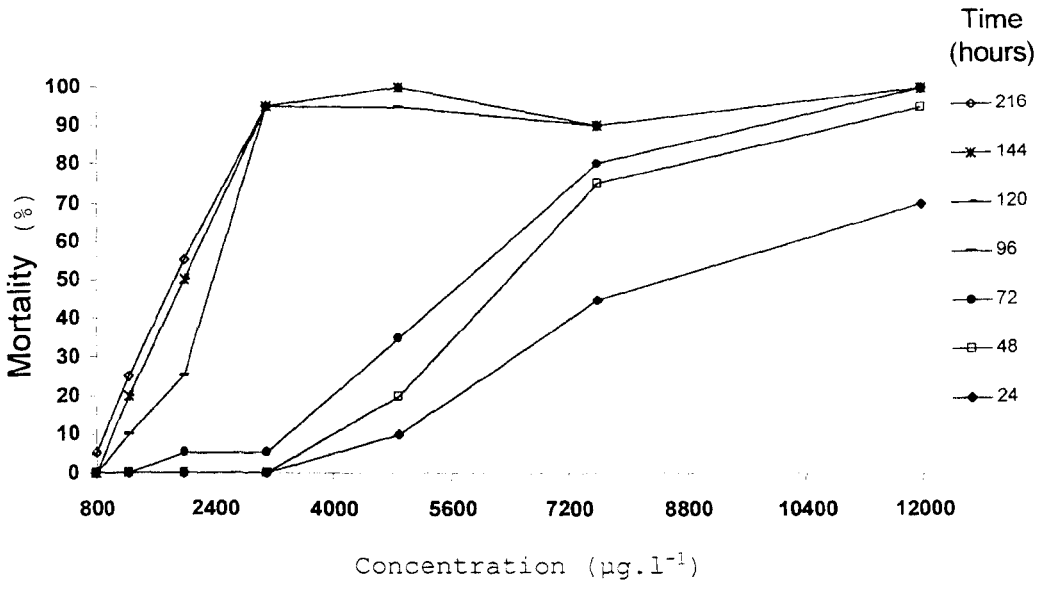


Fig. 3.10. Mortality pattern of *Litopenaeus vannamei* exposed to propiconazole. Group size = 20.

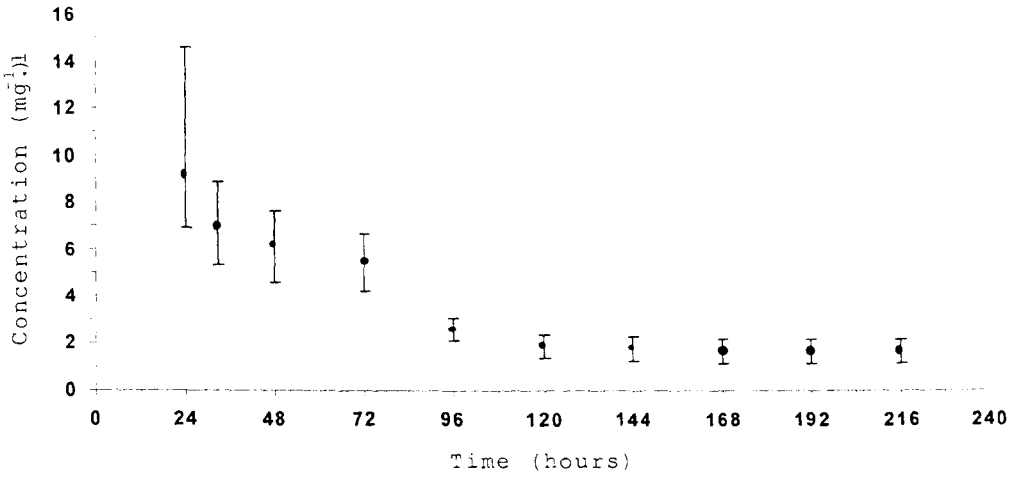


Fig. 3.11. LC50 values and 95% confidence limits of propiconazole for *Litopenaeus vannamei*. Threshold LC50 of 1716 $\mu g.l^{-1}$.

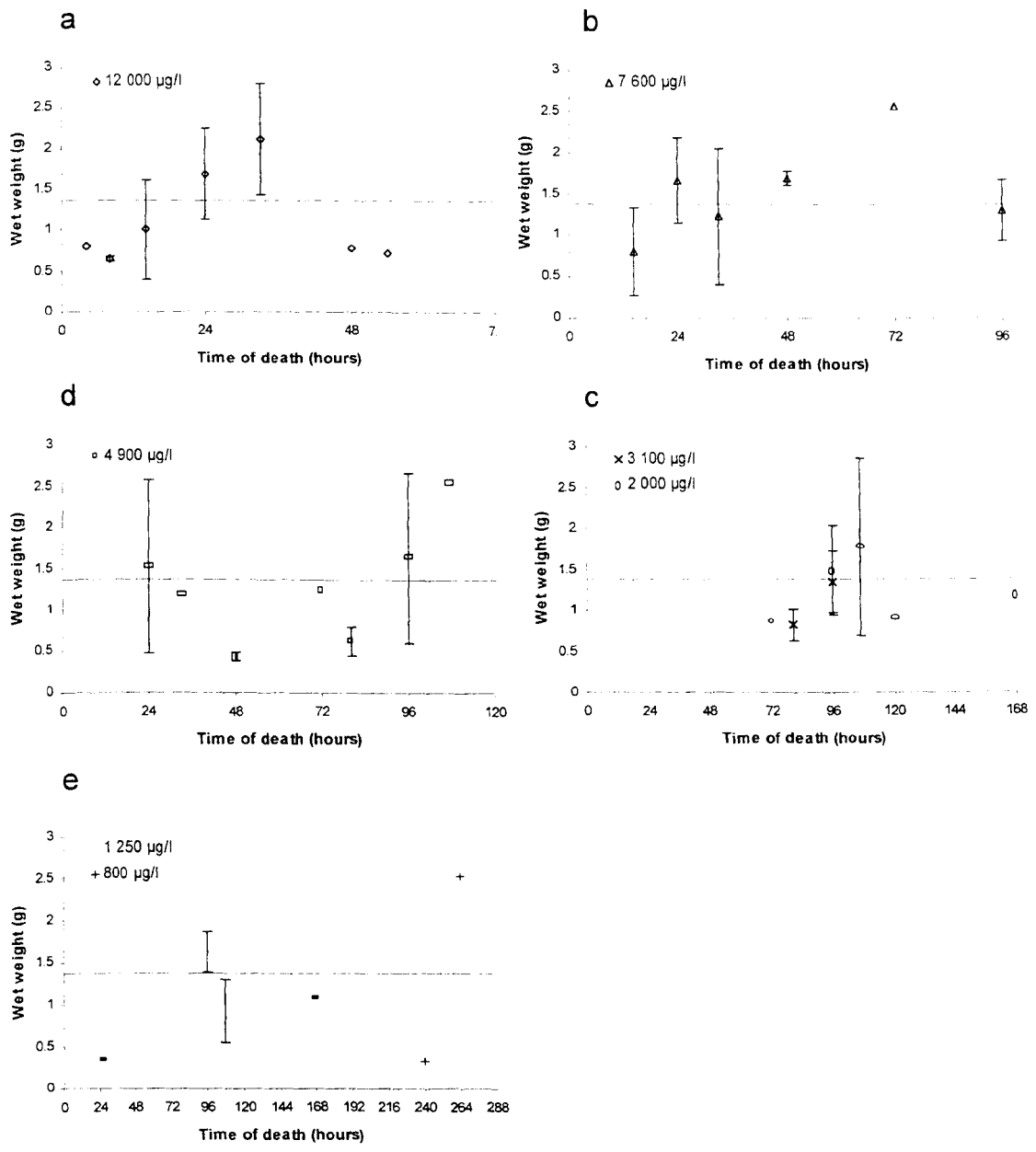


Fig. 3.12 (a to e). Average wet weight of organisms in relation to time of death for the propiconazole acute experiment. Bars represent standard deviation and horizontal line the average of all organisms used in the experiment.

shown in Table 3.11. The average intermoult duration during acclimation resulted in 177 hours (≈ 7days). The frequency distribution of the intermoult data from all organisms (Fig. 3.13) failed the normality test (P = 0.002).

During the exposure, the control channel showed a moulting frequency of 1.07 times every ten days, while the animals in the acetonitrile control (155.5 $\mu l.l^{-1}$) moulted 0.79 times in the same period. The moulting frequency in the exposure channels is shown in Table 3.12. The average intermoult duration during exposure was of 214 hours (≈ 9 days) for the control, of 130 hours for the acetonitrile control and of 281 hours (≈ 12 days) for the 800 $\mu g.l^{-1}$ concentration. The distribution of frequencies of intermoult duration data is presented in Fig. 3.14. The graphic comparison between control and 800 $\mu g.l^{-1}$ (Fig. 3.15) suggests a possible increase in the intermoult duration in the animals exposed to the fungicide.

The behavioural responses of organisms exposed to propiconazole were similar than the ones shown in the Tilt exposure. A period of less appetite was also observed after the initial days of exposure, returning to normality at the end of the experiment. The animals in the solvent control did not show this behaviour.

3.4. DISCUSSION

The results obtained confirmed that the experimental conditions achieved with the flow-through system permitted reliable exposures in a time-independent fashion. This approach has some obvious advantages over short-term acute

Table 3.11. Moulting frequency per channel during ten days of acclimation for propiconazole definitive experiment. Conf. int. = 95% confidence interval for the population median.

Channel	1	2	3	4	5	6	7	8
n	9	10	9	10	10	10	9	10
Moults in 10 days	1.44	1.4	1.11	1.2	1.1	1.4	1.11	1.4
Median	2	1	1	1	1	1	1	1
Conf. int.	1-2	1-2	1-1	1-2	1-2	1-2	1-1	1-2
Channel	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
n	9	8	8	9	9	10	9	10
Moults in 10 days	1.22	1.38	1.13	1.11	1.22	1.2	1.11	1.3
Median	1	1	1	1	1	1	1	1
Conf. int.	1-2	1-2	1-2	1-1	1-2	1-2	1-1	1-2

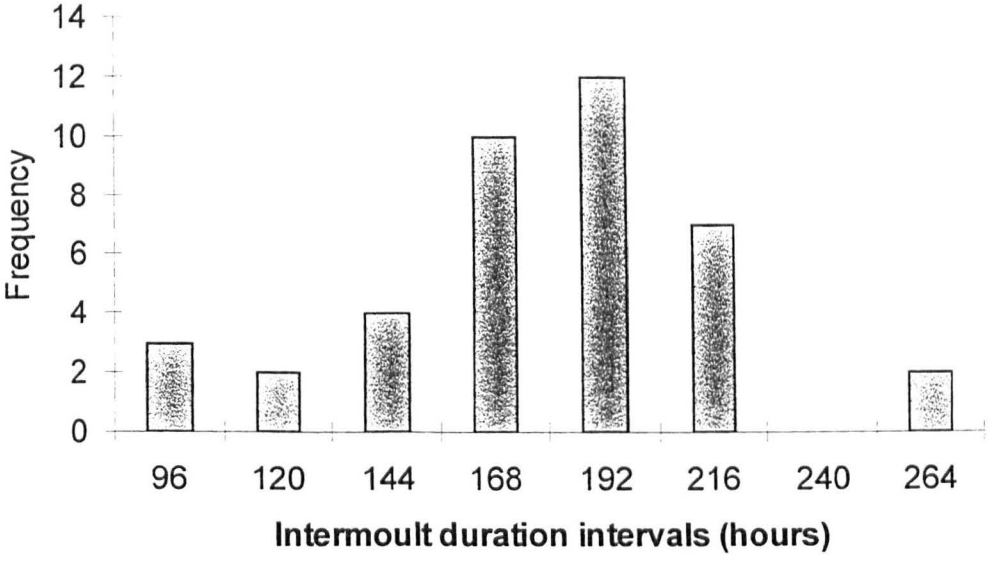


Fig. 3.13. Frequency distribution of intermoult duration during acclimation for the propiconazole definitive experiment.

Table 3.12. Moulting frequency per channel during ten days of exposure to propiconazole. Concentrations based in the active compound content. Conf. int. = 95% confidence interval for the population median.

Channel	1	2	3	4	15	16
n	9	9	7	6	10	10
Conc. ($\mu g.l^{-1}$)	800	800	1250	1250	acetonitrile control (155.5 $\mu l.l^{-1}$)	0
Moults in 10 days	1.11	0.71	0.61	0.6	0.79	1.07
Median	2	1	1	1	1	2
Conf. int.	1-2	1-1	0-1	0-2	1-2	1-2

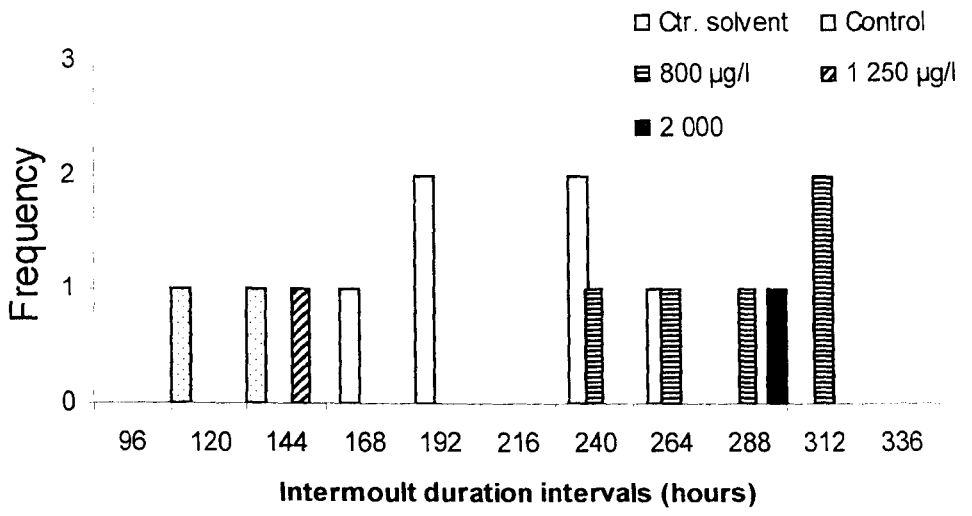


Fig. 3.14. Frequency distribution of intermoult duration data during exposure to propiconazole.

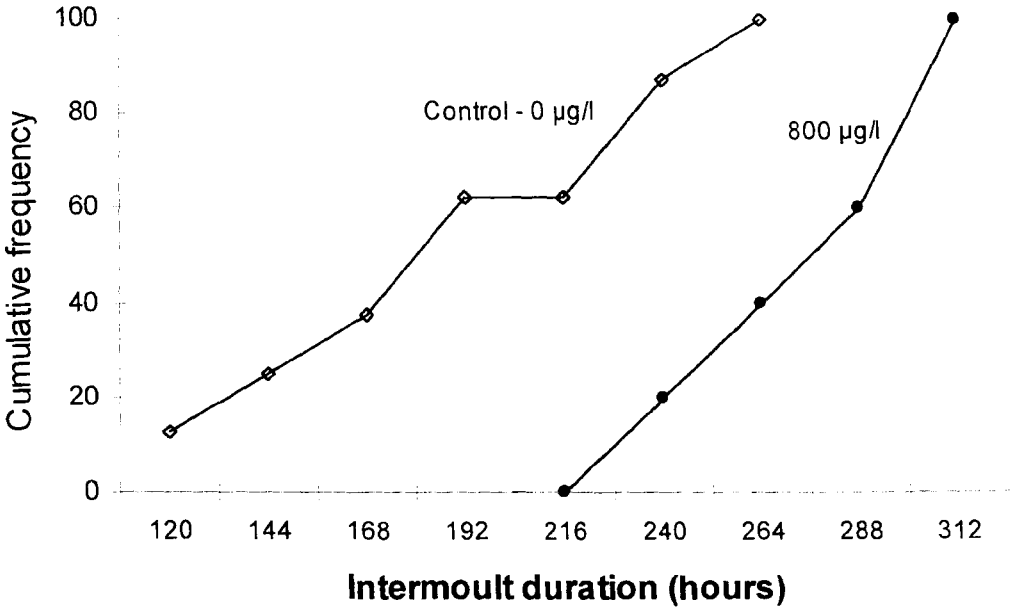


Fig. 3.15. Distribution of cumulative frequencies of intermoult duration after exposure to propiconazole. Intermoult data from solvent control included in the control. Concentrations based in the active compound content.

exposures. For practical or economic reasons, standard toxicity tests set exposure times of 24, 48, 72 and 96 hours, which can underestimate the toxicity of chemicals (Abel, 1991b; van Leeuwen, 1995). From the toxicity data presented, it can be appreciated that median lethal concentrations at early exposure times are considerably higher in comparison with those determined later in the experiment. For instance the toxicity curves for methyl-parathion and propiconazole stabilised only after 96 hours in relation to the concentration axis. In a time-independent test, the exposure is allowed to continue until the toxic effect ceases or nearly ceases (van Leeuwen and Hermens, 1995). This approach is probably the most reliable method of determining a threshold LC50. The threshold LC50 is, by definition, the concentration below which more than 50% of the organisms are not expected to die (Abel, 1991b; van Leeuwen, 1995). A threshold parameter is considered to have more theoretical significance than an LC50 at an arbitrary time period (van Leeuwen, 1995), and is particularly important in the study of slow-acting poisons (Abel, 1991b), although it does not necessarily have ecological significance (Newman and Dixon, 1996). Although there is some controversy in the analysis of toxicity data with respect to the use of time-to-death responses (Newman and Dixon, 1996) or concentration-time relationships, the later is frequently selected because it requires a simpler analysis and can be parameterized with existing acute toxicity data (Barata *et al.*, 1999). In this study, the threshold LC50's determined were used as baseline to design the mixture toxicity experiments (see chapter 5).

The toxicity levels observed in this study indicate that methyl-parathion is "highly toxic" to *L. vannamei*, eliciting its acute toxicity in concentrations below than 100 $\mu g.l^{-1}$. This corresponds with the toxicity reported for other aquatic invertebrates (WHO, 1993; see table 3.1). On the other hand, Tilt and propiconazole can be considered as "moderately toxic" to *L. vannamei*, with acute toxicity levels between 1000 to 10 000 $\mu g.l^{-1}$, generally coinciding with the toxicity reported for aquatic invertebrates (see table 3.2). The difference on threshold LC50 values between technical grade propiconazole and Tilt can be attributed to the ingredients in the formulation of the later (see section 2.3).

The experimentation with Tilt showed the differences in exposure conditions between static and flow-through systems. The results obtained within the range finding tests using static systems were unable to be reproduced in the flow-through system definitive experiments, where no mortalities were observed. This over-estimation of the toxicity was probably caused by anoxic conditions induced by Tilt in static conditions, which tend to form a film of oil in the water surface (no mortalities were recorded in control chambers). In fact, the static system consistently estimated a Tilt lethal toxicity between 150 and 500 $\mu g.l^{-1}$, which coincide with the toxicity reported for Tilt on *Litopenaeus vannamei* postlarvae of 500 $\mu g.l^{-1}$ (48-h LC50) in static conditions and similar temperature and salinity (Enriquez *et al.*, 1996). However, in the present study, the appropriate estimation of the toxicity was achieved only when the range finding test was performed in the flow-through system. After this experience, all the range finding tests were performed in flow-through system conditions. The reliability of the exposure conditions achieved in the flow-through system was

tested in the definitive experiments. The lack of correlation between weight of organisms and their time of death indicates that exposure conditions in the flow-through system were homogeneous for all organisms in the exposure chambers. This was also observed in further experiments described in Chapter 4 and 5.

From the results obtained, it can be appreciated that the moulting data was inappropriate for comparative statistical analysis. The main reason is that the moult frequency of the shrimp juveniles used in these experiments was too low in relation to the exposure time. The expected mortality levels in acute exposures were also a contributing factor for the few registered data. Considering these circumstances, moulting was considered only as baseline information on the effect of pesticides for further experimentation.

Reddy *et al.* (1985) report degrees of moult-inhibition in the crab *Oziotelphusa senex senex* in relation with sublethal concentrations of methyl-parathion (0.1 to 1 ppm). They suggest that while effects could be related to the action of methyl-parathion on the neuroendocrine control of moulting, these are also linked to the apparent lack of appetite and reduced food consumption. As described in section 3.3.1, effects on food consumption were noted in the present study under exposure to methyl-parathion, but further experimentation would be necessary to evaluate the effects on moulting of *L. vannamei.*

No published reports were found on the effects of Tilt or propiconazole on moulting behaviour of crustaceans. Claims that propiconazole can act as a

moulting-inhibitor in crustaceans (Intriago *et al.*, unpublished) by inhibiting calcium and/or phosphate metabolism in cuticular epithelial cells has never been substantiated by data. In the present study, the moult frequency data for Tilt and propiconazole acute exposures provided no definitive evidence of moult inhibition. However, although experimental data were few, these studies do not rule out the possibility of moult retardation during exposure to propiconazole, either as Tilt or technical grade compound. Although far from conclusive, these results suggest a need for further research in this area. Analysis of moulting data in an exposure prolonged during 32 days to Tilt is presented in chapter 4 and some experimental considerations are discussed.

CHAPTER 4.

SUBCHRONIC EXPOSURE TO TILT

4.1. INTRODUCTION

The biological response of an organism to the action of chemicals is determined by the integrated phenotypic response of all its systems working in concert (Hebel *et al.*, 1997). Such responses may range from imperceptible changes to death (Hellawell, 1989). Depending on the concentration and time of exposure, chemicals can elicit physiological changes and impairment of function when the organism's response mechanisms, in terms of detoxification or excretion, are overcome (Hellawell, 1989). This imbalance in the "normal" equilibrium of the organisms can lead to impaired behavioural responses, altered fecundity, pathological tissue changes and formation of lesions, anatomical and genetic changes, disturbed metabolism, reduced growth rate, weakening of defence mechanisms, and predisposition to viral and bacterial infections (Mix, 1988; Hellawell, 1989; Malins and Ostrander, 1991).

When disease occurs, it represents a clear indication of negative deviation from a normal state of the organism (Kinne, 1980) and is a likely consequence of adverse environmental conditions (Murchelano, 1982). Laboratory and field studies have indicated that exposure to toxic chemicals induces the development of characteristic pathological disorders in aquatic organisms (Huggett *et al.*, 1992), although evidence of increased disease prevalence related with environmental degradation are frequently circumstantial (Murchelano, 1982; Mix, 1988; Ouellet *et al.*, 1997). Some studies have

attempted to relate cellular and tissue aberrations (especially neoplasia) occurring in fish (Malins *et al.*, 1988; Overstreet, 1988), bivalve molluscs and other invertebrates (Mix, 1988; Van Beneden, 1996) with body burdens of toxicants such as polynuclear aromatic hydrocarbons (PAH) and polychlorinated biphenyls (PCB), pesticides and chlorine. Pathological abnormalities in marine organisms could be related with problems in human health. Although the information is fragmentary, and no direct proof of etiology exists, epidemiological studies have correlated a high incidence of ovarian cancer in human populations in the same areas with high incidence of tumours in bivalves (Van Beneden, 1996).

In crustaceans, the processes of moulting and regeneration are linked under the control of the neuroendocrine system (Weis *et al.*, 1992). Several studies have assessed the effects of chemicals on moult and regeneration, mostly in laboratory conditions: Dithiocarbamates showed moult-related toxicity and affected the initiation and progression of limb regeneration, at levels well below the LC50, for the grass shrimp *Palaemonetes pugio* (Conklin and Rao, 1982), while chlorinated phenols caused dose-related inhibition of limb regeneration in the same species, but did not alter the intermoult duration (Rao *et al.*, 1979). In studies with the blue crab *Callinectes sapidus*, Horst and Walker (1995) suggest that diflubenzuron can disrupt the functioning of the Golgi complex and inhibit the incorporation of ^{3}H-N-acetylglucosamine into cuticular chitin in postmoult crabs. The anionic detergent, alkylbenzene sulphonate, shortened the moult cycle and reduced the body weight gain after ecdysis in the shrimp *Crangon crangon* (Drewa *et al.*, 1988). Similarly, *Oziotelphusa senex* crabs underwent

ecdysis at much slower rate under exposures to malathion and methyl-parathion (Reddy *et al.*, 1985). In the marine shrimp, *Penaeus monodon*, shell softening was observed after exposure to the pesticide gustation (Baticados and Tendencia, 1991) and to rotenone and saponin (Cruz-Lacierda, 1993). Additionally, *Uca pugilator* crabs have shown reduced number of pigment cells, setae or tubercles in regenerated limbs in exposures to metals such as mercury and cadmium (Weis *et al.*, 1992), and major morphological abnormalities after exposure to tributyltin (TBT), indicating teratogenic effects in developing tissues (Weis *et al.*, 1987; Weis and Kim, 1988). Several authors have associated pollution with a bacterial disease (called 'shell disease') that affects the exoskeletom of shrimp, crabs and lobsters (see Murchelano, 1982; Overstreet, 1988), and with the incidence of "black gill" in crabs and lobsters (Young and Pearce, 1975; Sawyer, 1982). Besides the potential environmental consequences, adverse effects may be of significance in aquaculture due to the highly unregulated application of natural and synthetic agents for pest control (Baird, 1994). Moreover, conditions normally found in shrimp culture such as low oxygen concentration, drastic changes in salinity and temperature and transmission of viral and bacterial diseases, could predispose the animals to acute and chronic effects of pollutants. Although it is generally assumed that disease outbreaks and massive mortalities in shrimp farming are linked with environmental degradation, the current state of the knowledge on disease-pollutant interactions is very limited (Couch, 1974; Couch and Courtney, 1977; Roque *et al.*, unpublished).

The information presented in this chapter deals with a 32-days subchronic exposure to the fungicide Tilt to juveniles of *Litopenaeus vannamei* in flow-through conditions. The effects of Tilt exposure were evaluated on the moult cycle (moulting frequency and intermoult duration), and on feeding and general behaviour. Tilt exposure also produced several morphological pathologies (deformities) on the test organisms. The deformities were characterised and quantified depending on their occurrence in several parts of the shrimp in order to link their incidence with the level of exposure.

The results obtained were compared with the preliminary observations obtained in the acute exposure experiments to the fungicide (Chapter 3), and permitted to assess the performance of the flow-through system in a longer exposure period.

4.2. MATERIAL AND METHODS

4.2.1. Experimental set-up

As explained in section 3.3.2, different concentrations of Tilt were tested in the flow-through system resulting in low incidence of mortalities after 32 days of exposure. The flow rate of the exposure system in relation to organism loading was calculated using the average wet weight from all the organisms in the experiment and the daily consumption of seawater. The nominal concentrations for this experiment were: 0, 400, 500, 600, 690, 770, 850 and 900 $\mu g.l^{-1}$. Following the bioassay procedures described in chapter 2, the endpoints evaluated on the test organisms were moult frequency and intermoult duration. General patterns of behaviour during the experiment were also recorded.

4.2.2. Observation of morphological characteristics

At the end of the exposure, the animals were preserved in Davidson's AFA fixative (Bell and Lightner, 1988). During this procedure, several morphological abnormalities were noticed on rostrum, pereiopods and uropods (hereafter called deformities) and quantified (see statistical analysis). Additional deformities were found in the setal development of maxillipeds and uropods, and antennal scale and telson, but no quantification was performed. Fig. 4.1 shows a schematic representation of the morphology of *L. vannamei* indicating the structures examined for deformities.

4.2.3. Statistical analysis

As in previous experiments, moulting was evaluated by registering the number of times each animal moulted during the experiment (moulting frequency), and also by estimating the number of hours between consecutive moults (intermoult duration).

For the acclimation period, moulting frequency was calculated per channel and used to calculate the overall moulting frequency. Intermoult duration was calculated per individual and per channel, and then used to estimate the overall intermoult duration. Subsequently, the intermoult data was ordered in class intervals to graphically assess its distribution of frequencies, and normality by a Kolmogorov-Smirnov test.

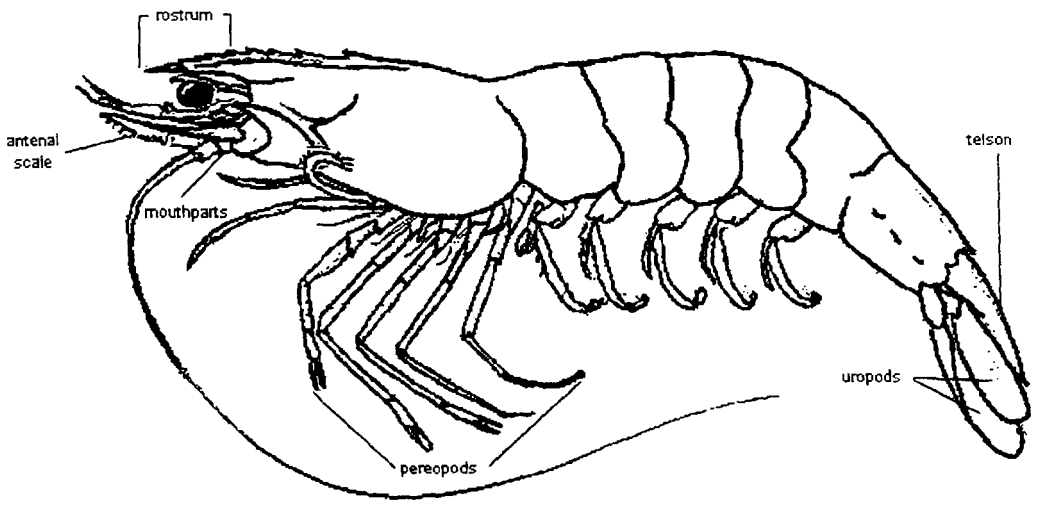


Fig. 4.1. General anatomy of adult *Litopenaeus vannamei* (from Wyban and Sweeney, 1991). The morphological characteristics examined for deformities are indicated.

Since moulting frequency and intermoult duration data are discrete and non-normally distributed, they were analysed for correlation with Tilt treatments using non-parametric rank correlation statistics (Zar, 1996; Hollander and Wolfe, 1999). For exposure, moulting frequency was calculated per channel and tested for correspondence with Tilt concentration by a non-parametric *Gamma* correlation analysis (Statsoft, 1995). The intermoult duration was calculated per individual and per exposure channel, and analysed for correspondence with Tilt concentration by a Kendall-Tau non-parametric correlation (Statsoft, 1995; Hollander and Wolfe, 1999). The Kendall-Tau coefficient represents the probability that in the observed data the two variables are in the same order versus the probability that the two variables are in different orders, while the *Gamma* statistic is equivalent to Kendall-Tau, but preferable when the data contain many tied observations (Statsoft, 1995). Additionally, the intermoult data was ordered in class intervals to assess frequencies and cumulative relative frequencies per exposure channel. This allowed the comparison of intermoult data distributions per Tilt treatment through a log-logistic transformation and regression fitting (see Hewlett and Plackett, 1979) followed by analysis of covariance (ANCOVA) and Tukey's multiple comparison among slopes.

As deformities were detected at the end of the experiment, a detailed microscopic inspection allowed an ordinal categorisation of morphological observations, followed by a quantification of the occurrence of deformities in three structures: the rostrum, the pereopods and the uropods (Fig. 4.1; Table 4.1). For rostrum and uropods, three arbitrary response categories were

Table 4.1. Description of morphological observations in *Litopenaeus vannamei* juveniles.

Category	Rostrum	Pereopods	Uropods
0	Thick, strong and long, ending well beyond the eye	Well defined shape and tips clearly functional, often with sharp ends	Clear, transparent, uniform shape. Setae clear and visible.
1	Crooked, bent or twisted	Lumps present; ends blunt and clear; deformed	Small amounts of deposits, usually on tips
2	Short, ending before eye begins; deformed		Large amounts of deposits, superimposed on each other forming a lump. Deformed with no uniform shape and setae not developed or appear in grooves.

established: no deformity (0), slight deformity (1) and severe deformity (2), while for pereopods only absence (0) or presence (1) of deformities was determined. The incidence of deformities was tested for correspondence with Tilt concentrations by *Gamma* non-parametric correlation analysis.

Statistical analyses were performed using SigmaStat for Windows (version 1.0) and Statistica for Windows (release 4.2).

4.3. RESULTS

Mortality levels below 20% were observed in all concentrations of Tilt during 32 days of exposure. Table 4.2 shows the mortality at the end of the experiment. The average wet weight of all organisms used in the experiment was of 1.14 ± 0.42 g. This yields a estimated daily flow rate of 1.58 $l.g^{-1}$ per channel in the flow-through system, with a daily consumption of 18 litres of sea water, based on ten organisms per exposure chamber (see section 2.9).

Moulting.

The overall moulting frequency was of 0.96 times during ten days of acclimation. Table 4.3 shows the moulting frequency per channel. For the same period, Fig. 4.2 presents the frequency distribution of the intermoult duration data, which failed the normality test. The average of intermoult duration for all organisms was 147 hours (≈ 6 days).

Moulting frequencies per channel during exposure to Tilt are shown in Table 4.4. The non-parametric *Gamma* correlation analysis showed a significantly

Table 4.2. Mortality of test organisms after 32 days of exposure to Tilt (concentrations based in propiconazole as active compound).

Channel	1	2	3	4	5	6	7	8
Concentration ($\mu g.l^{-1}$)	400	400	500	500	600	600	690	690
Mortality (%)	20	0	0	0	0	0	0	0
Channel	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Concentration ($\mu g.l^{-1}$)	770	770	850	850	900	900	0	0
Mortality (%)	0	10	0	0	10	20	0	0

Table 4.3. Average of moulting frequency per channel during 10 days of acclimation for the Tilt subchronic experiment. Conf. int. = 95% confidence interval for the population median.

Channel	1	2	3	4	5	6	7	8
N	10	10	10	10	10	10	10	10
Moults in 10 days	0.90	1.00	1.10	0.80	0.90	1.20	0.80	1.10
Median	1	1	1	1	1	1	1	1
Conf. int.	1-1	0-2	1-1	0-1	0-1	1-2	0-1	1-2
Channel	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
N	10	9	10	10	10	10	10	10
Moults in 10 days	0.70	0.89	1.30	0.90	0.90	1.20	0.70	1.00
Median	1	1	1	1	1	1	1	1
Conf. int.	0-1	0-1	1-2	1-1	0-2	1-2	0-1	0-2

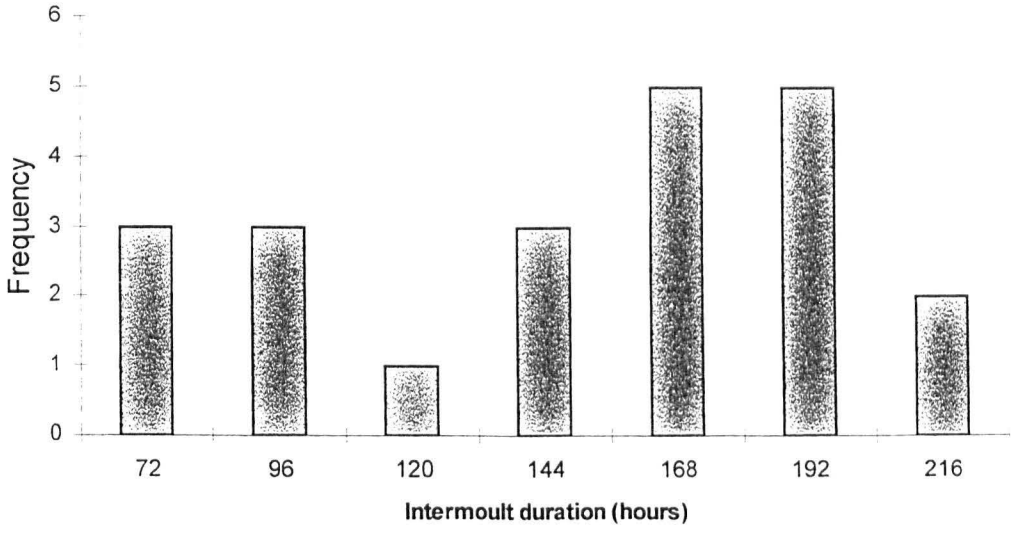


Fig. 4.2. Frequency distribution of intermoult duration during acclimation for the Tilt subchronic experiment.

Table 4.4. Average of moulting frequency per channel in 32 days of exposure to Tilt. Concentrations based in the active compound content. Conf. int. = 95% confidence interval for the population median.

Conc. ($\mu g.l^{-1}$)	400	400	500	500	600	600	690	690
N	8	10	10	10	10	10	10	10
Moults in 10 days	0.86	0.91	0.84	0.97	0.78	0.88	0.94	1.03
Median	3	3	3	3	3	2.5	3	3
Conf. int.	2-3	2-3	2-4	2-4	2-3	2-4	2-4	3-4
Conc. ($\mu g.l^{-1}$)	770	770	850	850	900	900	0	0
N	10	9	10	10	9	8	10	10
Moults in 10 days	0.97	0.83	0.78	0.84	0.76	1.02	1.00	1.03
Median	3	3	2	3	2	3	3	3
Conf. int.	2-4	2-3	2-4	2-3	2-3	3-4	3-4	3-4

negative correlation between concentration and moult frequency (*Gamma* = -0.1524, P = 0.0342). The intermoult duration for the control channels during exposure averaged 231 hours (≈ 10 days). The Kendall-Tau non-parametric correlation showed no significant correspondence between concentration and intermoult duration (τ = 0.074, P = 0.175). However, the comparison of the distribution of frequencies of intermoult duration showed more disperse distributions in most of the exposure treatments (compared to the control), with a general increase in intermoult duration (Fig. 4.3 – a to h). This is better appreciated by comparing the distribution of cumulative frequencies of intermoult duration for the different Tilt treatments (Fig. 4.4 – a to h). Subsequently, the log-logistic transformation of the cumulative frequencies (Fig. 4.5) coupled with the ANCOVA and the Tukey's multiple comparison among slopes found Tilt treatments 500, 600, 770 and 900 $\mu g.l^{-1}$ significantly different than control, but did not find differences for the treatments 400, 690 and 850 $\mu g.l^{-1}$ (Table 4.5).

Morphological observations.

Deformities were observed in several parts of the organisms exposed to Tilt and graded in relation to control organisms. In the rostrum, malformations were classified as slight deformities when this appendix was bent or crooked, while severe deformities were recorded for those organisms showing considerable shortening in the rostrum (Fig. 4.6 – a to d). The observed protuberances (lumps) and blunt tips in pereopods were considered as deformities (Fig. 4.7 – a to d). The lumps appeared to occur only in the 3rd, 4th and 5th pereopods, mainly in the carpopodites, meropodites and propodites, while blunt ends were

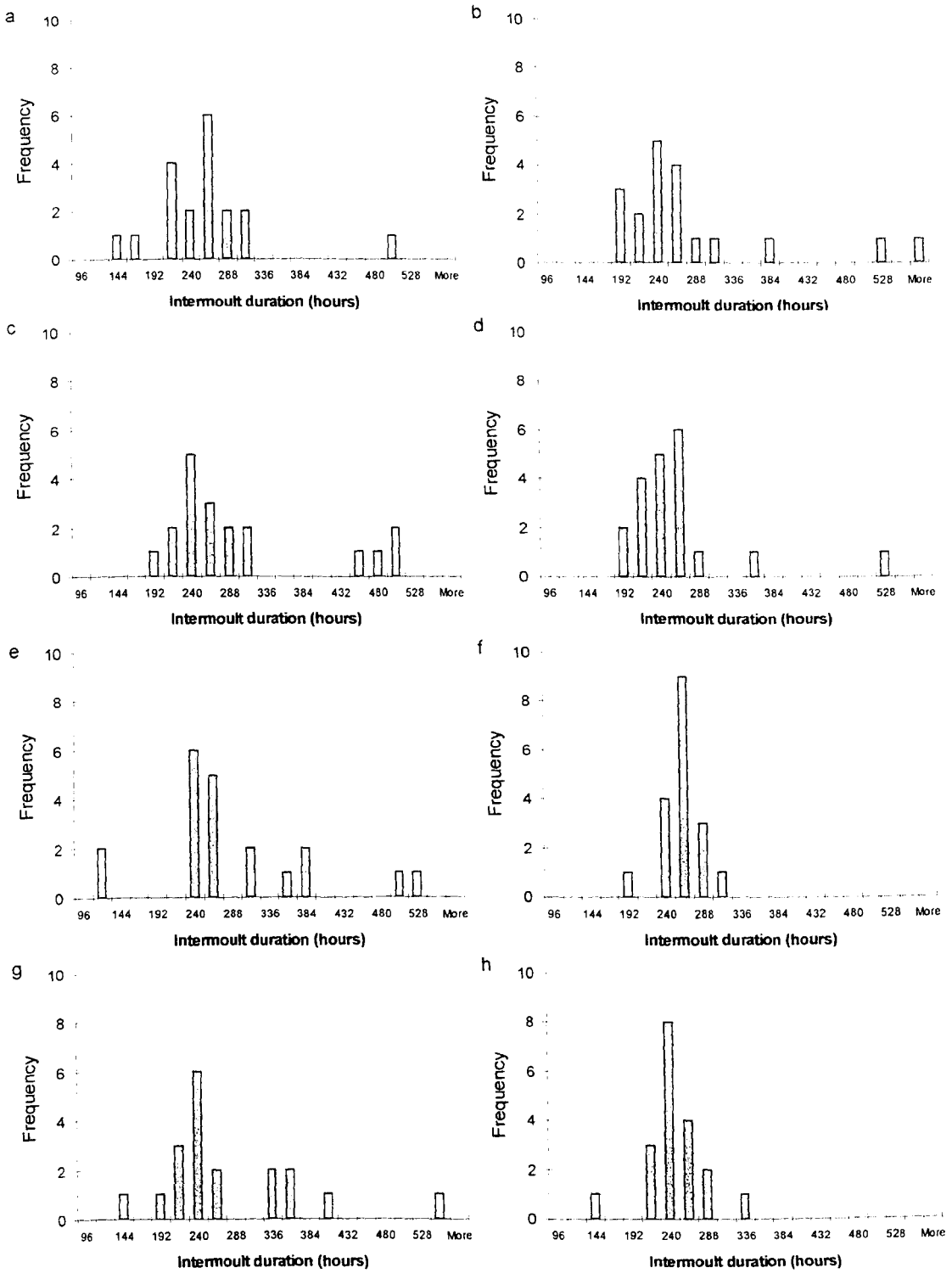


Fig. 4.3. Frequency distribution of intermoult duration during 32 days of exposure to Tilt: a) 400 $\mu g.l^{-1}$, b) 500 $\mu g.l^{-1}$, c) 600 $\mu g.l^{-1}$, d) 690 $\mu g.l^{-1}$, e) 770 $\mu g.l^{-1}$, f) 850 $\mu g.l^{-1}$, g) 900 $\mu g.l^{-1}$, h) 0 $\mu g.l^{-1}$. Concentrations based in active compound content.

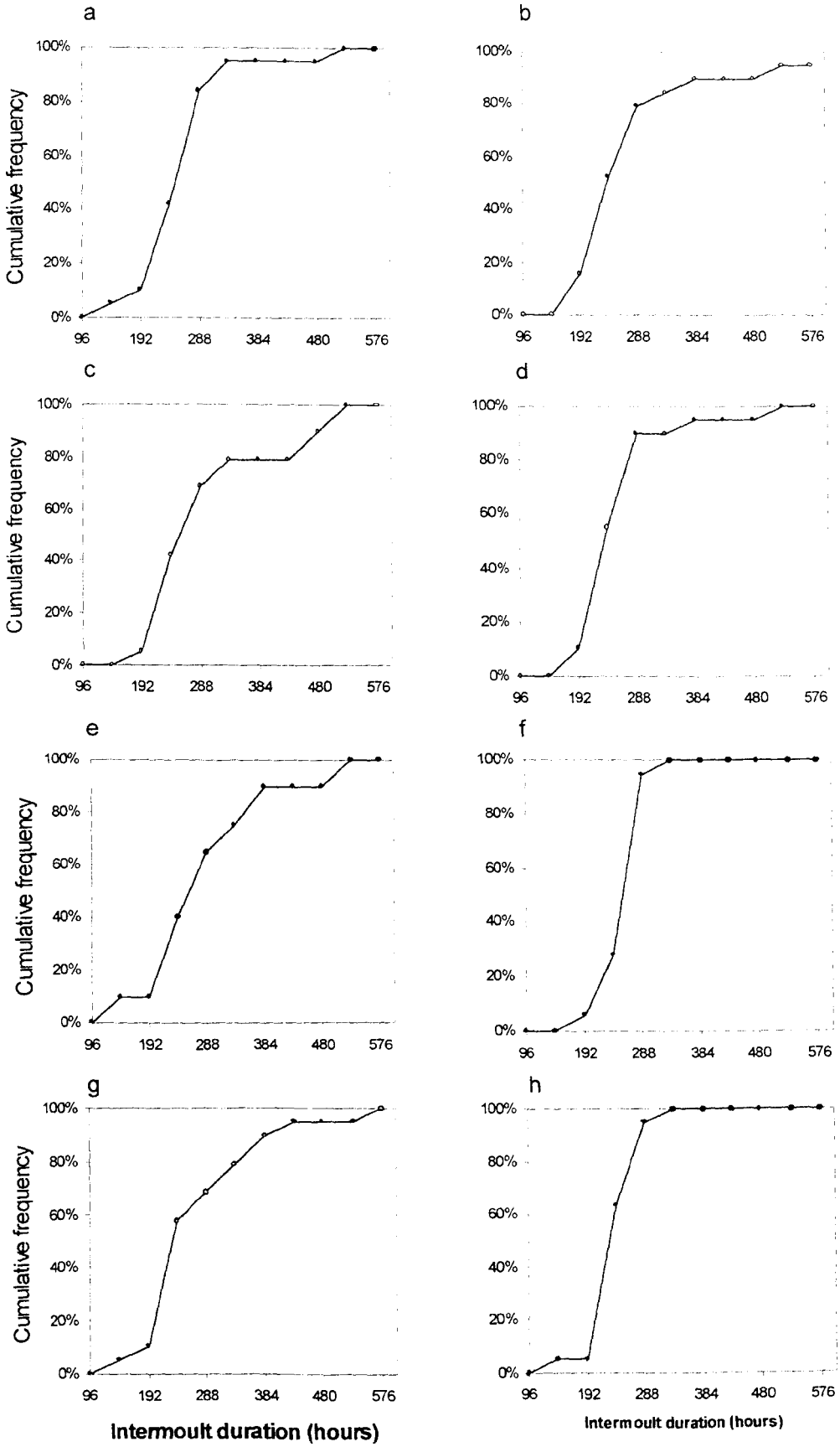


Fig. 4.4. Distribution of cumulative frequencies of intermoult duration during 32 days of exposure to Tilt: a) 400 $\mu g.l^{-1}$, b) 500 $\mu g.l^{-1}$, c) 600 $\mu g.l^{-1}$, d) 690 $\mu g.l^{-1}$, e) 770 $\mu g.l^{-1}$, f) 850 $\mu g.l^{-1}$, g) 900 $\mu g.l^{-1}$, h) 0 $\mu g.l^{-1}$. Concentrations based in the active compound content.

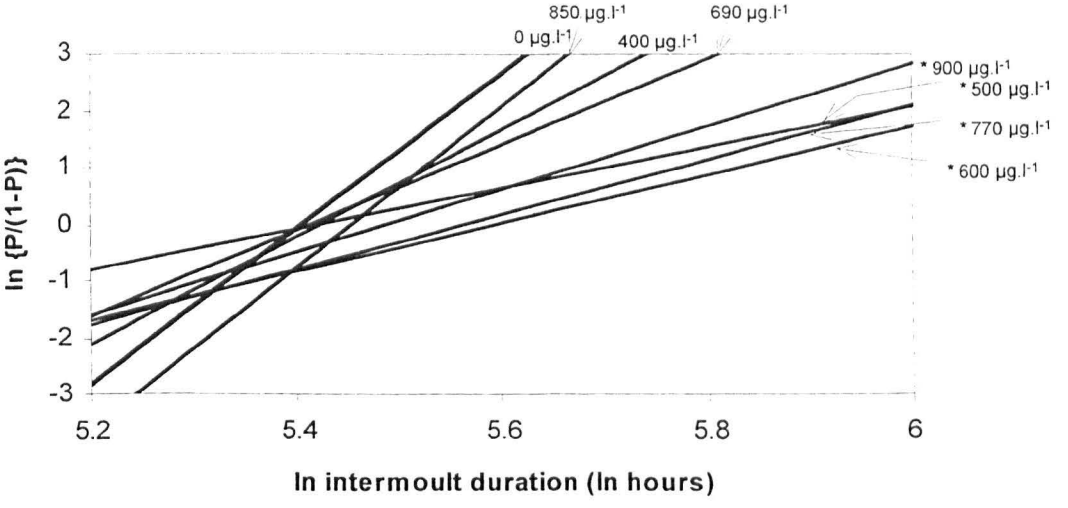


Fig. 4.5. Log-logistic transformation of cumulative frequencies of intermoult duration for Tilt treatments. * denotes significantly different than control. Concentrations bases in active compound content.

Table 4.5. Results from the Tukey's multiple comparison among slopes of intermoult duration distributions for Tilt treatments. Different symbols were assigned to denote significantly differences between treatments.

Tilt treatments ($\mu g.l^{-1}$)	Homogeneous treatments		
0	♣		
400	♣		
500			♦
600			♦
690	♣	♠	
770		♠	♦
850	♣		
900		♠	♦

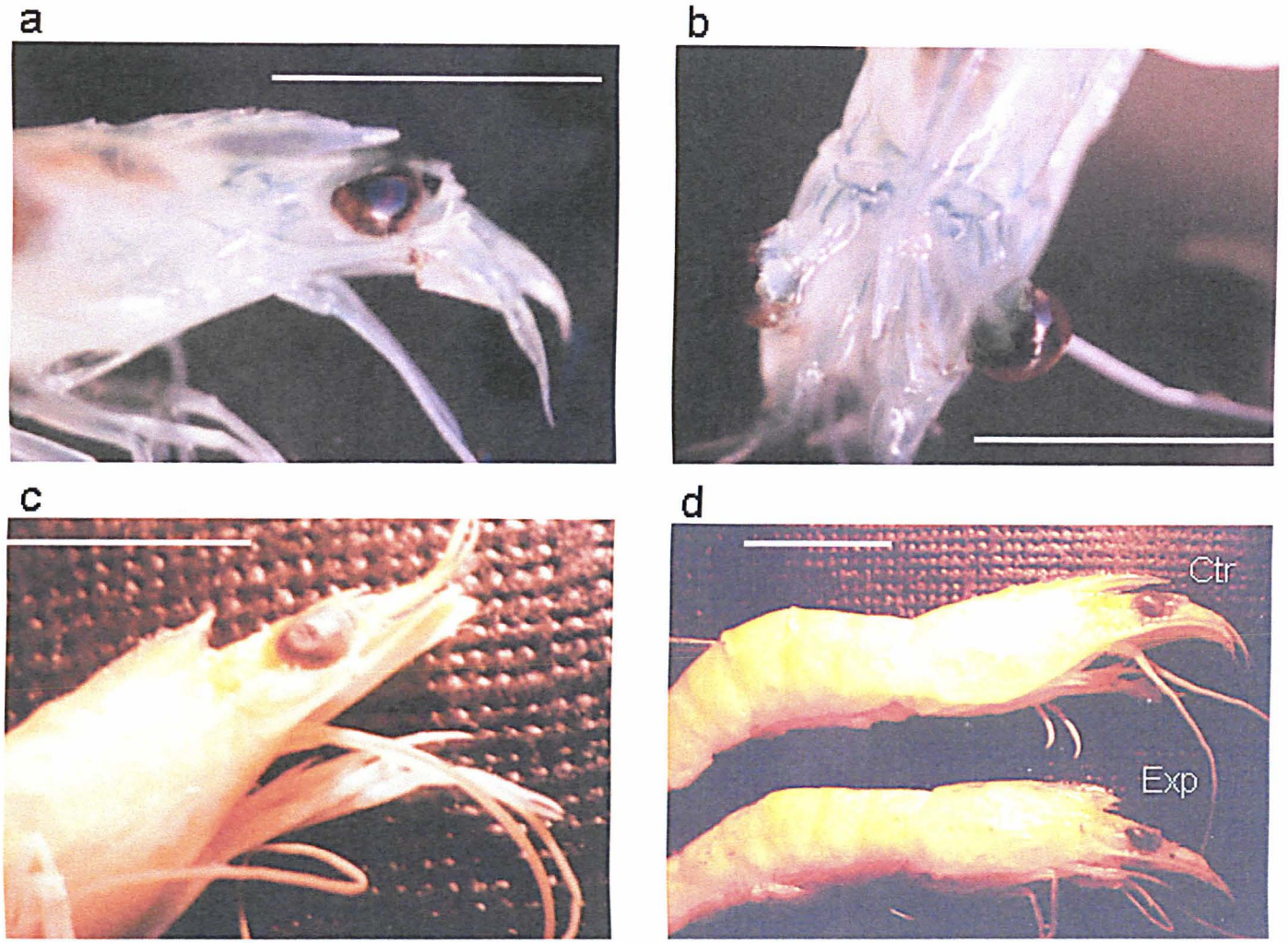


Fig. 4.6. Deformities present in the rostrum of *Litopenaeus vannamei* after exposure to Tilt (refer to table 4.2). a) normal rostrum from a non-exposed control; b) bent rostrum; c) shorten rostrum; d) comparison between non-exposed (Ctr) and exposed (Exp) shrimps. Bars represent 1 cm approximately.

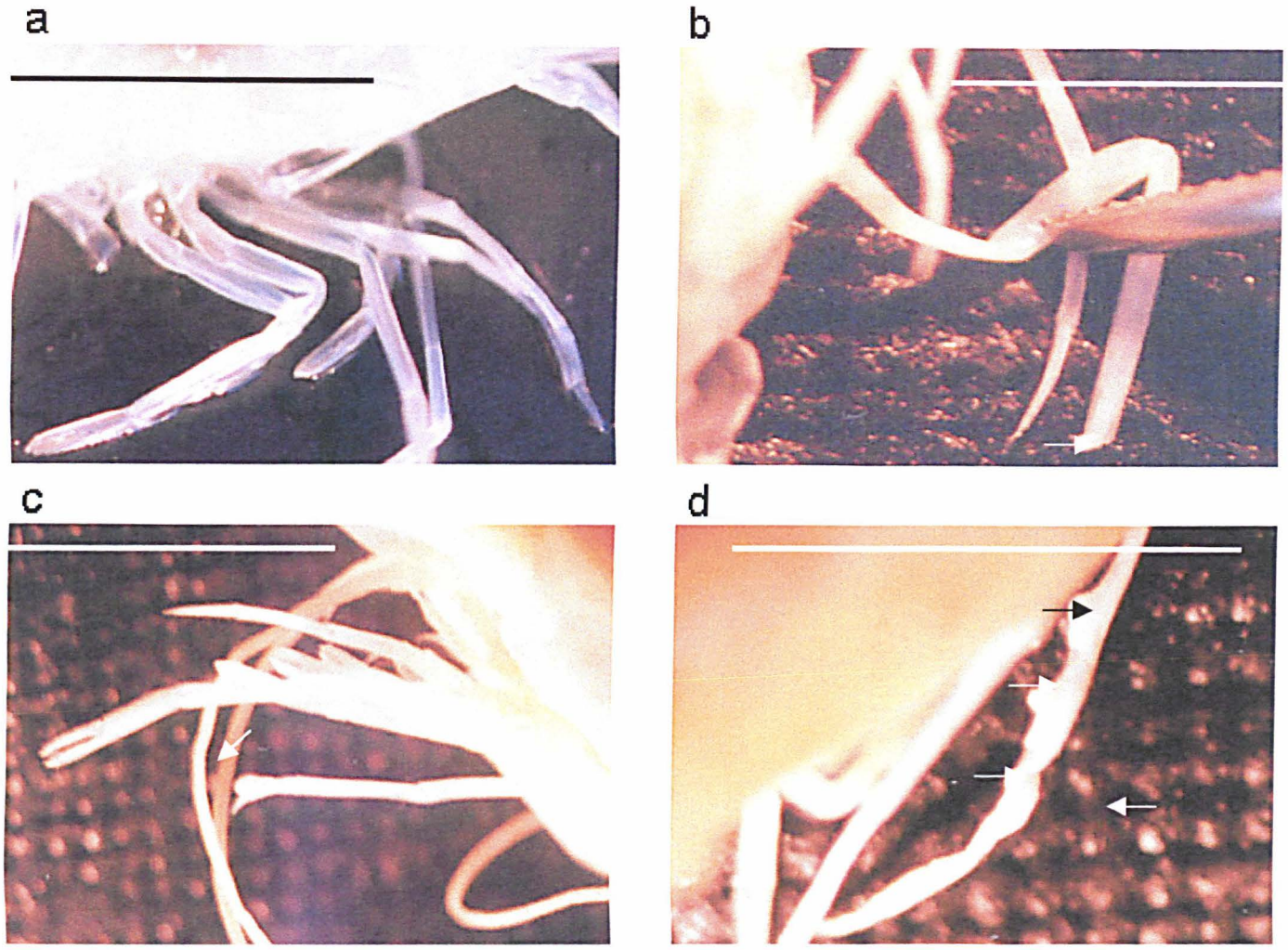


Fig. 4.7. Deformities present in pereopods of *Litopenaeus vannamei* after exposure to Tilt (refer to table 4.2). a) normal pereopods from a non-exposed control; b,c and d) presence of tumors and blunt ends. Arrows indicating blunt tips and tumour-like protuberances. Bars represent 1 cm approximately.

indistinctly present in dactylopodites (see reference schemes in Fig. 4.10 - a, b). Presence of small amounts of white deposits in the tips of uropods was classified as slight deformity. When deposits occurred in large amounts, frequently with changes in shape and lack of setae, the deformity was considered as severe (Fig. 4.8 – a to d).

The incidence and severity of deformities per characteristic is presented in Fig. 4.9 (a, b and c). The *Gamma* non-parametric correlation analysis showed significant positive correspondence between concentration of Tilt and deformity of the rostrum (*Gamma* = 0.454, $P < 0.001$), the pereopods (*Gamma* = 0.698, $P < 0.001$) and the uropods (*Gamma* = 0.425, $P < 0.001$).

The setal development of maxillipeds (mouthparts) and uropods also showed some deformities after exposure to Tilt, but no quantification was performed. Fig. 4.10(c) presents a diagram showing the general morphology of a maxilliped. A closer inspection of maxillipeds (Fig. 4.11 – a to d) revealed some differences in the setal base of exopodites from exposed animals (900 $\mu g.l^{-1}$, presented in 5 of 8 animals analysed) in relation to those from the control (no deformities were observed in any of the 12 organisms analysed). Some deformities observed in the setae of the uropods are presented in Fig. 4.12 (a to h). They occurred in all Tilt treatments and did not seem to follow a concentration-related pattern.

The well-defined and pointed telsons in control organisms were conspicuously different from the rounded and malformed telsons in some exposed animals

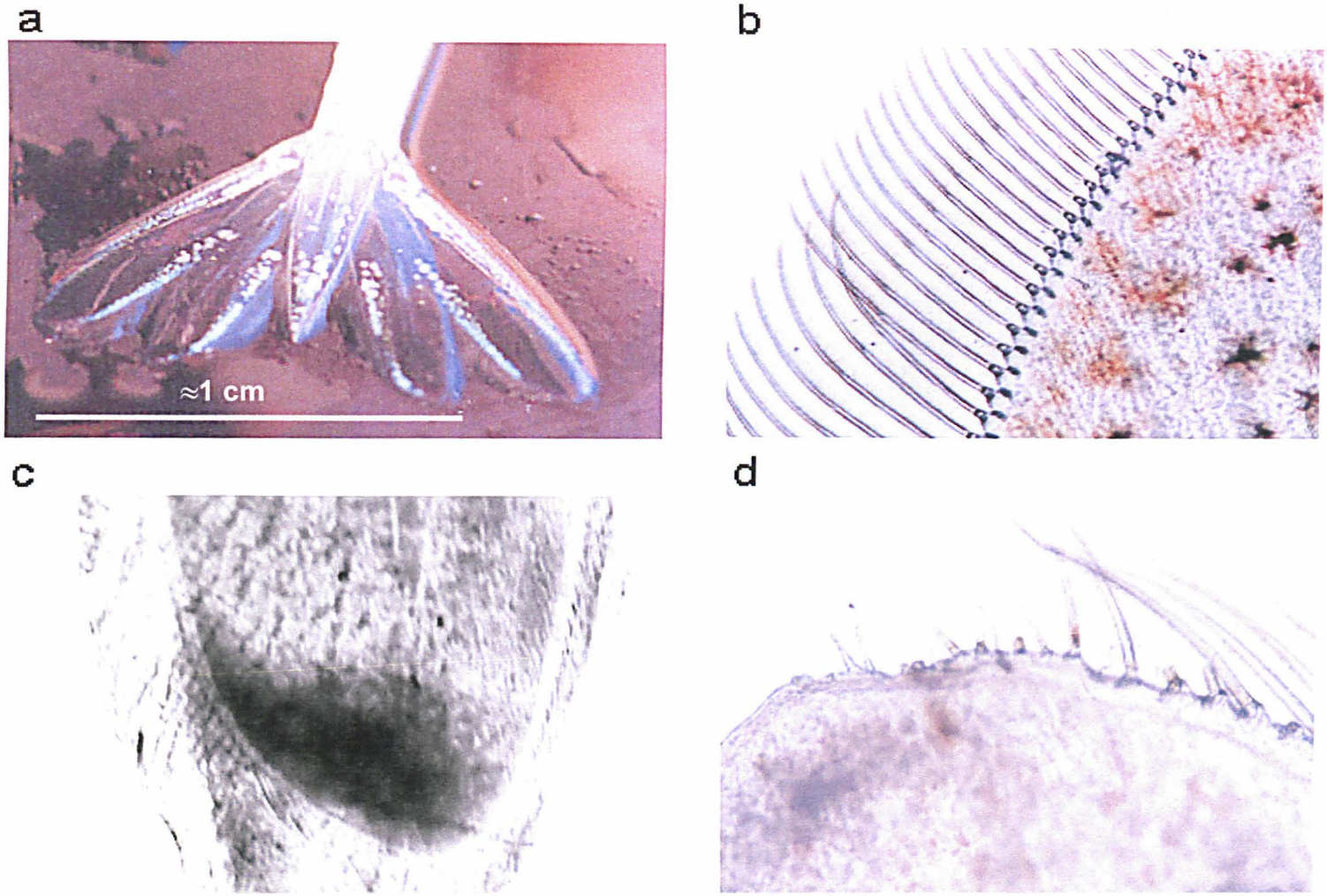


Fig. 4.8. Morphological characteristics present in uropods of *Litopenaeus vannamei* in the Tilt subchornic experiment (refer to table 4.2). a) normal uropods from a non-exposed control; b) setae clear and visible in control (x400); c) presence of deposit in tips (x 100); d) deformed or non developed setae (x400).

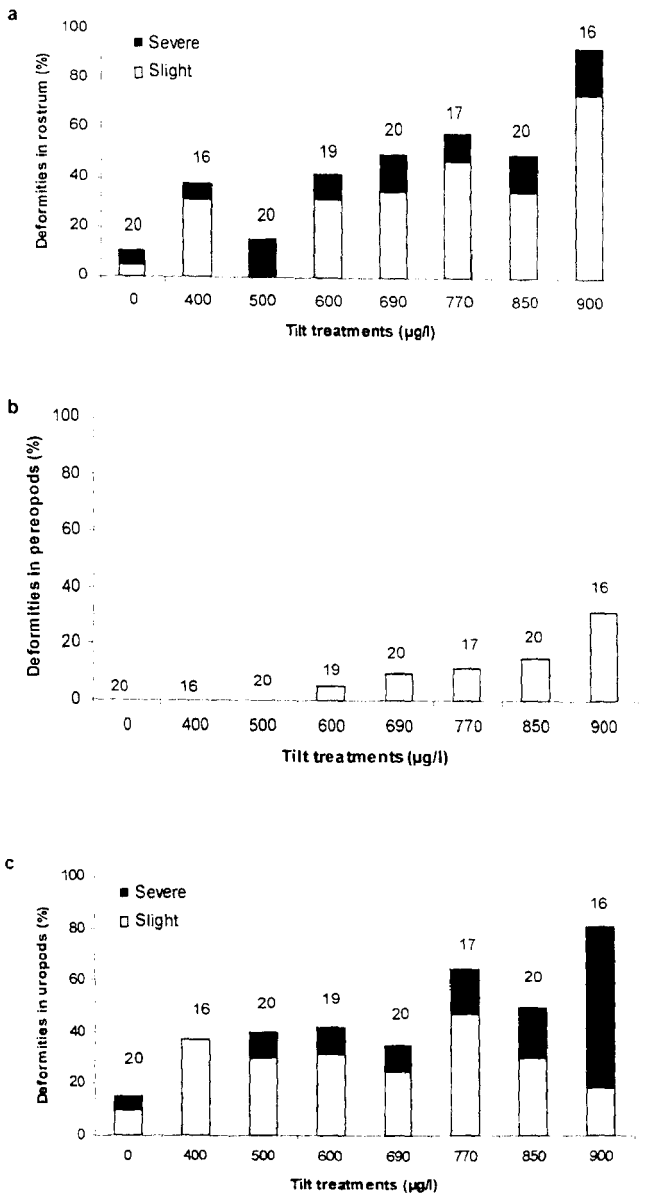


Fig. 4.9. Incidence of deformities in morphological structures of shrimps exposed 32 days to Tilt: a) rostrum, b) pereopods and c) uropods. Numbers above bars represent the organisms analysed (n).

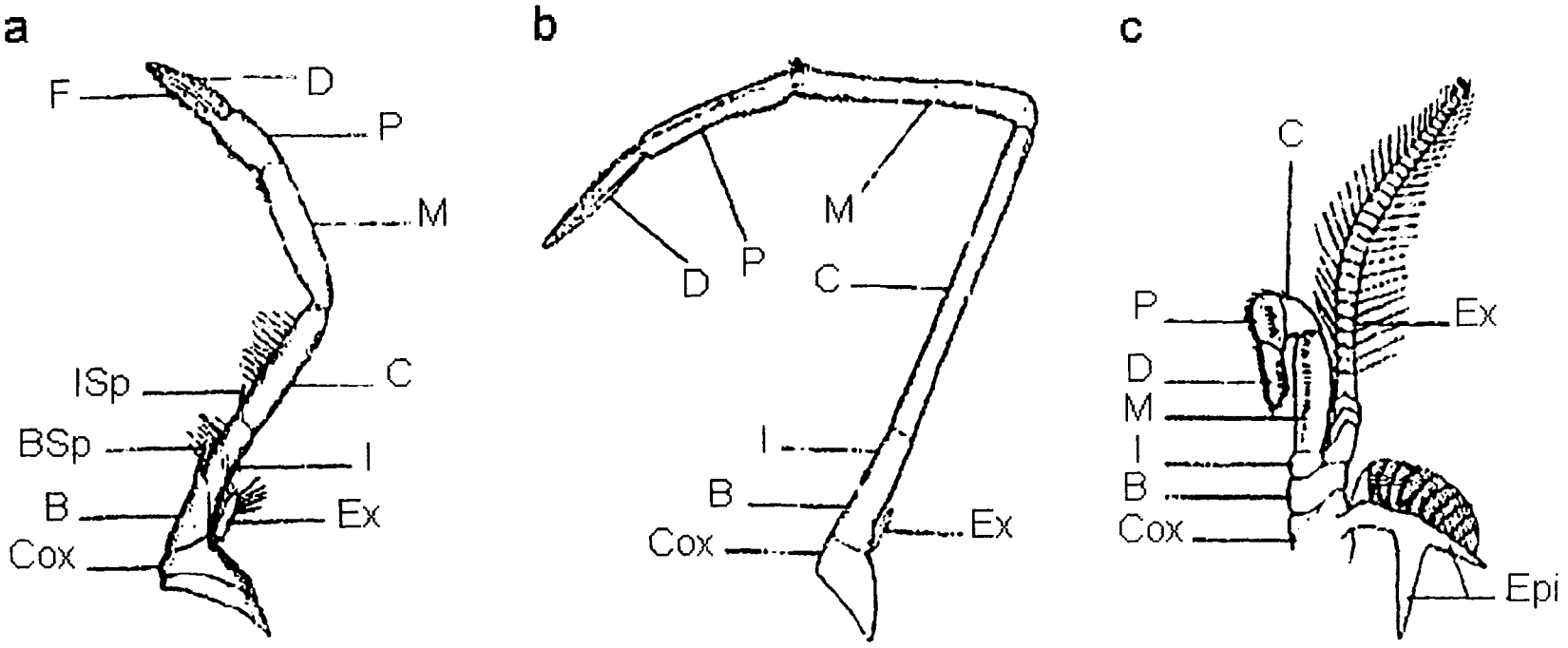


Fig. 4.10. Schematic representation of 1st (a) and 5th (b) pereopods, and maxilliped (c). F) finger; D) dactylopodite; M) meropodite; C) carpopodite, I) ischiopodite; B) basipodite; P) propodite; ISp) ischial spine; BSp) basial spine; Ex) exopodite; Cox) coxopodite; Epi) epipodite (from Dall *et al.*, 1990).

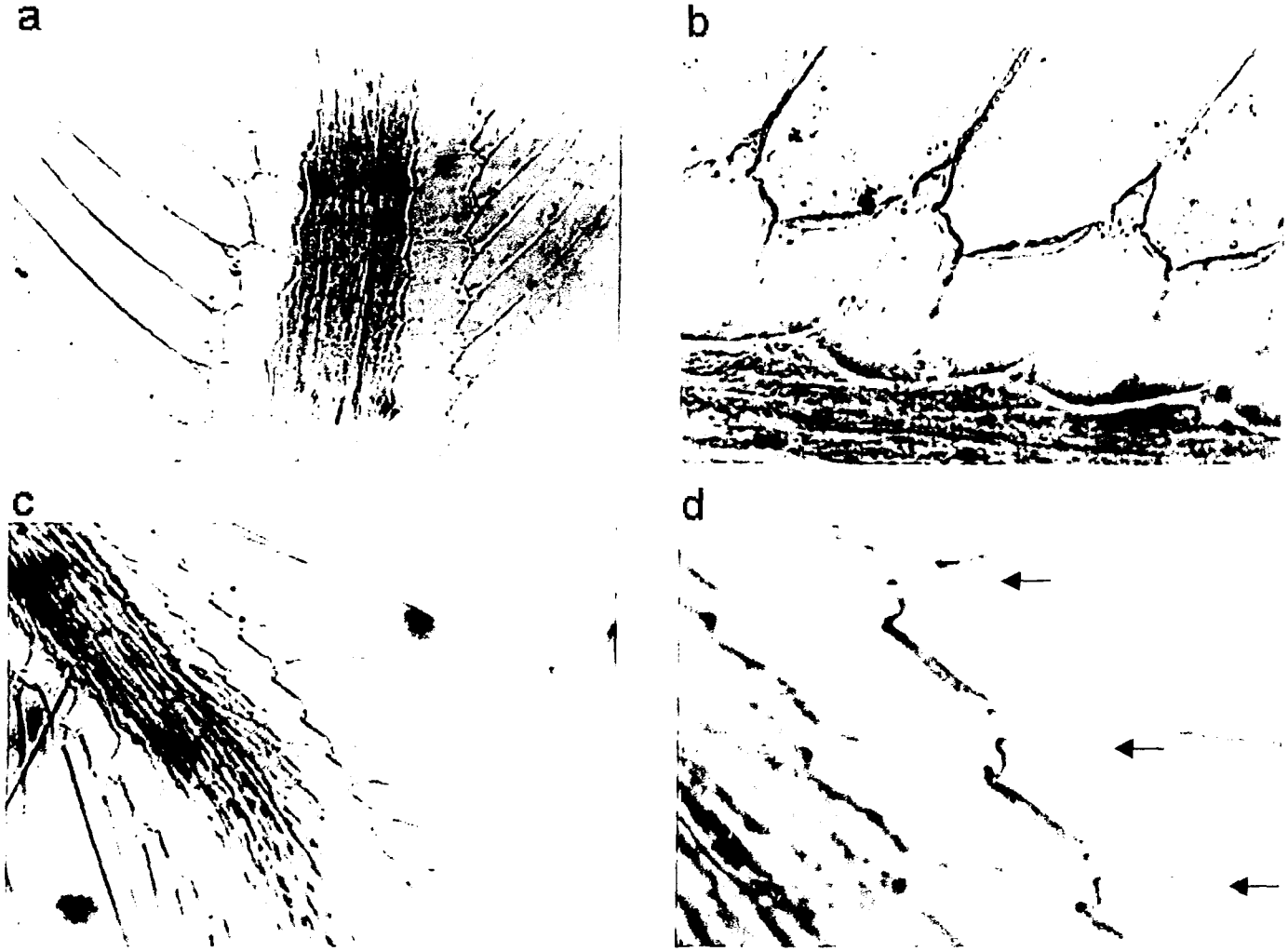


Fig. 4.11. Deformities presented in maxillipeds of *Litopenaeus vannamei* after exposure to Tilt. a) exopodite from a non-exposed control (x 400); b) detail of setal base from a normal exopodite; c) exopodite from an exposed animal (x 400); d) detail of setal base from a deformed exopodite (abnormal structures indicated by arrows).

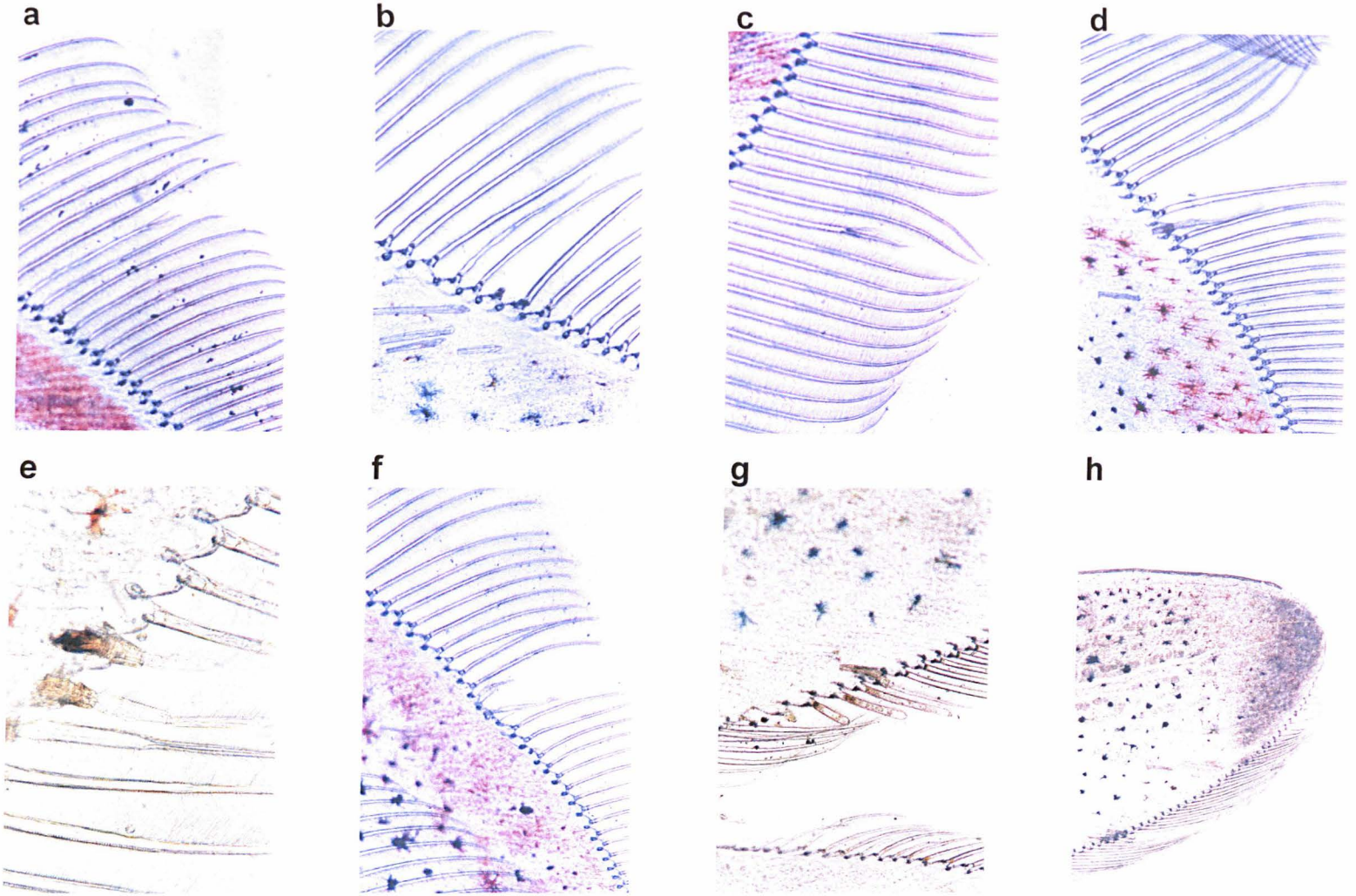


Fig. 4.12. Deformities observed in setal development of uropods of *Litopenaeus vannamei* after 32 days exposure to Tilt: a, b and c) shortening and irregular sizes; d, e and f) shortening and deformation; g) thickening and deformation; h) lack of setaes. All pictures were taken at x400, except h (x100).

(Fig. 4.13 – a, b). This occurred incidentally in all the range of concentrations. Similarly, the antennal scale showed deformities similar to the uropods, with occurrence of white deposits usually on tips, occasionally forming lumps, with setae not developed or appearing in grooves (Fig. 4.13c). Additionally, at the end of the exposure some shrimps were noted with swollen-black eyes, a bluish body colour and rigid exoskeleton (only in 850 and 900 $\mu g.l^{-1}$ concentrations). These shrimps showed no response to light, but were able to locate and ingest the food pellets.

Behaviour.

In general, during the first days of exposure to Tilt, shrimps showed erratic movements and hypersensitivity, but were able to locate the food pellets when offered. However, after 10 days of exposure, most of the exposed organisms became inappetent and rejected the pellets. This lack of appetite lasted for two or three days and returned to normal feeding until the end of the experiment.

4.4. DISCUSSION

From the results obtained, it can be appreciated that the analysis of moulting data suggests only limited effects of Tilt upon moulting. For instance, the moulting frequency of exposed organisms showed signs of being negatively correlated with Tilt concentration. Also, the distribution of intermoult duration frequencies in some Tilt treatments was in fact significantly different than the control. However, the intermoult duration data did not follow a concentration-dependence pattern. Moreover, the organisms exposed to 850 $\mu g.l^{-1}$ (second

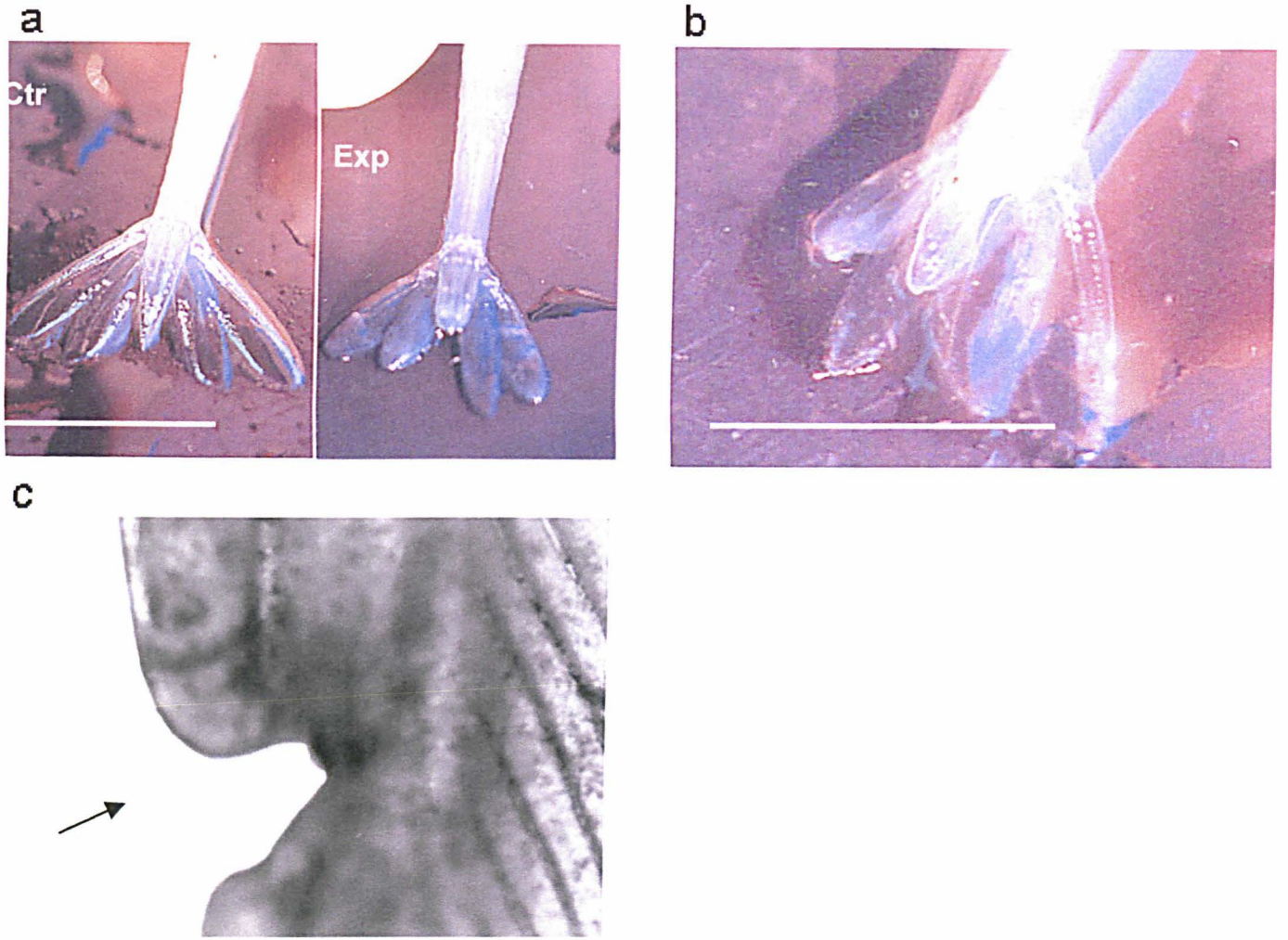


Fig. 4.13. Additional deformities presented in *Litopenaeus vannamei* after exposure to Tilt. a) comparison between telsons of non-exposed (Ctr) and exposed (Exp) shrimps; b) severely deformed telson and uropods; c) groove in antennal scale (indicated by arrow, x400). Bars represent 1 cm approximately.

highest concentration) showed the closest intermoult duration distribution to the control, while the organisms in 500 and 600 $\mu g.l^{-1}$ (2^{nd} and 3^{rd} lowest) were significantly different.

Some factors could have contributed to the "inconsistency" in the moulting data. As explained in section 3.3.2, this experiment was not initially aimed to perform a subchronic exposure, but an acute one instead. Problems with the extrapolation from static to flow-through conditions explain why the concentrations were not selected with reference to the threshold LC50, as it would be expected in a chronic experiment. The resulting concentrations were, therefore, probably too narrowly spaced for comparative analysis of a sublethal endpoint such as moulting. On the other hand, the high variability in the moulting response complicated the recognition of possible differences among treatments after a relatively short exposure period. However, Forbes and Depledge (1996) point out that pollutants may induce an increase in the variability of biological responses in benthic invertebrates, and suggest that this could represent a general symptom of environmental stress. Considering the above arguments and the analysis of results, it is still uncertain whether Tilt can lower the moulting frequency (or increase the intermoult time), but it is clear that it can affect the variability in the moult response.

No precise reports of propiconazole residues in estuarine or marine environments were found in the literature. The range of concentrations used in this experiment (from 400 to 900 $\mu g.l^{-1}$) may not represent the expected levels in the environment. For example, an expected environmental concentration

(ECC) of 83 $\mu g.l^{-1}$ for propiconazole was determined considering the application rates for agricultural use in Canada (Peterson *et al.*, 1994).

To better evaluate the effects of Tilt and other toxicants on the moulting of *L. vannamei*, some modifications in the experimental procedures should be considered. The tested concentrations should be determined in relation to the threshold LC50, down to levels including expected levels of the toxicant in the environment, and longer exposure times should be achieved. One option could be to start the experiment using shrimps with the same moult stage (i.e. intermoult stage), although this could be difficult to achieve in large-scale experiments and it does not reduce the inherent variability. The use of larval stages, which moult more frequently (every 30-40 hours) in a synchronised fashion (Wyban and Sweeney, 1991), could represent a more reliable and faster way to evaluate the effects on moulting. Another approach could be to study the effects of pollutants on chitin synthesis (Horst and Walker, 1995), shell quality (Baticados and Tendencia, 1991; Cruz-Lacierda, 1993) or circulating ecdysteroids during the moult cycle (Chan *et al.*, 1988; Blais *et al.*, 1994).

From the study of morphological characteristics, it was clear that Tilt caused morphological abnormalities in exposed shrimp. The incidence of deformities showed a clear concentration-related incidence in relation to the exposure levels. Yet, further research is needed to identify the agent, or combination of agents, responsible of producing the deformities. Evidence from previous reports associate the PAHs (contained in the formulation of Tilt, see section 2.3)

with neoplasia and other abnormalities in fish, molluscs and invertebrates (Malins *et al.*, 1988; Mix, 1988), although no specific reports were found in the literature for crustaceans. Also, PAHs have been associated with reduction of feeding responses in detritivorous fish, deposit-feeding polychaetes and filter-feeding bivalves (Anderson, 1992). McCain and Malins (1992), report a decrease in feeding behaviour, particularly those involving searching, on the spot shrimp *Pandalus platyceros* after exposure to crude oil and water-soluble fractions of crude oil. A similar pattern of alteration in feeding activities was noted in the shrimp exposed to Tilt, although in this case similar behaviour was also registered by the organisms exposed to technical grade propiconazole (see section 3.3.3). No reports were found in the literature about feeding responses of organisms exposed to Tilt or propiconazole.

As stated previously, the effects observed were obtained in laboratory conditions, with concentrations that could exceed the levels and persistence of the fungicide in a realistic environmental scenario. It is therefore necessary to investigate which would be the expected environmental concentrations in Mexican coastal waters in order to achieve chronic exposures. For this, additional approaches are advisable such as the study of effects at biochemical, histological and physiological level.

Nevertheless, the results presented in this study indicate potentially serious effects of Tilt on crustaceans. The effects of pollutants on moulting and regeneration can be used to examine disruption in the neuroendocrine system, or on development and growth (Weis *et al.*, 1992), and its possible relation with

the incidence of deformities. The deformities shown in maxillipeds and pereopods are particularly relevant due to the high concentration of chemoreceptor cells in these appendages (Lee and Meyers, 1997). Dactyls of pereopods and maxillipeds play a role in recognition or perception of chemical signals (Lee and Meyers, 1997). Therefore, their affectation can have behavioural consequences in activities such as avoidance, locomotion, feeding and reproduction (Hebel *et al.*, 1997). Beside pereopods and maxillipeds, other important chemoreceptor sites are the antennules, mandibles, maxillules and maxila. The presence of finely tuned chemoreceptors in several anterior appendages may be adaptive because it is not uncommon for crustaceans to lose appendages as a result of predation and physical damage (Devine and Atema, 1982). However, some toxicants may be able to disrupt all chemoreceptor sites and pose a threat to the organism's overall feeding and reproductive performance and thus survival. For instance, the crab *Pachygrapsus crassipes* showed inhibition of feeding responses and failed to display male mating responses after exposure to oil extracts (Takahashi and Kittredge, 1973), while Farr (1978) reported a relative increase in the consumption of the grass shrimp *Palaemonetes pugio* by the fish *Fundulus grandis*, when the shrimp was exposed to sublethal concentrations of methyl parathion, suggesting that the pesticide could decrease its ability to avoid predation.

It is also possible, of course, that the shrimp cages used to record individual observations could have caused some of the lesions presented in antennal scale, telson and uropods of exposed organisms. These lesions, however,

were clearly different or otherwise absent in the control organisms, indicating a possible effect in cuticular repairing or limb regeneration. The incidence of morphological abnormalities and reduction in the number of setae in regenerated limbs were reported in the fiddler crab *Uca pugilator* after exposure to TBT, suggesting a developmental toxicity (rather than physiological) since no deformities occurred in limbs that were not autotomysed (Weis *et al.*, 1987), probably affecting the processes of differentiation and morphogenesis (Weis and Kim, 1988). This is not the case for the present study as deformities occurred even in appendages that were not likely subjected to damage or friction. The biological significance of toxicant-induced abnormalities in minute structures such as setae of maxillipeds and uropods is difficult to determine with the information available. However, since these structures regenerate in each moult cycle, the appearance of deformities could have value as an early indicator of moulting or regeneration disruption.

CHAPTER 5.

TOXICITY TESTS: MIXTURES.

5.1. INTRODUCTION

As previously mentioned in Chapter 1, it is generally accepted that mixtures of pollutants are more likely to occur than single compounds alone. However, research in aquatic ecotoxicology is still largely based in studies of single chemicals, little is known about the potential toxic interactions of such mixtures, and new combinations of chemicals are released into ecosystems faster than research procedures can be developed (Hermens *et al.*, 1984a; Luoma, 1996). The cost of testing potential combinations of pollutants and the lack of a general agreement on the most appropriate procedures to measure joint toxicity are important issues in modern ecotoxicology. The characterisation and understanding of the effects of toxic mixtures are considered a priority in contemporary human health (Bolt and Mumtaz, 1996; Kappus and Yang, 1996) and environmental issues (Gardner *et al.*, 1998; Walker *et al.*, 1998).

The information presented in this chapter is an approach to assess the interactive toxicity of the organophosphorous insecticide methyl-parathion in combination with the triazole-derivative fungicide propiconazole – technical grade and commercial formulation (Tilt) – on the marine shrimp *L. vannamei*. Based on the concentration ranges used in the acute toxicity tests for each toxicant, mixtures of toxicant pairs were placed in the flow-through system using

mortality as an endpoint. Moulting and behaviour were also evaluated during the experiments.

5.2. MATERIAL AND METHODS

5.2.1 Experimental set-up

Based in the results of the lethal toxicity tests presented in chapter 3, each fungicide (Tilt and propiconazole) was tested as a methyl-parathion – fungicide pair in time-independent exposures under flow-though conditions. For each experiment, the test organisms were acquired and maintained as described in section 2.2 and introduced in the flow-through system for acclimation followed by exposure. The experimental conditions for these experiments were the same as described for the flow-through system in section 2.7 and 2.8. For exposure, seven methyl-parathion – fungicide mixtures were prepared with half of each concentration within the range used to determine the acute toxicity.

5.2.2. Mixture toxicity test: methyl-parathion and Tilt

After acclimation, test solutions were placed in the flow-through system reservoirs by pipetting the appropriate amounts of stock solution of methyl-parathion and Tilt (both toxicants prepared as described in 2.2.2). The two toxicants were simultaneously mixed by refilling the reservoirs up to 18 litres with seawater. For this test, the acclimation lasted 240 hours, followed by 336 hours of exposure. The tested concentrations are shown in Table 5.1. During exposure, time of death was recorded and deceased organisms were weighed. At the end of the experiment, the surviving animals were weighed and

Table 5.1. Concentrations of methyl-parathion and Tilt in the FTS for the mixture toxicity test (based in the active compound content). Ten organisms in each channel.

Channel	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Methyl-parathion ($\mu g.l^{-1}$)	0.5	0.5	0.9	0.9	1.6	1.6	3	3
Tilt ($\mu g.l^{-1}$)	475	475	500	500	527	527	555	555
Channel	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Methyl-parathion ($\mu g.l^{-1}$)	5.3	5.3	9.7	9.7	17.5	17.5	0	0
Tilt ($\mu g.l^{-1}$)	585	585	617	617	650	650	0	0

preserved in Davidson's AFA fixative (Bell and Lightner, 1988). Moulting and behaviour were recorded during the acclimation and exposure.

5.2.3. Mixture toxicity test: methyl-parathion and propiconazole

The acclimation time for this test was 288 hours, followed by an exposure of 264 hours. In the exposure period, the test solutions were prepared by placing methyl-parathion and propiconazole simultaneously in the reservoirs of the flow-through system, and mixed through the refilling (stock solutions prepared as described in section 2.3). Table 5.2 shows the concentrations used of both toxicants. Deceased organisms were weighed and the time of death recorded. All remaining organisms were weighed at the completion of the test and then preserved in Davidson's AFA fixative (Bell and Lightner, 1988). Moulting and behaviour were recorded during the acclimation and exposure.

5.2.4. Statistical analysis

For each combination of fungicide - methyl-parathion, the pattern of mortalities per concentration was plotted. The mortality data was used to calculate the median lethal concentration (LC50) of the components of the mixture at different times, so the toxic units could be calculated (Marking, 1985). The concept of toxic units involves the sum of toxic contributions of various components in a mixture, according to the formula: $\frac{Am}{Ai} + \frac{Bm}{Bi} = S$; where *A* and *B* are chemicals, *i* and *m* are the toxicities (LC50) of *A* and *B* individually and in mixture, and *S* is the sum of activity (see chapter 3 for the single-compound toxicities). The toxicity of the mixture was evaluated by calculating the additive index (*AI*), which assigns zero as a reference point for simple additive toxicity and

Table 5.2. Concentrations of methyl-parathion and propiconazole in the FTS for the mixture toxicity test (based in the active compound content). * Acetonitrile control (150 $\mu l.l^{-1}$). Ten organisms per channel.

Channel	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Methyl-parathion ($\mu g.l^{-1}$)	0.5	0.5	0.9	0.9	1.6	1.6	3	3
Propiconazole ($\mu g.l^{-1}$)	400	400	625	625	1000	1000	1550	1550
Channel	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16***
Methyl-parathion ($\mu g.l^{-1}$)	5.3	5.3	9.7	9.7	17.5	17.5	0	0
Propiconazole ($\mu g.l^{-1}$)	2450	2450	3800	3800	6000	6000	0	0

linear positive and negative values for greater and less than additive effects respectively (Marking and Dawson, 1975). *AI* values were obtained using the following procedure:

For $S \leq 1.0 \rightarrow AI = \frac{1}{S} - 1$, and for $S \geq 1.0 \rightarrow AI = S(-1) + 1$.

The toxicity data was also used to evaluate the effects of the mixtures on the median survival time and the threshold toxicity based on the procedure proposed by Calamari and Marchetti (1973), and Axiak and Abel (1991). The authors utilised the toxic units (here called "threshold toxic units or TTU") to denote the quantity of the toxicants present as a fraction of the lethal threshold concentration. The threshold toxic units were plotted for toxicants, individually and in mixture, versus the median lethal time, and the resulting toxicity curves compared. The toxicity curves for individual toxicants were constructed with data from the single-compound toxicity tests (chapter 3).

The flow rate in the flow-through system was calculated in relation to the organisms loading using the average wet weight (± standard deviation) from the organisms used in the experiment and the daily consumption of seawater.

Moulting was evaluated by registering the number of times each animal moulted during the experiment (moulting frequency) and also by estimating the number of hours between consecutive moults (intermoult duration).

For acclimation, moulting frequency was calculated per channel and used to calculate the overall moulting frequency. Intermoult duration was calculated per

individual and per channel, and then used to estimate the overall intermoult duration. Subsequently, the intermoult data was ordered in class intervals to graphically assess the distribution of frequencies and normality by a Kolmogorov-Smirnov test.

For exposure, moulting frequency was calculated per channel and tested for correspondence with concentration of the mixtures by a non-parametric *Gamma* correlation analysis. The intermoult duration was calculated per individual and per exposure channel, and analysed for correspondence with the concentration of the mixtures by a Kendall Tau non-parametric correlation. Additionally, the intermoult data was ordered in class intervals to graphically assess the distribution of frequencies per exposure channel.

5.3. RESULTS

5.3.1. Methyl-parathion – Tilt mixture test

The combined toxicity of methyl-parathion and Tilt is showed in the Fig. 5.1. The mixture with the highest concentration of methyl-parathion – Tilt (17.5 – 650 $\mu g.l^{-1}$ respectively) produced 40% of lethal responses at 336 hours of exposure (14 days). The resulting data was not suitable to calculate the LC50 and toxic units at any exposure time.

Table 5.3 shows the resulting mortality at the end of the exposure period in relation with the threshold toxic units used for the mixture. The low mortality suggests a less than additive effect in the interaction of the toxicants. An observed mortality of 0% with TTU values near 1 is far below the response

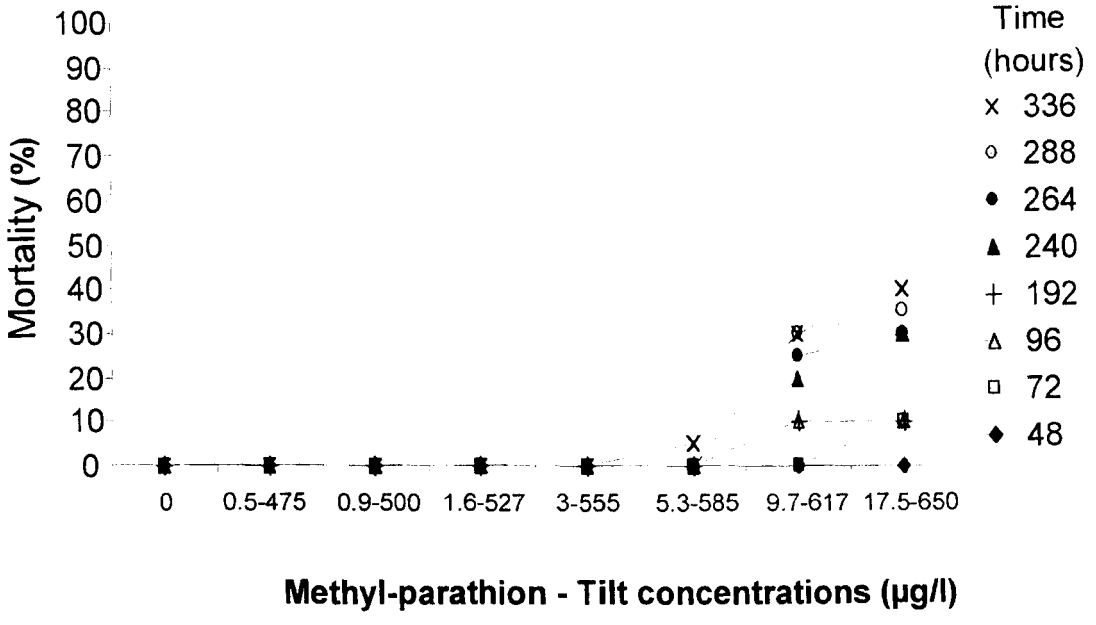


Fig. 5.1. Mortality pattern of *Litopenaeus vannamei* exposed to mixtures of methyl-parathion and Tilt. Group size =20.

Table 5.3. Observed mortality of *Litopenaeus vannamei* after 336 hours of exposure to mixtures of methyl-parathion and Tilt. *Threshold LC50 of 4.38 $\mu g.l^{-1}$. $^{+}$Threshold LC50 of 1137 $\mu g.l^{-1}$. Concentrations based in the active compound content.

Toxicants ($\mu g.l^{-1}$)		Threshold toxic units			
Methyl-parathion *	**Tilt^{+}**	**Methyl-parathion**	**Tilt**	**Total**	**% mortality**
0.5	**475**	0.11	0.42	0.53	0
0.9	**500**	0.21	0.44	0.65	0
1.6	**527**	0.37	0.46	0.83	0
3	**555**	0.69	0.49	1.17	0
5.3	**585**	1.21	0.51	1.73	5
9.7	**617**	2.22	0.54	2.76	30
17.5	**650**	4.00	0.57	4.57	40

expected on the basis of an additive effect alone, which would be around 50% lethality.

The average wet weight of organisms used in this experiment was of 0.81 ± 0.26 g, yielding a daily flow rate of 2.22 $l.g^{-1}$ per channel, considering ten organisms per exposure chamber and a daily supply of 18 litres per channel in the flow-through system (see section 2.9). Fig. 5.2 shows the wet weight of the organisms that died per treatment during the exposure period. The correlation analysis showed no correspondence between the time of death and wet weight in the methyl-parathion – Tilt mixtures (r = correlation coefficient; $P<0.05$): 17.5 – 650 $\mu g.l^{-1}$ ($r = -0.573$, $n = 8$) and 9.7 – 617 $\mu g.l^{-1}$ ($r = -0.713$, $n = 6$).

The average moulting frequency of all groups was of 1.2 times during 10 days of acclimation. The moulting frequency per flow-through system channel is shown in Table 5.4. The intermoult duration for this period was of 157 hours (≈ 7 days). Fig. 5.3 shows the frequency distribution of the intermoult duration data, which fits a normal distribution.

The moulting frequency in the control channels during exposure was of 1.04 times in ten days. Table 5.5 shows the moulting frequency per channel. The *Gamma* correlation analysis showed evidence of a negative correspondence between the concentration of pesticides in the mixtures and moult frequency (*Gamma* = -0.163, P = 0.032). For the same period, the estimated average for intermoult duration was of 180 hours (≈ 7 days) for the control channels. However, in this case the Kendall-Tau correlation did not reveal a

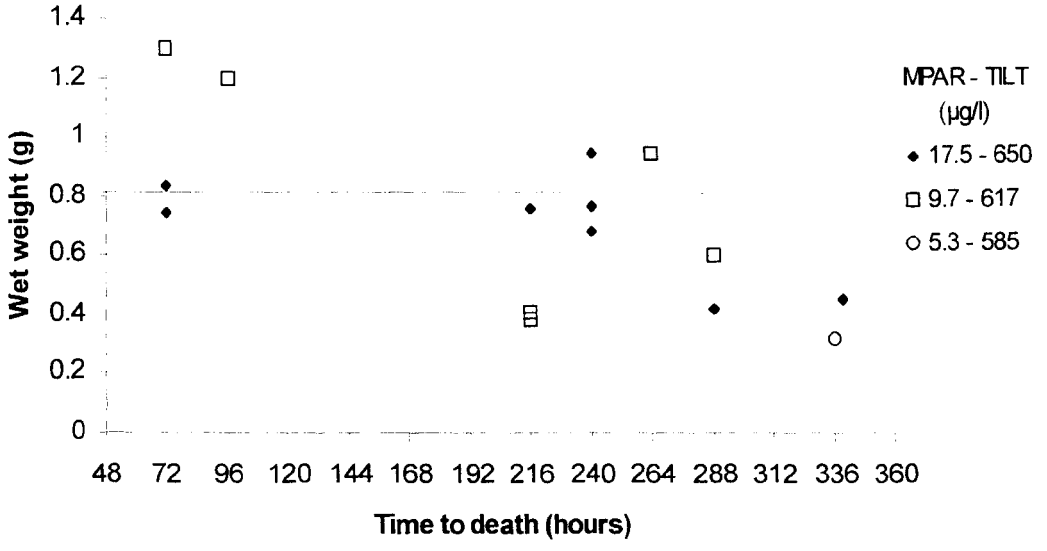


Fig. 5.2. Wet weight of organisms in relation to time of death for the mixtures of methyl-parathion – Tilt. The line denotes the average of all organisms used in the experiment.

Table 5.4. Moulting frequency per channel during ten days of acclimation for the methyl-parathion - Tilt mixture experiment. Conf. int. = 95% confidence interval for the population median.

Channel	1	2	3	4	5	6	7	8
N	10	10	10	10	10	10	10	10
Moults in 10 days	1.2	1.1	1.0	1.3	1	1.3	1.2	1.3
Median	1	1	1	1	1	1	1	1
Conf. int.	1-2	1-1	1-2	1-2	0-2	1-2	1-2	1-2
Channel	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
N	9	10	10	9	10	10	10	10
Moults in 10 days	1.11	1.3	1.2	1.22	1	1.1	1.3	1.3
Median	1	1	1	1	1	1	1	1
Conf. int.	1-2	1-2	1-2	1-2	1-1	1-2	1-2	1-2

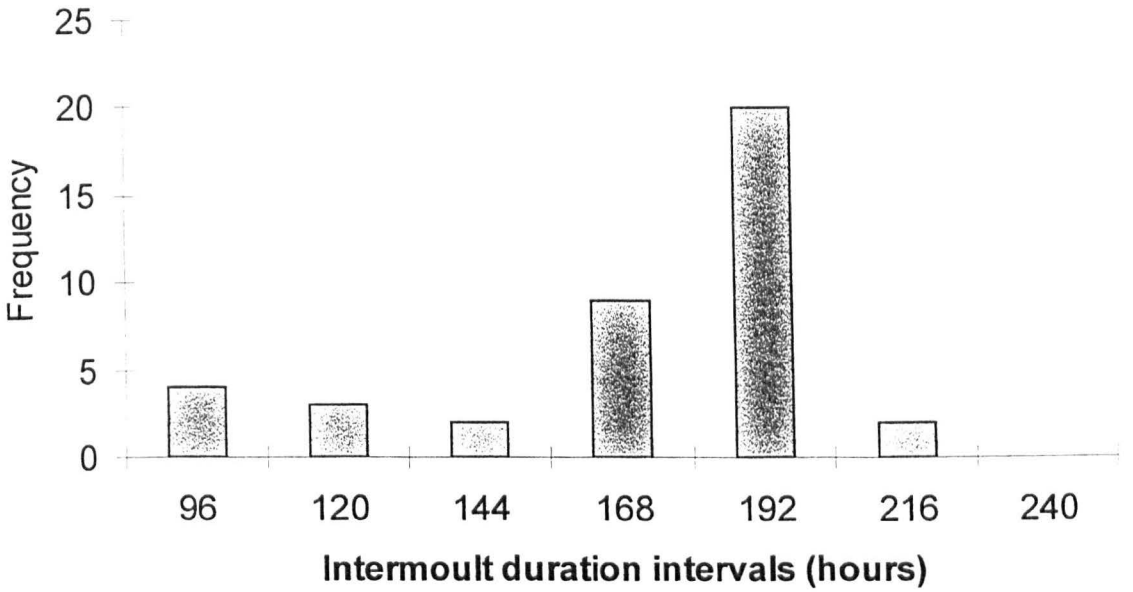


Fig. 5.3. Frequency distribution of intermoult duration during acclimation for the methyl-parathion – Tilt mixture experiment.

Table 5.5. Moulting frequency per channel in ten days of exposure to mixtures of methyl-parathion and Tilt. Conf. int. = 95% confidence interval for the population median.

Channel	1	2	3	4	5	6	7	8
n	10	10	10	10	10	10	10	10
Moults in 10 days	1.14	1.36	1.07	0.93	0.86	0.93	1.14	1
Median	1.5	2	1.5	1	1	1.5	2	1.5
Conf. int.	1-2	1-3	1-2	1-2	1-2	0-2	0-2	1-2
Channel	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
n	9	10	8	6	6	6	10	10
Moults in 10 days	1.19	1.07	0.8	0.6	0.83	0.95	1	1.07
Median	2	1	1	1	1	1.5	2	1.5
Conf. int.	1-2	1-2	0-2	0-1	1-2	0-2	0-2	1-2

significant correspondence between concentration of the mixture and intermoult duration (τ = 0.096302, P = 0.235). The distribution of frequencies of intermoult duration is presented in Fig. 5.4 (a to h). Due to the relatively short exposure time, few data was collected. It is therefore difficult to assess whether the mixture has any effect on moulting or not.

In general, the shrimps were able to locate and ingest the food pellets during the exposure. However, the two highest exposure levels produced lack of co-ordination, immobilisation of pereopods and erratic movements in the organisms before death, although some of them recovered and showed normal behaviour until the end of the experiment.

5.3.2. Methyl-parathion – propiconazole mixture test

Fig. 5.5 shows the pattern of mortalities in relation to the concentrations and time of exposure. At the first 24 hours, 50 % lethality was observed in between the mixtures 5.3 – 2450 $\mu g.l^{-1}$ and 9.7 – 3800 $\mu g.l^{-1}$ (methyl-parathion – propiconazole respectively). By the end of the experiment, 50 % of mortality occurred in between the mixtures 0.9 – 625 and 1.6 – 1000 $\mu g.l^{-1}$.

The combined toxicity of the mixture was evaluated at different times by calculating the toxic units from the LC50's of the toxicants, individually (see Table 3.6) and in the mixture, to subsequently calculate the AI's. The graphic representation of the AI's, for different times of exposure (Fig. 5.6), suggests that there was a greater than additive toxic interaction at higher concentrations during the first four days of the experiment. For the three following days, the AI

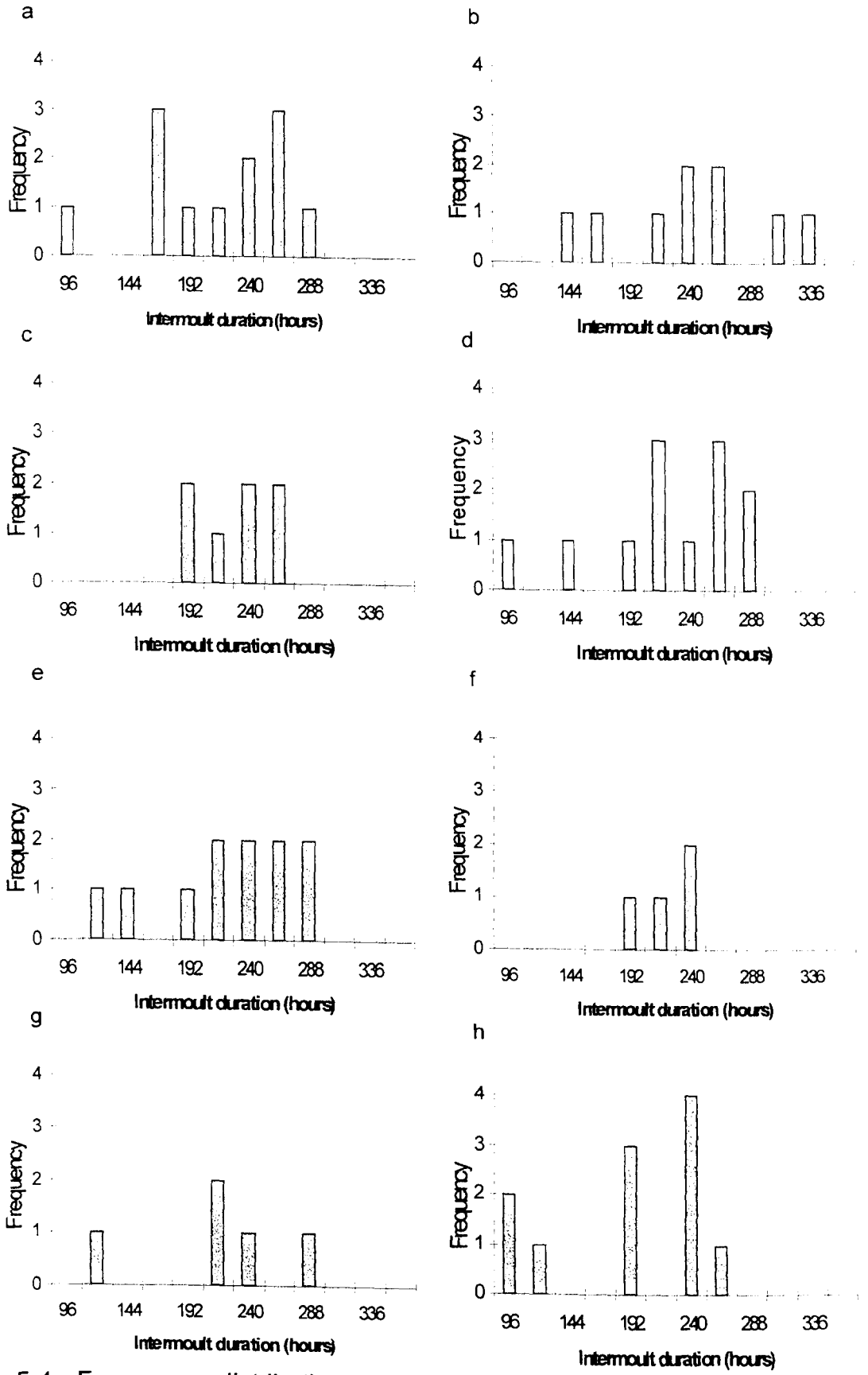


Fig. 5.4. Frequency distribution of intermoult duration during the methyl-parathion - Tilt mixture experiment: a) 0.5 - 475 $\mu g.l^{-1}$, b) 0.9 - 500 $\mu g.l^{-1}$, c)1.6 - 527 $\mu g.l^{-1}$, d) 3 - 555 $\mu g.l^{-1}$, e) 5.3 - 585 $\mu g.l^{-1}$, f) 9.7 - 617 $\mu g.l^{-1}$, g) 17.5 - 650 $\mu g.l^{-1}$, h) 0 $\mu g.l^{-1}$. Concentrations based in active compound content.

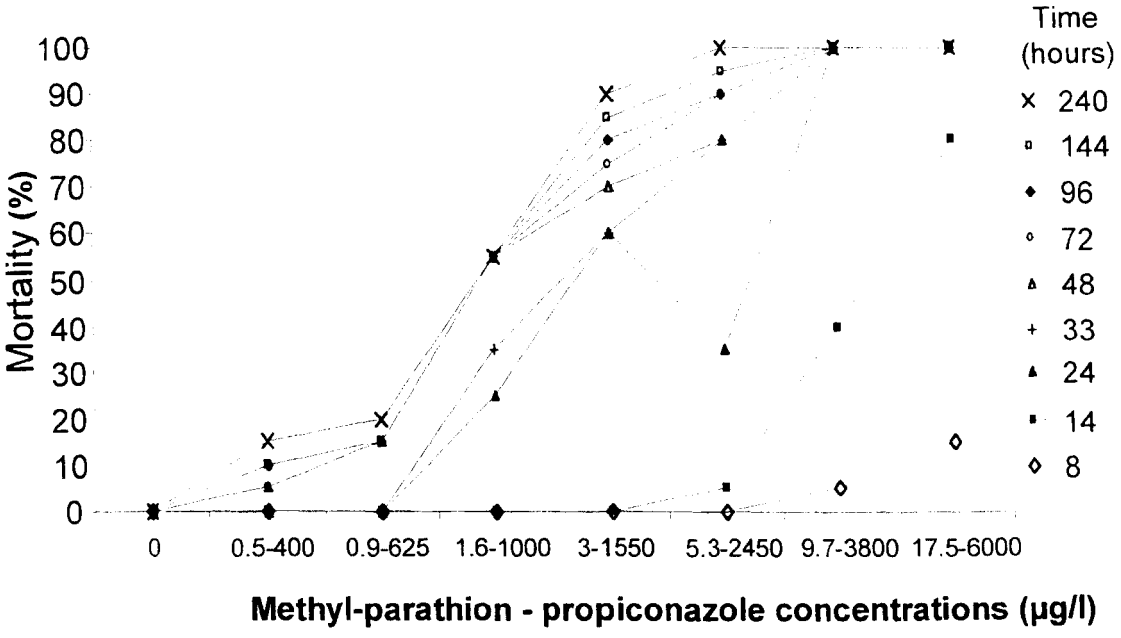


Fig. 5.5. Mortality pattern of *Litopenaeus vannamei* exposed to mixtures of methyl-parathion and Propiconazole. Group size =20.

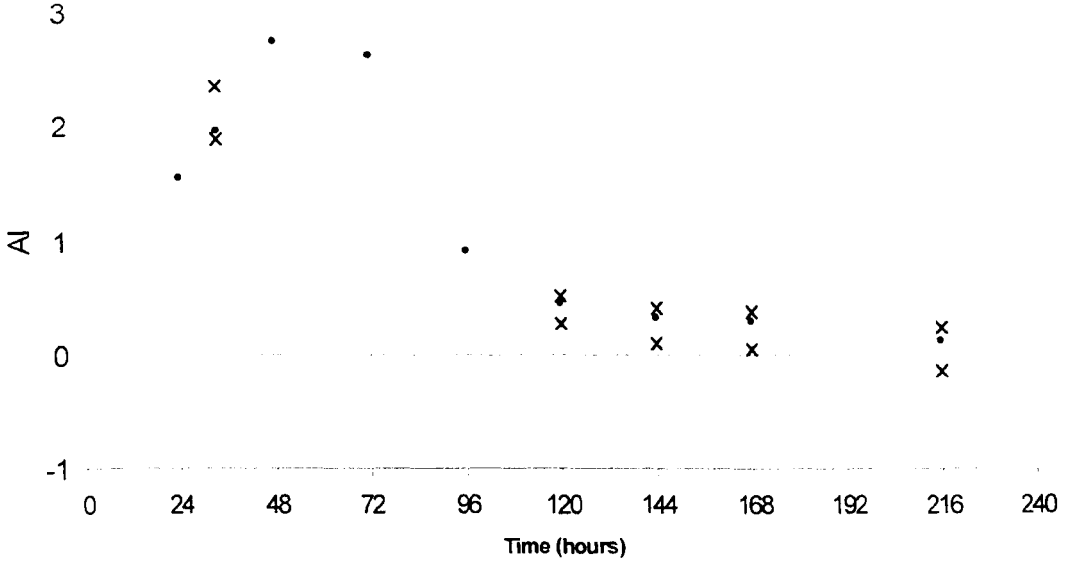


Fig. 5.6. Additive index (AI) at different times of exposure for the methyl-parathion – propiconazole mixture experiment. Crosses denote the AI's for the 95% confidence intervals of LC50's. Dashed line at zero represents the simple additive toxicity.

values decreased notably, but their ranges still denote a greater than additive effect. It is until the 216 hours of exposure (9 days) when the AI ranges overlapped zero, which indicates a simple additive combined toxicity (see Marking and Dawson, 1975) in the mixtures with lower concentrations.

The effect of the mixtures was also evaluated on the threshold toxicity. Fig. 5.7 shows that the toxicity curve for the mixtures of methyl-parathion – propiconazole lay below those representing the single compounds. Calamari and Marchetti (1973) defined this as a more than additive effect, since a simple additive effect would have the curve of the mixture bounded by the curves of the single-compounds. This graphical analysis was unable to evaluate the effect of mixtures at lower concentrations (near or below the threshold levels or end of experiment) where the LT50's could not be determined. However, by the end of the experiment (240 hours), a value of 0.95 in the combined threshold toxic units corresponded to an observed mortality near 50% (Table 5.6). This supports the idea of a simple additive toxicity effect at the end of the exposure period (or close to the threshold toxicity levels).

The average wet weight of all the organisms was of 0.81 ± 0.26 g, which corresponded to a daily flow rate of 2.21 $l.g^{-1}$ per channel in the flow-through system (daily consume of 18 litres per channel, for ten organisms per exposure chamber, see section 2.9). Fig. 5.8 (**a**, **b** and **c**) shows the average wet weight of the organisms per treatment in relation of their time of death. No correspondence was found in the mixture treatments: 17.5 – 6000 $\mu g.l^{-1}$, 9.7 – 3800 $\mu g.l^{-1}$, 5.3 – 2450 $\mu g.l^{-1}$, 3 – 1550 $\mu g.l^{-1}$ and 1.6 – 1000 $\mu g.l^{-1}$, with

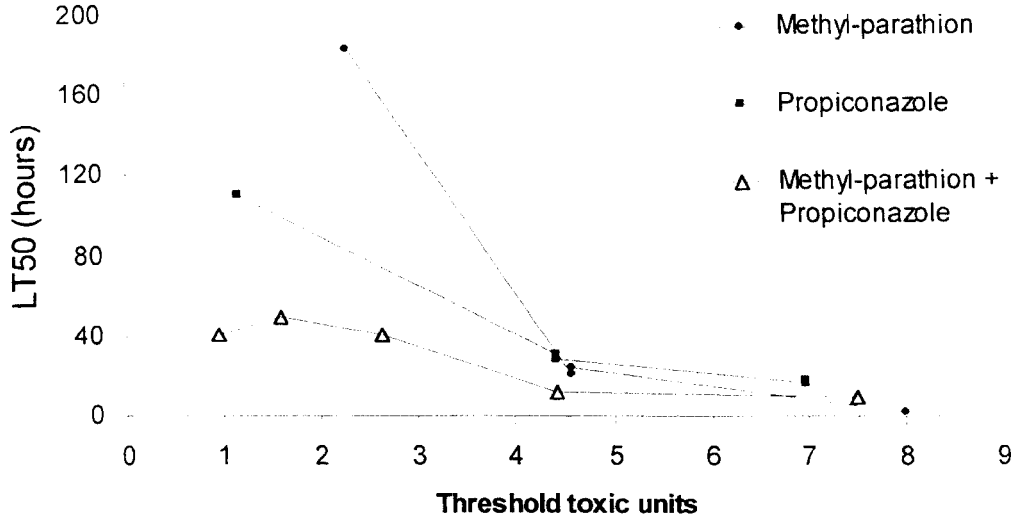


Fig. 5.7. Toxicity curves of methyl-parathion alone, propiconazole alone, and the mixture of methyl-parathion – propiconazole.

Table 5.6. Observed mortality of *Litopenaeus vannamei* after 240 hours of exposure to mixtures of methyl-parathion and propiconazole. *Threshold LC50 of 4.38 $\mu g.l^{-1}$. +Threshold LC50 of 1716 $\mu g.l^{-1}$. Concentrations based in the active compound content.

Toxicants ($\mu g.l^{-1}$)		Threshold toxic units			
Methyl-parathion *	**Propiconazole+**	**Methyl-parathion**	**Propiconazole**	**Total**	**% mortality**
0.5	**400**	0.11	0.23	0.35	15
0.9	**625**	0.21	0.36	0.57	20
1.6	**1000**	0.37	0.58	0.95	55
3	**1550**	0.69	0.90	1.59	90
5.3	**2450**	1.21	1.43	2.64	100
9.7	**3800**	2.22	2.21	4.43	100
17.5	**6000**	4.00	3.50	7.49	100

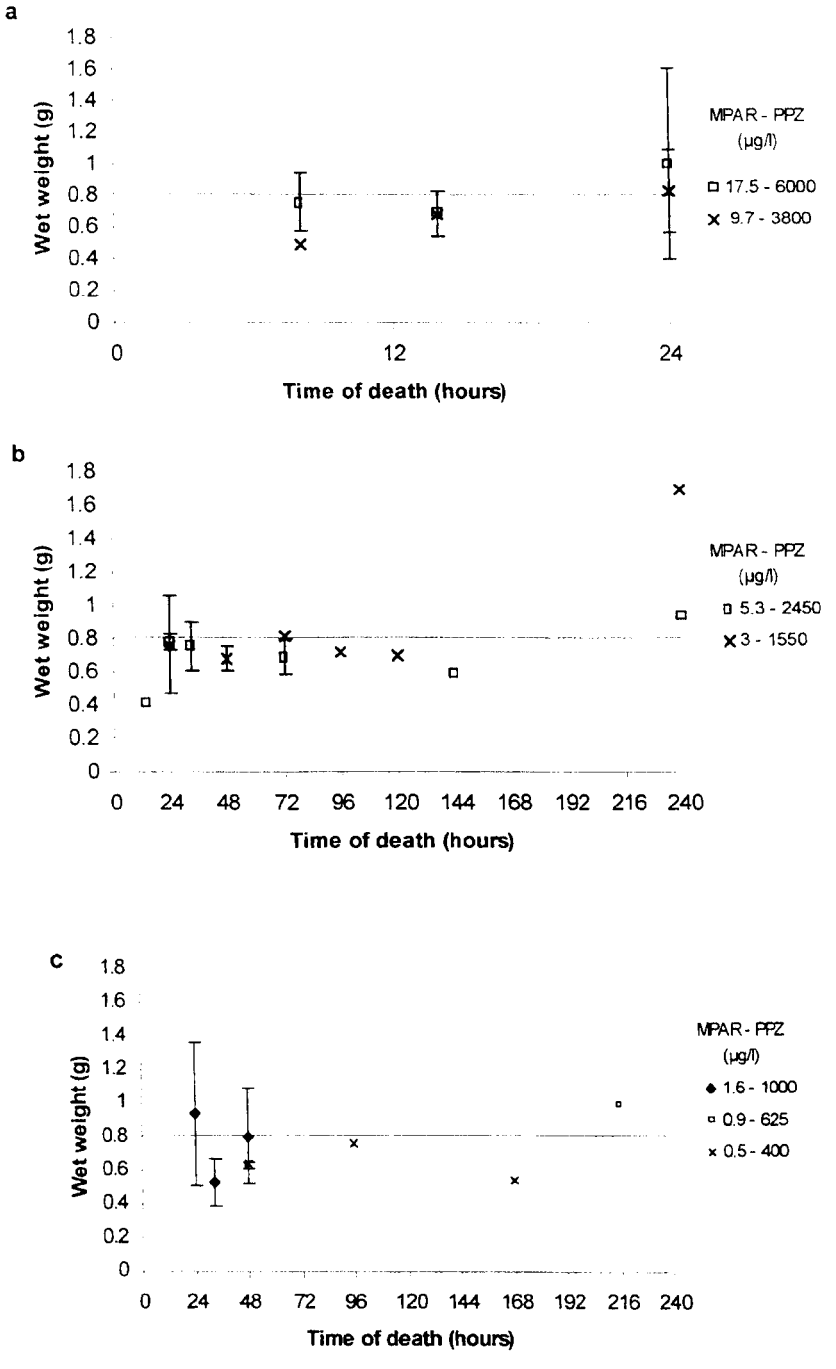


Fig. 5.8 (**a**, **b** and **c**). Average wet weight of organisms in relation to time of death for the mixtures of methyl-parathion – propiconazole. Bars represent standard deviation. The line denote the average of all organisms used in the experiment.

correlation coefficients (r) of 0.34 (n = 20), 0.382 (n = 20), 0.179 (n = 20), 0.561 (n = 18) and -0.138 (n = 13) respectively ($P<0.05$).

Table 5.7 shows the moulting frequency per channel during acclimation. In this period, the average moult occurrence estimated for ten days was of 0.91 times. For the intermoult duration data, the average for acclimation was of 155.3 hours, and its distribution of frequencies fits a normal distribution (Fig. 5.9).

Due to high mortality and few organisms moulting during the exposure period, moulting frequency and intermoult duration were not determined.

No appreciable differences were noted in the localisation and ingestion of pellets on the exposed organisms in relation with the controls. At the end of the experiment, few organisms were observed with black gills in both control and exposed treatments.

5.4. DISCUSSION

The theoretical explanation of joint effects of toxicants in a mixture is particularly complicated when each component acts on a different physiological or biochemical system but contributes to a common response (EIFAC, 1987). The chemicals used in this study are known to have different modes of action. The primary biochemical effect of methyl-parathion, as organophosphorous pesticide (OP), based its neurotoxicity on the inhibition of acetylcholinesterase (AChE) (WHO, 1993). Its metabolism is explained by the oxidative enzymatic

Table 5.7. Moulting frequency per channel during ten days of acclimation for the methyl-parathion - propiconazole experiment. Conf. int. = 95% confidence interval for the population median.

Channel	1	2	3	4	5	6	7	8
n	10	10	9	10	10	10	9	10
Moults in 10 days	1.17	1.42	0.93	1	0.83	0.92	1.3	1
Median	1.5	2	1	1	1	1	2	1
Conf. int.	1-2	1-2	0-2	0-2	0-2	1-2	1-2	0-2
Channel	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
n	10	10	10	10	10	10	10	10
Moults in 10 days	0.75	0.58	0.92	1	0.75	0.58	1	0.5
Median	1	1	1	1	1	0.5	1	0
Conf. int.	0-2	0-1	0-2	0-2	0-2	0-2	0-2	0-2

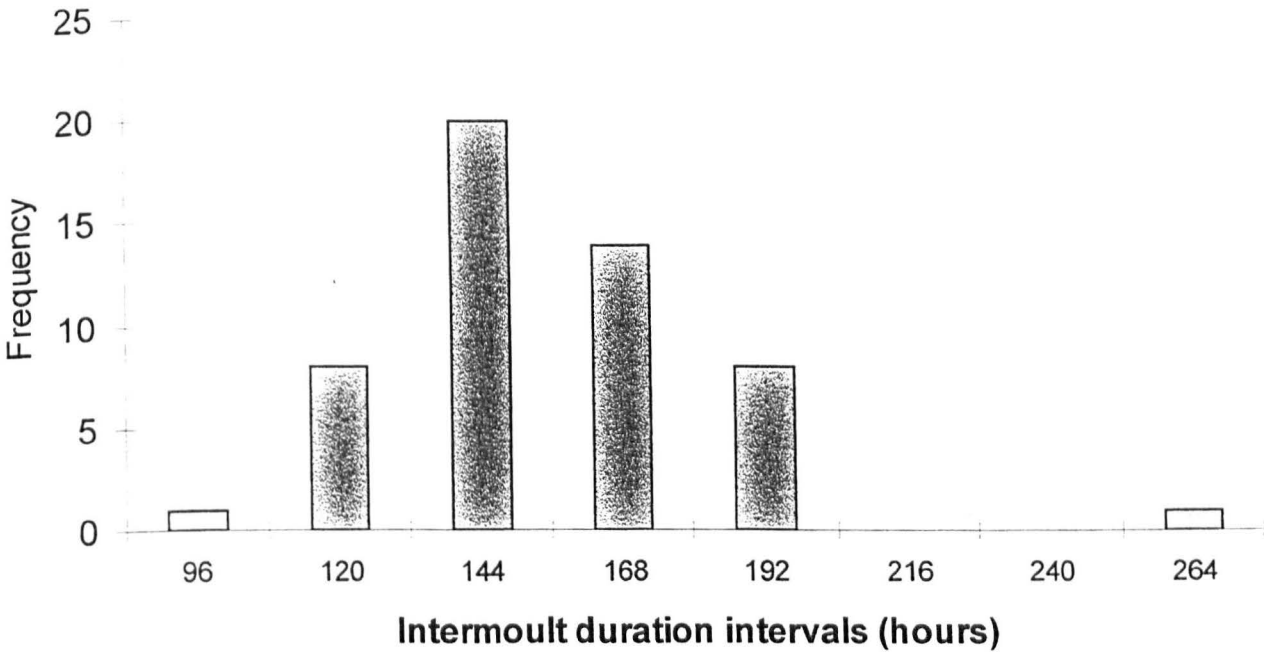


Fig. 5.9. Frequency distribution of intermoult duration during acclimation for the methyl-parathion – propiconazole mixture experiment.

attack on the P-S linkage in the original molecule, which derives a more potent acetylcholinesterase inhibitor called methyl-paraoxon (Hassall, 1990). In studies with the shrimp *Penaeus japonicus*, the increase of toxicity of organophosphorothionates with the progress of larval stages was attributed to the simultaneous increase in the oxidative desulfuration activity (Kobayashi *et al.*, 1991). The neurotoxic activity of methyl-parathion can cause impairment in the immunological responses of *Penaeus monodon* (Bodhipaksha and Week-Perkins, 1994). On the other hand, propiconazole, contained also in Tilt as active compound, is a systemic fungicide belonging to the group of triazoles (Hassall, 1990). Its mode of action is attributed to the interference with sterol synthesis by preventing C-14 demethylation by the cytochrome P-450 system, being generically catalogued as ergosterol biosynthesis inhibitor (EBIs) (Hartley and Kidd, 1987; Hassall, 1990; Kwok and Loeffler, 1993). Propiconazole has been found to induce cytochrome P-450 in quail and to interfere with the avian sterol metabolism (Hassall, 1990; Johnston *et al.*, 1994, Ronis *et al.*, 1994). No reports were found on the metabolism of propiconazole on crustaceans.

The results obtained in this study indicated important differences in the way methyl-parathion interacted with propiconazole depending on the way the fungicide was incorporated in the mixture. When Tilt – (the formulated presentation of propiconazole was used), the combined toxicity with methyl-parathion was less than additive, as the mortality responses were lower than the expected for a simple additive action. On the other hand, when technical grade propiconazole was used in the mixture with methyl-parathion, the initial effect was greater than additive as the highest concentrations readily killed the

organisms. However, as the exposure continued, the lethal responses at lower concentrations stabilised to levels that could be considered as simple additive. All together the results coincide with the common consensus that as concentrations of toxicants are reduced towards the level of no effect, their potential for addition is similarly reduced (EIFAC, 1987; Lloyd, 1991). This also highlights the importance in performing time-independent exposures that, as described in the lethal tests in chapter 3, can help to define toxicity curves with more accuracy than traditionally short-duration exposures.

Although observed only in lethal levels, the greater than additive toxicity in the combination of methyl-parathion and technical grade propiconazole opens important questions on the way the metabolism of these chemicals interacts in crustaceans. The fact that the combined toxicity showed simple additivity in threshold levels is equally interesting as it could involve the participation of mechanisms of compensation. In general, the toxicity of a pesticide may be increased as a result of the induction of an enzyme that activates it, or the inhibition of an enzyme that detoxifies it (Johnston *et al.*, 1990). Unfortunately, the scarce information available on the metabolisms of these chemicals at toxicokinetic level in crustaceans makes it difficult to explain the possible mechanisms of interaction. Some studies have reported an enhancement of toxicity in mixtures between EBI fungicides and OP pesticides in vertebrates. Laboratory studies with the hybrid red-legged partridge, Japanese quail and pigeon showed that a pre-treatment with the EBI fungicide, prochloraz, resulted in an increased toxicity of the OP insecticides malathion, parathion, and dimethoate (Riviere *et al.*, 1985; Johnston *et al.*, 1989, 1990, 1994). The

mechanism behind this interaction was attributed to the induction of the hepatic cytochrome P450 dependent monooxygenase system by the fungicide, resulting in a more active oxidation of the OP insecticides to more toxic oxon products which in turn inhibit the butyryl and acetyl cholinesterases (Johnston *et al.*, 1989). However, this hypothesis has been described as too simplistic for vertebrates since, in addition to activation, OP's are also inactivated via P450-dependent oxidative dearylation and esterolytic cleavage in liver microsomes, cytosol and serum (Ronis and Badger, 1995). For instance, worst-case scenario trials in semi-field conditions with the red-legged partridge failed to show potentiation of toxicity between prochloraz and malathion, even after it was proved that the fungicide was able to significantly induce the monooxygenase system (Johnston *et al.*, 1996). Similarly, even though significant elevations in paraoxon formation were observed *in vitro* following propiconazole or vinclozolin treatment of rats, and propiconazole induced malaoxon formation in quail microsomes, neither propiconazole nor vinclozolin enhanced parathion toxicity *in vivo* in the rat, nor did propiconazole affect *in vivo* malathion toxicity in quail (Ronis and Badger, 1995). Also, experiments with red-legged partridges pre-treated with propiconazole showed the same inhibition of serum butyryl cholinesterase activity than control organisms pre-treated with corn oil following administration with malathion (Johnston *et al.*, 1994). Recent findings have shown that the EBI fungicides produce mixed patterns of induction and inhibition of P450 enzymes which are fungicide, species and tissue specific (Ronis *et al.*, 1998). The synergism observed between EBI fungicides and pyrethroid insecticides in the honeybee *Apis mellifera* (Pilling and Jepson, 1993) has been related to the inhibition of microsomal oxidation caused by the

fungicides, which delays the metabolism, detoxication, and excretion of the insecticide (Pilling *et al.*, 1995).

More studies are needed in order to predict the possible effects of fungicides and insecticides in crustaceans. For instance, the inducibility of monooxygenases in invertebrates is still a subject of controversy. While the hepatic biotransformation enzyme cytochrome P4501A (CYP1A) in vertebrates is known to be specifically induced by PAH, PCBs and dioxins, much less is known of the existence, fundamental properties and gene regulation of such system in marine invertebrates (Livingstone, 1991; Livingstone *et al.*, 1994). Apparent induction of the mixed-function oxidases (MFO) system with exposure to model inducers has been reported in the marine crabs (*Callinectes sapidus, Sesarma cinerum, Uca pugilator* and *Maja crispata*) and polychaetes (*Nereis virens*) (Singer *et al.*, 1980; Bihari *et al.*, 1984), freshwater crayfish *Astacus astacus* (Lindström-Seppä and Hänninen, 1986) and the starfish *Asterias rubens* (Den Besten *et al.*, 1993), while other studies report lack of induction in the blue crab *Callinectes sapidus* (Singer *et al.*, 1980) and spiny lobster *Panilurus argus* (James and Little, 1984). Although exceptions exist, the responses are generally variable and, to an extent, absent (Livingstone, 1991), and certainly much lower than for fish (Livingstone *et al.*, 1994). The limited response could reflect a less sophisticated mechanism of induction than in vertebrates, which can be dampened down by interactions with other environmental variables, such as season and reproductive state (Livingstone *et al.*, 1994). On the other hand, several invertebrates, including some crustaceans, are well able to metabolise PAH to hydroxylated metabolites. In

the spiny lobster, this is because the hepatopancreas contains cytochrome P450 isozymes in the 2 family, which have broad substrate selectivity (Margaret James, personal communication). However, the inducibility of these isozymes is not known in crustaceans.

As mentioned in section 2.3, PAHs represent around 60% of the volume in the Tilt 250E formulation. Tilt, however, far from enhancing the toxicity of methyl-parathion (as would be expected if either propiconazole or the PAH were able to induce the MFO system), showed a less than additive effect instead. With the information available it is unclear how the combination methyl-parathion with the components in the Tilt formulation produced a substantially different toxicity pattern than the observed in the methyl-parathion – propiconazole mixture. Further experiments are necessary to determine the reproducibility of these toxicity patterns using novel combinations of the components of the mixture, and including the evaluation of sublethal responses at the toxicokinetic, physiological and organismal level.

The analysis of moulting data in the methyl-parathion – Tilt mixture test produced only limited evidence of possible moult retardation due to the mixture treatments. Using this as preliminary information, further research could be undertaken considering the modifications in the experimental procedures mentioned in chapter 4, i.e. longer exposure times with sublethal concentrations. This also applies to the methyl-parathion – propiconazole combination, which did not generate enough data to be analysed. The differences in behaviour appreciated in both experiments indicate that there is a

considerable potential to use behavioural responses in the ecotoxicological evaluation of the mixtures.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

The results obtained in this study indicated that, given the appropriate conditions, the marine shrimp *Litopenaeus vannamei* has considerable potential as a test species in tropical marine ecotoxicology. Due mainly to its use in aquaculture, this organism has been extensively studied in many aspects of its ecology and biology, while the optimal environmental conditions are well known for the rearing of the different life stages. The reproduction of this species has been successfully achieved under laboratory conditions, and there is a good understanding of the endocrinological mechanisms involved in key processes such as moulting and reproduction. Similar approaches have been taken with other penaeid species found in Mexican waters such as *L. stylirostris, L. californiensis, Farfantepenaeus californiensis* and *F. brevirostris* (Pacific coast), and *L. occidentalis, L. setiferus, F. aztecus. F. brasiliensis, F. duorarum* and *F. notialis* (Atlantic coast). The fact that some aquaculture laboratories maintain reference stocks of some penaeid species (e.g. pathogen-free strains) could be useful for standardisation purposes in ecotoxicology.

The literature review revealed that little attention has been paid to ecotoxicological research in Mexico, particularly in the Northwest coast that is known for extensive agricultural practices. While some studies have been focused to determine the levels of most common contaminants, the information on the effects of such chemicals upon local species is scarce.

Taking this in consideration, this study was aimed at developing a tropical marine bioassay to assess contaminant effects on *L. vannamei* in flow-through conditions. A flow-through system was designed to provide the optimal conditions for the test organisms (Chapter 2), which were individually arranged in cages to evaluate the moulting rate during the experiments. When these conditions were achieved, the system was used to perform acute toxicity experiments of the organophosphorous insecticide methyl-parathion, and the triazole derivative fungicide propiconazole (technical grade and commercial formulation – Tilt) (Chapter 3). Based on the toxicity data of the single-compound experiments, the interactive toxicity between methyl-parathion and the fungicides was assessed (Chapter 5). Additionally, the effects of a 32-days subchronic exposure to Tilt were evaluated in moulting, morphology and behaviour of the shrimps (Chapter 4).

The flow rate utilised in the flow-trough system was an important factor to achieve 100% survival in all control channels (the longest experimental period was of 42 days). The daily consumption of the system was of 288-litres equally distributed among the flow channels, allowing a 90% of test solution replacement every 15 hours in the exposure chambers (4.5-litres). This represented a flow rate ranging from 1.32 to 2.22 $l.g^{-1}.day^{-1}$, which is close to the values recommended of 2 to 3 $l.g^{-1}.day^{-1}$ for fish in continuous-flow tests (Sprague, 1969). During exposure, the reliability of the exposure conditions was tested by recording the wet weight of deceased organisms in relation to their time of death. None of the experiments performed in the flow-through system showed a correlation between exposure time and size of organisms,

which indicates that the exposure was homogeneous within the exposure chambers and that size was not a factor in the observed toxic response. With some modifications, the system could be readily adapted for lower or higher organisms loading due to the use of the variable-speed peristaltic pump.

Apart from the peristaltic pump and the Teflon FEP tubing, the flow-through system consisted of inexpensive off-the-shelf components. The simple and modular construction of the flow-through system allowed an easy operation and uncomplicated daily maintenance. This feature makes possible further modifications in the system to accommodate other test species with different requirements.

The continuous replacement of the test solution minimised the negative effects of feeding in toxicological bioassays such as accumulation of waste metabolic products and oxygen deprivation. However, it is still necessary to perform a comprehensive evaluation of the environmental variables in the flow-through conditions such as oxygen uptake, ammonia concentration, pH, bacterial content, for assurance that test conditions are suitable for interpretation of toxicity effects. For exposure, it is also essential to perform analytical determinations of the toxicants tested. Prior to any experiment, a histopathological and bacteriological analysis of the test organisms may be desirable to discard batches with high prevalence of disease. Altogether, this will allow the isolation of the toxic response from other environmental variables that can potentially affect the test organisms. Nevertheless, the conditions achieved in the flow-through system permitted to appropriately perform time-

independent exposures, which normally are more prolonged than traditional exposures of 24, 48, 72 or 96 hours. From single-compound acute toxicity tests, it could be appreciated that median lethal concentrations at early exposure times were substantially higher than those obtained later in the experiment. This is clearly evident in the toxicity curves of methyl-parathion and propiconazole that reached the asymptote respect to concentration only after 96 hours. Similarly, in the methyl-parathion – propiconazole mixture test, the toxicity curves suggested a greater than additive toxic interaction during the first four days of the experiments, but ultimately resulted in a simple additive toxicity after 9 days of exposure.

In general, the acute toxicity shown by the pesticides used in this study corresponded to the toxicity levels reported in the literature for aquatic invertebrates. Toxicological information for methyl-parathion is more readily available than for propiconazole. With a threshold LC50 of 4.4 $\mu g.l^{-1}$, methyl-parathion is comparatively more toxic than propiconazole and its commercial formulation, Tilt, with threshold LC50's of 1 716 and 1 137 $\mu g.l^{-1}$ respectively. This could be explained by noting that crustaceans generally show high sensitivity to insecticides due to their close phylogenetic relationship with insects (Williams and Duke, 1979). As an organophosphate insecticide, methyl-parathion elicits its effects by binding to the cholinesterases and interrupting the normal neural transmission (Hassal, 1990). On the other hand, the principal mode of action of the fungicide propiconazole is attributed to the interference with sterol synthesis by preventing C-14 demethylation by the cytochrome P-450 system, being generically catalogued as an ergosterol biosynthesis inhibitor

(EBIs) (Hartley and Kidd, 1987; Hassall, 1990; Kwok and Loeffler, 1993). The higher toxicity of Tilt in relation with technical grade propiconazole could be mainly attributed to the components of the formulation, in particular the polyaromatic hydrocarbons (PAH) that represent 60.2% of the total content.

Experimentation with Tilt revealed major differences in toxicity estimates between the static system and the flow-through system. The static system was initially utilised in the range finding test aimed to broadly estimate the toxicity of the toxicant. These estimates, however, could not be reproduced in subsequent definitive experiments carried in flow-through conditions. For instance, the lethal toxicity estimated for Tilt in the static system was consistently estimated between 150 and 500 $\mu g.l^{-1}$ (propiconazole as active ingredient), coinciding with that reported by Enriquez *et al.* (1996) for the same fungicide with *L. vannamei* postlarvae: 48-h LC50 of 500 $\mu g.l^{-1}$ in a static system (28° C, 35°/oo). The over-estimation of the toxicity was probably caused by anoxic conditions induced by Tilt in static conditions, which tends to form a film of oil in the water surface, inhibiting oxygen exchange. After this experience, further range finding tests were always carried out in flow-through conditions.

As described above, some definitive experiments failed to produce the expected mortalities as the tested concentrations were selected based on static system toxicity estimates. Eventually, one of these experiments was prolonged for 32 days of exposure to assess the effects of Tilt on the test organisms (see section 3.3.2 and Chapter 4). Although few mortalities were observed after exposure, the shrimps showed several morphological abnormalities (deformities) which

were directly associated with the exposure to Tilt. For instance, the incidence and severity of deformities on the rostrum, pereopods and uropods showed a clear concentration-correspondence with the fungicide. In particular, the presence of lumps on the pereopods may be of ecotoxicological significance as these appendages have an important concentration of chemoreceptor cells (Lee and Meyers, 1997). The disruption of chemosensory capabilities may have behavioural consequences in activities such as avoidance, locomotion, feeding and reproduction. The histopathological analysis of the lumps (data not shown) revealed local areas of inflammation with signs of aggregation, degranulation, necrosis and some melanisation (Dr. J. Turnbull, pers. comm.). Moreover, additional deformities were detected in antennal scale and maxillipeds that are also known as chemosensory appendages. The presence of chemoreceptors in several parts of the organisms may be adaptive as it is not uncommon in crustaceans to lose appendages as a result of predation and physical damage (Devine and Atema, 1982). However, general disruption of chemoreceptor sites by toxicants could pose a significant threat to the organism's overall performance and survival. It is probable that some lesions occurring in antennal scale, telson and uropods could have been caused by friction with the cages where the shrimps were confined. These lesions, however, were clearly different or totally absent in control organisms, indicating a possible effect in cuticular repairing or limb regeneration. It cannot be concluded that the active ingredient of Tilt, propiconazole, could solely be responsible for the observed effects. The presence of PAH in the formulation could be an important factor as they have been associated with abnormalities in fish and invertebrates. The biological significance of toxicant-induced abnormalities in minute structures

such as setae of maxillipeds and uropods is difficult to determine with the information available. However, since these structures regenerate in each moult cycle, the appearance of deformities could have value as an early indicator of moulting or regeneration disruption. The observed effects of methyl-parathion, propiconazole and Tilt on behaviour and moulting will be further discussed.

The prediction of the joint effects of toxicants with different modes of action is particularly difficult considering the physiological and biochemical mechanisms potentially implicated. A simplistic theoretical explanation of the interactive effects between methyl-parathion and propiconazole can be outlined from their known toxicological metabolism. Johnston *et al.* (1989) has described a mechanism for this interaction in vertebrates. They suggest that the induction of the hepatic cytochrome P450 dependant monooxygenase system (MFO) by the fungicide can eventually generate a more active oxidation of the OP insecticide to more toxic oxon products which in turn inhibit the cholinesterases. This explanation however does not consider that, in addition to activation, OP's can also be inactivated via P450-dependant oxidative dearylation and esterolytic cleavage in liver microsomes, cytosol and serum (Ronis and Badger, 1995). Moreover, EBI fungicides have been found to produce mixed patterns of induction and inhibition of P450 enzymes which are fungicide, species and tissue specific (Ronis *et al.*, 1998). The biochemical effects of such pesticide combinations on crustaceans are still a pending question. For instance, although some researchers have reported apparent induction of the MFO system on invertebrates with model inducers, a limited response is normally observed. This is probably reflecting a less sophisticated mechanism of

induction than vertebrates, which can be dampened down by interactions with other environmental variables, such as season and reproductive state (Livingstone *et al.*, 1994).

The preliminary approach to assess the toxicity of the mixtures between methyl-parathion and the fungicides revealed interesting differences depending on the way the fungicide was introduced in the mixture. The combination between methyl-parathion and technical grade propiconazole showed an initial greater than additive effect as the highest concentrations tested readily killed the organisms. However, as the exposure continued, the lethal responses stabilised in the subsequent lower concentrations to levels that could be considered as simple additive. This coincides with a general pattern of reduction in the potential of addition of toxicants as their concentrations are reduced towards the level of no effect (EIFAC, 1987; Lloyd, 1991). In the other hand, the mixture of methyl-parathion and Tilt showed a less than additive toxicity as the lethal responses were substantially lower than the expected for a simple addition action. With the information available, it is unclear how the components of Tilt interacted with methyl-parathion to produce a different toxicity pattern than the observed by propiconazole as methyl-parathion pair. As mentioned before, Tilt contains PAHs, which along with propiconazole are known to induce the oxidative metabolism, and can potentially enhance methyl-parathion toxicity. A possible explanation is that some of the other components in the Tilt formulation could have chemically reacted or trapped the insecticide, therefore reducing its bioavailability. The effects observed in the mixture toxicity tests need to be corroborated by testing novel combinations (including non-

simultaneous exposure) of the chemicals involved. This also illustrates the necessity of further studies on the metabolism of such chemicals in crustaceans to understand mechanisms of interactive toxicity.

Behavioural observations can be a valuable tool to assess the effects of pollutants in ecotoxicological experiments. Test organisms are able to show specific responses depending on the type and mode of action of pollutants, which include avoidance reactions, changes in feeding and mating behaviour, as well as altered locomotory activity (Hebel *et al.*, 1997). Some of these responses were observed in the experiments carried out in this study. In the acute toxicity tests, the neurotoxicity of methyl-parathion was indicated by the irritability that the organisms showed to light and handling. Symptoms of disorientation and spasms were observed prior to death in the highest concentrations of exposure, while in general the animals seemed to show reduced food consumption in relation with control organisms. A sign of acute intoxication was characterised by some organisms able to detect the food when offered, but unable to grab the pellets with the pereopods or retain the food for long periods. The exposure to Tilt apparently had a narcotic effect on the shrimps as they showed less activity in non-perturbed conditions than controls, although also reacted more vigorously to stimuli such as light and handling. While an apparent lack of appetite was observed initially when the exposed animals ignored or were unable to detect the food pellets, the organisms were able to regain normal food consumption at the end of the experiment. Similar feeding behaviour occurred with lower exposure levels during the 32 days subchronic exposure to Tilt, and the acute toxicity test with propiconazole. This

supports the idea that the active ingredient, propiconazole, is responsible for the temporary variation in the feeding response. In the mixture experiments, both Tilt and propiconazole were apparently able to counteract at some degree the neurotoxic effects of methyl-parathion. For instance, in the methyl-parathion – Tilt experiments, the organisms exposed to the highest concentrations showed the effects associated with OP exposure like lack of co-ordination, immobilisation of pereopods and erratic movements prior death. However, the surviving organisms were able to recover and showed normal behaviour until the end of the experiment. In turn, the exposed animals in the methyl-parathion – propiconazole test did not show appreciable alteration on the ability to locate and ingest the pellets. Altogether, the behaviour patterns of the mixture experiments suggest that, at this level, there is a probable antagonistic interaction between the neurotoxicicity of methyl-parathion and the likely narcotic effects of propiconazole. Although the behavioural observations were only recorded qualitatively and *per se* were not considered as endpoints in the experiments, they point out important differences on the way the test organisms faced the exposure scenarios. The results obtained in this study indicate a considerable potential to use behavioural responses in ecotoxicological research with penaeid shrimps.

The moulting rate was also studied during the experiments. The importance of crustacean moulting as ecotoxicological endpoint derives from its influence on essential processes such as growth, reproduction, physiology and behaviour (Skinner, 1985; Dall *et al.*, 1990). In this study, the evaluation of the moulting rate as response to the pesticides was approached quantitatively. Moult casts

were accurately registered during the experiments by using designated cages to confine the shrimps individually. The confinement also prevented incidental cannibalism that can occur when shrimps are placed all together. The cast collection was easily performed during the first hours of photoperiod light as moulting occurred mostly during the "night" and shrimps are normally inappetent following ecdysis. When tagging is impractical, this cage-design may also be useful to study other endpoints that need periodic measurements from a given organism (e. g. growth, development, reproductive parameters, etc.), or non-destructive sampling procedures (e.g. of haemolymph). However, further analytical corroboration of the toxicant levels in the flow-through system is necessary, as the cages may have contributed with a greater binding surface in the system.

Although the moulting data obtained in the acute toxicity tests were generally insufficient for comparative statistical analysis, the information provided some preliminary evidence of the effect of pesticides that need to be confirmed in further studies. In general, the amount of data was limited due to the expected mortalities during exposure, but also because the moult frequency of the shrimp juveniles were too low in relation to the experimental period. Nevertheless, the information obtained in the Tilt and propiconazole experiments seems to indicate slight moult retardation as a consequence of exposure to both fungicides. Similar evidence was found in the 32 days exposure experiment with Tilt, where the moulting frequency showed a negative correspondence with the fungicide concentration and the distribution of intermoult duration frequencies of some treatments differed significantly from the control. In

general, it was appreciated that Tilt treatments increased the variability in the moulting response. Such increase in the variability of biological responses have been previously observed in benthic invertebrates exposed to pollutants, and has been considered as a general symptom of environmental stress (Forbes and Depledge, 1996). From the mixture toxicity tests, only the methyl-parathion – Tilt combination generated some data due to high survival rate in all treatments. The analysis suggested a slight concentration-related decrease of moult frequency, but failed to find a similar pattern in the intermoult duration data.

Far from conclusive, the results obtained address the need to carry out more studies on the effects of these toxicants on the moulting process of *L. vannamei*. Still, it is necessary to determine whether the possible moult retardation can be attributed to a biochemical or physiological disruption or due to feeding inhibition. The utilisation of moulting rate as an endpoint in toxicological experiments could be improved if some procedures are considered to reduce the variability in the response. One option could be to pre-select test organisms with the same moult stage, although this could be difficult to achieve in large-scale experiments and it does not reduce the intra-specific variability. The use of larval stages, which moult more frequently (every 30-40 hours) in a synchronised fashion (Wyban and Sweeney, 1991), may be a more reliable and faster way to assess the effects of toxicants on the moulting rate. Other approaches could also be considered to study the moulting process such as the effect of pollutants on chitin synthesis (Horst and Walker, 1995), shell quality (Baticados and Tendencia, 1991; Cruz-Lacierda, 1993) or circulating

ecdysteroids during the moult cycle (Chan *et al.*, 1988; Blais *et al.*, 1994). In general, studies of sublethal responses in laboratory conditions can be designed following some basic recommendations. For example, when choosing exposure concentrations, they can be based in relation to the threshold LC50, down to levels including the expected levels of toxicants in the environment, achieving longer experimental periods. Also, the simultaneous measurement of selected sublethal responses (behavioural, cellular or physiological) may be desirable for a better understanding of the biological processes involved.

Several research directions can be suggested considering the results from the current findings. While some questions remained unanswered and it is still necessary to improve certain experimental procedures, the current study illustrates the need of ecotoxicological research in Mexico. Based in the experiences with *L. vannamei*, new bioassays can be designed to assess the toxicity of pesticides and other pollutants released in coastal waters of Mexico. The flow-through system can be adapted to evaluate the comparative sensibility to toxicants using different life stages of penaeid shrimps, and in the same way, other local species of invertebrates and fish. Some research lines that were also explored during this study are described in Appendix C, such as the study of an alternative route of exposure (oral administration of pesticides, section C.1), and the implementation of techniques to measure biochemical responses like as ecdysteriods levels, mixed-function oxidases and acetylcholinesterase activity (section C.2). Altogether, the present findings stress the need of future research regarding the utilisation of relevant endpoints in ecologically sound

bioassays in order to study the effects of pollutants in realistic scenarios such as pulse exposures or environmentally relevant concentrations.

The lack of accurate information on pesticide use in Mexico makes difficult to predict whether residues of the pesticides used in this study may or not interact in field conditions. However, it can be assumed that due widely use of methyl-parathion and other organophosphates, they are likely to be present in most agricultural areas where fungicides are applied. For instance, the estimated annual agricultural use of propiconazole in the USA overlaps in most regions and type of crops with the estimated use of methyl-parathion (USGS– Pesticide National Synthesis Project, 1992). Pesticide usage patterns, however, are likely to change constantly due to pest resistance, change in crops or environmental regulations. In fact, the use of methyl-parathion can be greatly diminished after the Environmental Protection Agency in the USA announced restrictions on its use on many of the most significant food crops within the country, after the insecticide was found to pose unacceptable dietary risks to children (EPA, 1999). This illustrates the need of ecotoxicological research in tropical areas where, in many cases, the application of pesticides remains largely unregulated. Studies on the effects of pollutants on local tropical species, both in field and in laboratory, are necessary for the environmental risk assessment of such areas.

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Appendix A

Mortality tables from the range finding tests

Table A.1. Percentage of mortalities in the range finding test for methyl-parathion. Concentrations based in the active compound content.

Time	Tested concentrations ($\mu g.l^{-1}$)					
(hours)	0	0.1	1	5	30	50
24	0	0	14	0	0	100
48	0	0	14	0	0	100
72	0	0	14	0	0	100
96	0	0	14	0	100	100
196	0	0	14	60	100	100

Table A.2. Percentage of mortalities in the range finding test for Tilt in static system. Concentrations based in the active compound content.

Time	Tested concentrations ($\mu g.l^{-1}$)								
(hours)	0	100	150	200	300	400	500	700	900
10	0	0	0	0	0	0	100	100	100
24	0	0	0	0	40	40			
48	0	0	0	20	100	100			
72	0	0	0	20					
96	0	20	0	20					

Table A.3. Percentage of mortalities in the range finding test for Tilt in the flow-through system. Concentrations based in the active compound content.

Time	Tested concentrations ($\mu g.l^{-1}$)					
(hours)	0	900	1 200	1 700	2 200	2 700
10	0	0	0	30	100	100
24	0	0	0	100		
48	0	0	100			
72	0	0				

Table A.4. Percentage of mortalities in the preliminary experiments with solvents (to be used as pesticide carriers) in the flow-through system.

Time	Tested concentrations ($\mu l.l^{-1}$)				
	Methanol			Acetonitrile	
(hours)	277	554	833	139	277
10	0	0	30	0	0
24	30	0	60	0	0
48	100	100	100	0	0
72				0	0
96				0	0
120				0	30
144				0	100

Table A.5. Percentage of mortalities in the range finding tests for propiconazole in the flow-through system. (S.C. – solvent control with acetonitrile). Propiconazole concentrations based in the active compound content.

Time (hours)	Tested concentrations ($\mu g.l^{-1}$)						S.C. ($\mu l.l^{-1}$)
	900	2 000	6 000	8 000	10 000	13 000	140
24	0	0	25	30	60	50	0
48	0	0	50	30	60	100	0
72	0	50	50	100	100		0

	Tested concentrations ($\mu g.l^{-1}$)				S.C. ($\mu l.l^{-1}$)
	800	1 900	4 300	10 000	140
24	0	0	0	40	0
48	0	0	20	60	0
72	0	0	40	80	0
96	0	0	60	100	0
120	0	0	60		0
144	0	0	60		0

Table B.1. Percentage of mortalities in the methyl-parathion definitive experiment. Concentrations based in the active compound content.

Time	Tested concentrations ($\mu g.l^{-1}$)													
(hours)	0.1	0.1	0.5	0.5	2	2	10	10	20	20	35	35	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.75	0	0	0	0	0	0	0	0	0	0	10	10	0	0
1	0	0	0	0	0	0	0	0	10	0	10	10	0	0
2	0	0	0	0	0	0	0	0	10	0	50	20	0	0
4	0	0	0	0	0	0	0	0	30	20	80	20	0	0
8	0	0	0	0	0	0	10	0	40	30	90	40	0	0
14	0	0	0	0	0	0	10	0	40	50	100	90	0	0
24	0	0	0	0	0	0	10	0	40	60	100	90	0	0
33	0	0	0	0	0	0	20	10	40	60	100	90	0	0
48	0	0	0	10	0	0	20	10	40	60	100	90	0	0
72	0	0	0	10	0	0	30	10	40	60	100	90	0	0
96	0	0	0	10	0	0	50	10	70	60	100	90	0	0
120	0	0	10	10	0	0	60	10	70	80	100	90	0	0
144	0	0	10	10	0	0	70	20	90	80	100	100	0	0
168	0	0	10	10	0	0	80	30	100	80	100	100	0	0
192	0	0	10	10	0	0	80	40	100	80	100	100	0	0
216	0	0	10	10	0	0	90	50	100	80	100	100	0	0
240	0	0	10	10	0	0	100	60	100	80	100	100	0	0

Table B.2. Percentage of mortalities in the Tilt definitive experiment. Concentrations based in the active compound content.

Time	Tested concentrations ($\mu g.l^{-1}$)															
(hours)	950	950	1000	1000	1055	1055	1110	1110	1170	1170	1235	1235	1300	1300	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0
8	0	0	0	0	10	0	10	10	0	10	0	0	10	20	0	0
14	0	0	0	0	20	0	40	20	0	20	10	0	10	60	0	0
24	0	0	0	10	30	10	50	40	0	50	30	20	50	90	0	0
33	0	0	0	10	30	10	50	40	0	50	30	20	50	90	0	0
48	0	0	0	10	30	10	50	40	0	50	30	90	100	90	0	0
72	0	0	0	10	30	10	50	40	0	50	100	100	100	100	0	0
96	0	0	0	10	30	10	50	40	0	50	100	100	100	100	0	0
120	0	0	0	10	30	10	50	40	0	50	100	100	100	100	0	0
144	0	0	0	10	30	10	50	40	0	50	100	100	100	100	0	0
168	0	0	0	10	30	10	50	40	0	50	100	100	100	100	0	0
192	0	0	0	10	30	10	50	40	0	50	100	100	100	100	0	0
216	0	10	0	10	30	10	50	40	0	50	100	100	100	100	0	0
240	0	10	0	10	30	10	50	40	0	50	100	100	100	100	0	0

Table B.3. Percentage of mortalities in the propiconazole definitive experiment. Concentrations based in the active compound content (S.C. – solvent control with acetonitrile).

Time	Tested concentrations ($\mu g.l^{-1}$)															
(hours)	800	800	1250	1250	2000	2000	3100	3100	4900	4900	7600	7600	12000	12000	0	S.C.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	10	20	0	0
14	0	0	0	0	0	0	0	0	0	0	20	0	50	20	0	0
24	0	0	0	0	0	0	0	0	20	0	50	40	80	60	0	0
33	0	0	0	0	0	0	0	0	20	20	60	70	90	90	0	0
48	0	0	0	0	0	0	0	0	20	20	70	80	100	90	0	0
72	0	0	0	0	0	10	0	10	30	40	80	80	100	100	0	0
96	0	0	10	10	20	30	90	100	100	90	100	80	100	100	0	0
120	0	0	20	20	30	70	90	100	100	100	100	80	100	100	0	0
144	0	0	20	20	30	70	90	100	100	100	100	80	100	100	0	0
168	0	0	20	30	40	70	90	100	100	100	100	80	100	100	0	0
192	0	0	20	30	40	70	90	100	100	100	100	80	100	100	0	0
216	0	0	20	30	40	70	90	100	100	100	100	80	100	100	0	0
240	0	10	20	30	40	70	90	100	100	100	100	80	100	100	0	0

Table B.4. Percentage of mortalities in the methyl parathion - Tilt definitive experiment. Concentrations based in the active compound content.

Time	Methyl parathion / Tilt tested concentrations ($\mu g.l^{-1}$)															
(hours)	475/0.5	475/0.5	500/0.9	500/0.9	527/1.6	527/1.6	555/3	555/3	585/5.3	585/5.3	617/9.7	617/9.7	650/17.5	650/17.5	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
72	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0	0
96	0	0	0	0	0	0	0	0	0	0	10	10	0	20	0	0
120	0	0	0	0	0	0	0	0	0	0	10	10	0	20	0	0
144	0	0	0	0	0	0	0	0	0	0	10	10	0	20	0	0
168	0	0	0	0	0	0	0	0	0	0	10	10	0	20	0	0
192	0	0	0	0	0	0	0	0	0	0	10	10	0	20	0	0
216	0	0	0	0	0	0	0	0	0	0	20	20	10	20	0	0
240	0	0	0	0	0	0	0	0	0	0	20	20	30	30	0	0
264	0	0	0	0	0	0	0	0	0	0	20	30	30	30	0	0
288	0	0	0	0	0	0	0	0	0	0	20	40	40	30	0	0
336	0	0	0	0	0	0	0	0	10	0	20	40	40	30	0	0
340	0	0	0	0	0	0	0	0	10	0	20	40	40	40	0	0

Table B.5. Percentage of mortalities in the methyl parathion - propiconazole definitive experiment. Concentrations based in the active compound content (S.C. – solvent control with acetonitrile).

Time	Methyl parathion / propiconazole tested concentrations ($\mu g.l^{-1}$)															
(hours)	400/0.5	400/0.5	625/0.9	625/0.9	1000/1.6	1000/1.6	1550/3	1550/3	2450/5.3	2450/5.3	3800/9.7	3800/9.7	6000/17.5	6000/17.5	0	S.C.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	10	0	10	20	0	0
14	0	0	0	0	0	0	0	0	0	10	40	40	60	100	0	0
24	0	0	0	0	10	40	30	90	50	20	100	100	100	100	0	0
33	0	0	0	0	10	60	30	90	70	90	100	100	100	100	0	0
48	10	0	0	30	50	60	50	90	70	90	100	100	100	100	0	0
72	10	0	0	30	50	60	60	90	80	100	100	100	100	100	0	0
96	10	10	0	30	50	60	70	90	80	100	100	100	100	100	0	0
120	10	10	0	30	50	60	80	90	80	100	100	100	100	100	0	0
144	10	10	0	30	50	60	80	90	90	100	100	100	100	100	0	0
168	20	10	0	30	50	60	80	90	90	100	100	100	100	100	0	0
192	20	10	0	30	50	60	80	90	90	100	100	100	100	100	0	0
216	20	10	10	30	50	60	90	90	90	100	100	100	100	100	0	0
240	20	10	10	30	50	60	90	90	100	100	100	100	100	100	0	0

APPENDIX C

ADDITIONAL RESEARCH

C.1. FEEDING EXPERIMENTS

C.1.1. Material and methods

The oral administration of pesticides was evaluated as bioassay model for toxicity tests. This set of experiments was carried-out in a static system, which consisted of 10-litre aquariums with aeration. The experimental conditions were the same described for the flow-through system in Chapter 2 (room temperature, photoperiod and seawater treatment). Toxicity feeding tests were carried out with methyl-parathion and technical grade propiconazole (see section 2.3).

Shrimps used in these tests had an average wet weight of 1 g. and were always randomly introduced in the system (7 organisms per aquarium). After an acclimation period of at least three days, the animals were starved for one day, and then fed with pellets (Rangen – 30% protein) containing different levels of pesticides (concentration range was previously determined by range finding tests). The contaminated pellets were prepared by immersing them in pesticide solutions of known concentration. Nominal concentrations (mg of pesticide / dry weight of pellet), were obtained by recording the differences of weight before and after the immersion. After that, the pellets were placed in an oven at 35° C for 1 hours to evaporate the solvent. The pesticide solutions were prepared in acetonitrile, which proved to preserve the stability of the pellets after the immersion. Negative and solvent controls were included as well. The pellets

were offered twice a day during 15 minutes followed by water exchange. Mortality was recorded before feeding and used to determine the median lethal dose (LD50) at different exposure times.

C.1.2. Results and conclusions

The mortality pattern from the methyl-parathion feeding experiment is shown in Fig. C-1. In the first two days of the experiment, the 50 % of lethality occurred between 140 to 170 $\mu g.g^{-1}$, and between 50 to 80 $\mu g.g^{-1}$ during the 4^{th} and 5^{th} day. The LD50 values calculated at different exposure times were used to construct the toxicity curve (Fig. C-2). During the first two days, the LD50's ranged around 250 $\mu g.g^{-1}$, decreasing to 145 $\mu g.g^{-1}$ at 72 hours, to be later situated at 98.5 and 71 $\mu g.g^{-1}$ at 96 and 120 hours respectively. This can be considered only a partial toxicity curve since no asymptotic line was reached.

The exposure to propiconazole absorbed in pellets failed to produce lethal responses. The animals completely rejected the pellets containing the highest dose of 27 $mg.g^{-1}$. Using pellets with 2 $mg.g^{-1}$, an initial consumption was observed, followed by pellet rejection later on. Doses of 0.2 and 0.017 $mg.g^{-1}$ were well accepted by the organisms, but no mortalities were recorded during five days of exposure. This could be explained due to the fact that shrimps are highly selective feeders, combined with the low toxicity of PPZ through oral administration.

Shrimp are therefore likely to taste and reject some toxicants at sublethal concentrations. The use of additional attractants (i.e. fish oil) or force-feeding

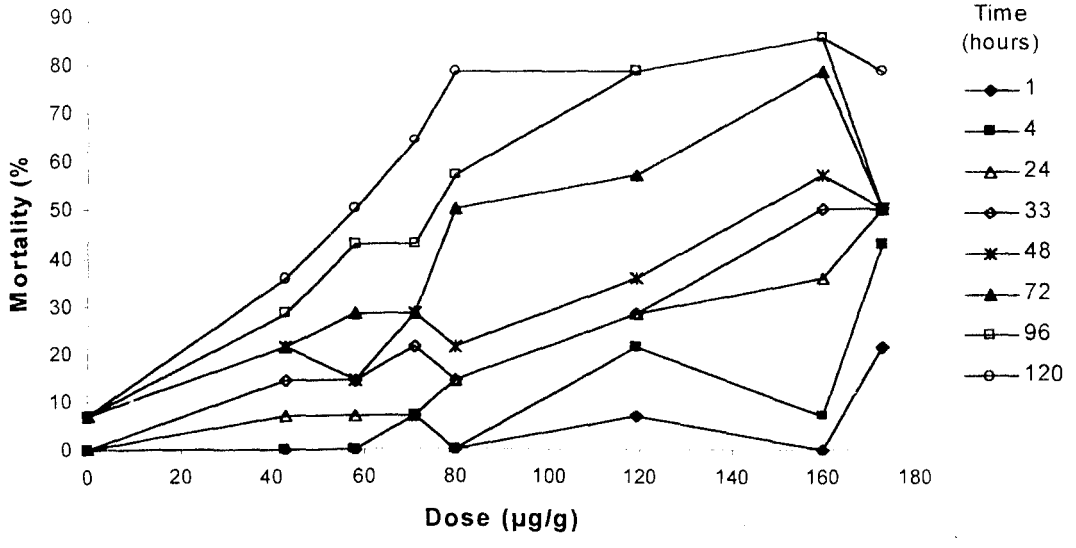

Fig. C-1. Mortality pattern of *Litopenaeus vannamei* exposed to methyl-parathion in feeding experiments. Doses expressed as µg of pesticide per gram of pellet (dry weight). Group size = 14.

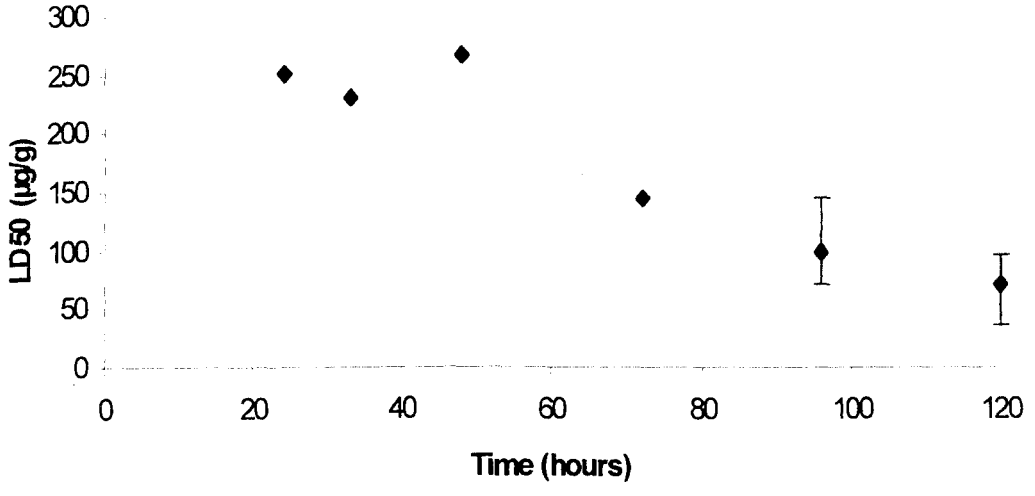

Fig. C-2. LD50 values and 95 % confidence limits of methyl-parathion for *Litopenaeus vannamei* in feeding experiments. Doses expressed as µg of pesticide per gram of pellet (dry weight).

could help to counteract this problem. The bioassay, however, could result inappropriate to test some chemicals. Shrimps could also stop eating when affected by the toxicant. For instance, in the methyl-parathion experiment, the animals exposed to the highest concentration (173 $\mu g.g^{-1}$) lost limb coordination (and probable chemosensorial capabilities) after the first doses, which prevented them to grab the pellets later on. This discontinuity in the exposure allowed some organisms to barely survive until the end of the experiment, and lowered the mortality record. This inconsistency could possibly be corrected by using immobilisation as endpoint instead of mortality, but further experimental corroboration should be performed. Also, a characterisation of the exposure in this bioassay model should be complemented with analytical procedures such as the determination of toxicant leaching from the pellets, the feasibility to achieve desired test concentrations, and actual intake and excretion of toxicants.

On the other hand, the oral administration of toxicants could provide a more realistic exposure method for benthic organisms than traditional water exposures, it minimises the effect of carriers in the toxic response, and it has the simplicity of a static system. The characteristics mentioned gives to this bioassay a potential application for the assessment of chemicals in chronic exposures and for interactive toxicity studies.

C.2. BIOCHEMICAL RESPONSES

C.2.1. Ecdysteroid levels

An enzyme immunoassay (EIA) technique to measure ecdysteriod levels in crustacean hemolymph was evaluated for its potential use in toxicological

studies. Preliminary trials were performed with hemolymph samples of freshwater crayfish at the laboratory of biochemistry of Ecole Normale Superieure. Paris. France. in collaboration with Prof. Rene Lafont (data not shown). The EIA used was a modification of the technique described by Porcheron *et al.* (1989). and used peroxydase as enzymatic tracer. This technique is known to detect three major immunoreactive components in *L. vannamei* hemolymph: 20-hydroxyecdysone. ecdysone and 3-dehydroecdysone (Blais *et al.*. 1994). Chan *et al.* (1988) and Blais *et al.* (1994) have reported the fluctuation on hemolymph ecdysteroid titers during the moult cycle of *L. vannamei*. The levels detected in such studies were considered low for early moult stages (metecdysis and anecdysis) or intermoult. to subsequently increase in later stages and reach a maximum during proecdysis. Similar profiles have been reported in other crustaceans (see Chan *et al.*. 1988). This pattern of circulating ecdysteroids could serve as baseline to study potential disruptive effects of pollutants on the moult process at endocrinal level.

C.2.2. Mixed-function oxidases (MFO)

Preliminary trials to measure MFO activities in shrimp were carried out at the Whitney laboratory. University of Florida. USA. in collaboration with Dr. Margaret James. Microsomal fractions were obtained from hepatopancreas of the marine shrimp *Farfantepenaeus. aztecus* using a homogenising buffer containing a mixture of proteases inhibitors to slow down the oxidation of monooxigenases in hepatopancreas. followed by ultracentrifugation (45 000 x g). Microsomal fractions were also obtained by aggregating the microsomes with calcium. which proved useful to increase the microsomal yield and only

required a high-speed centrifugation (25 000 x g), therefore eliminating the need of ultracentrifugation. Ethoxycoumarin O-deethylase (ECOD) activity was measured from the microsomal fraction with a fluorescence assay. ECOD activities recorded averaged 1.5 pmol/min/mg microsomal protein, which can be considered low if compared with previous results with the same species: 18 pmol/min/mg microsomal protein (M. James, personal communication). The microsomal protein content was determined using the Lowry protein bioassay.

The MFO assay has potential application to investigate the induciblility of the oxidative system in crustaceans by pesticides and other organic contaminants. However, the technique requires large amounts of hepatopancreas tissue and sample pooling is still necessary for juvenile or adult size shrimps.

C.2.3. Acetylcholinesterase activity (AChE)

Assay conditions were evaluated for AChE determination in *L. vannamei* to study the exposure to organophosphate pesticides, such as methyl-parathion. The methodology was adapted following the procedures suggested by Dr. Lucia Guilhermino (University of Porto, Portugal). The organisms were dissected to obtain the muscle, which is immediately homogenised in phosphate buffer and AChE activity was determined spectrophotometrically using acetylthyocholine as substrate. Protein determination was performed using the Lowry assay. The relation between AChE inhibition and pesticide levels has been previously demonstrated and has been used as a monitoring tool for marine pollution by organophosphates and carbamate pesticides (Bocquené *et al.* 1990). Moreover, AChE can also be performed in a non-destructive fashion and provide an index

of potentiation of organophosphate and carbamate insecticides by other pesticides (Walker. 1998) (see section 4.3). Preliminary trials showed inhibition of AChE in shrimps exposed to methyl-parathion (data not shown). but further experimentation is necessary to characterise and determine the optimal assay conditions. and test alternative tissues for enzyme activity (i.e. eye and haemolymph).